Molecular characterization of neuroprotective
β-amyloid interacting peptides and autoantibodies
relevant to Alzheimer’s disease

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For my family
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Introduction

1 INTRODUCTION

1.1 Alzheimer’s disease: pathophysiology, diagnosis and potential therapies

1.1.1 Biochemical pathways of neurodegeneration in Alzheimer’s disease

Alzheimer’s disease (AD) is the most frequent cause of dementia and affects more than 25 million people worldwide. It is presently an incurable, fatal neurodegenerative disease, which initially triggers episodic memory impairment and progressively affects other cognitive compartments responsible for visuospatial orientation, language, executive functions. Clinical manifestations of AD also include apathy, anxiety, agitation, irritability and even aggression [1, 2]. These symptoms are caused by synapse and neuron loss, which results in advancing hippocampal and cortical atrophy of the AD brain, combined with ventricular enlargement [3, 4]. Despite intensive research, there is still poor understanding of the molecular causes and lack of preventive or curative therapies.

The neurological disorder was named after Alois Alzheimer, the first one to provide, in 1906, a description of both symptomatic and pathological features of the disease. In his autopsy report on an early-onset case he noted the presence in brain of widespread deposits surrounding the neurons (senile plaques) and of twisted bands of fibers (neurofibrillary tangles) inside the nerve cells [5]. Detailed molecular research revealed that the extracellular deposits in AD brain are mainly composed of β-amyloid (Aβ) peptides, 38-43 amino acids in length [6], which are physiologically released from the amyloid precursor protein (APP) after successive cleavages by β- and γ-secretases [5, 7] (Figure 1).

APP is a transmembrane protein expressed in many types of cells and tissues. Alternative splicing of the APP gene yields as major isoforms APP-695, APP-751 and APP-770, out of which APP-695 represents the predominant isoform in brain [2, 8]. Normal processing of APP may follow two proteolytic pathways (Figure 1).
In the non-amyloidogenic pathway, proteolysis by α-secretase releases in the extracellular space a large N-terminal region (α-APPs). A subsequent cleavage by γ-secretase inside the transmembrane sequence of the remaining 83 amino acids long C-terminal fragment (C83 or α-APP CTF) generates a non-toxic N-terminally shortened variant of Aβ, called p3 and the APP intracellular domain (AICD). The amyloidogenic processing of APP comprises first proteolysis by β-secretase, with subsequent formation of the N-terminal ectodomain β-APPs and of a membrane-anchored carboxy-terminal region C99 or β-APP CTF, which is further cut by γ-secretase, leading to the extracellular release of Aβ peptides [5, 7]. Aβ(1-40) is the most abundantly produced isoform, followed by Aβ(1-42). However, Aβ(1-42) represents the major constituent of amyloid deposits, since the additional C-terminal residues Ile-41 and Ala-42 makes it more hydrophobic and hence more prone to aggregate than Aβ(1-40) [2, 7, 9]. The amino acid sequences of the two peptides are shown in Figure 3.

**Figure 1** Proteolytic pathways of APP processing. In the amyloidogenic pathway, proteolysis by β-secretase generates the N-terminal ectodomain β-APPs and a carboxy-terminal fragment (C99 or β-APP CTF), which is further cleaved by γ-secretase within its membrane-spanning region, leading to the extracellular release of Aβ peptides. In the non-amyloidogenic pathway, proteolysis by γ-secretase generates a large N-terminal fragment of APP (α-APPs), while the subsequent cleavage by γ-secretase of the remaining C-terminal sequence (C83 or α-APP CTF) generates an N-terminally shortened variant of Aβ, called p3. Both proteolytic pathways also lead to the final release of the APP intracellular domain (AICD) into the cytosol. Adapted with permission from Macmillan Publishers Ltd: Nat Rev Drug Discov [7], copyright 2002.
According to the “amyloid cascade hypothesis”, an increased production of Aβ and reduced clearance of the peptide trigger a pathological chain of events, including deposition of insoluble amyloid fibrils and eventually synapse and neuron loss [10-12]. Several soluble Aβ assemblies, such as small oligomers, annular oligomers and protofibrils have been identified in vitro and in vivo as intermediates in the process of Aβ fibrillization and accumulation into plaques [6, 13, 14]. The small Aβ oligomers, consisting of 2-50 monomer units, appear to be the most neurotoxic species, causing synaptic damage of cultured neurons [15] and memory deficits in rats [16, 17]. In AD patients, a strong correlation between the cortical levels of soluble Aβ species (monomers and oligomers) and the extent of synapse loss and associated memory impairment has been found [3, 18]. Aβ aggregates may damage nerve cells through multiple mechanisms: (i) disruption of calcium regulation by forming new calcium-conducting pores in the lipid bilayers of the plasma membrane [19, 20]; (ii) oxidative stress, due to the formation of hydrogen peroxide during Aβ association in the presence of metal ions [6, 19]; (iii) activation of signal transduction pathways triggering apoptosis [21]; (iv) activation of astrocytes and microglia, leading to inflammation [3, 21].

The formation of amyloid plaques (Figure 2) occurs early in the course of AD, years before the clinical symptoms [10]. Two types of Aβ accumulations have been found in the cortex of AD patients: dense-core fibrillar plaques and diffuse plaques. The dense-core fibrillar plaques are mainly composed of fibrillar Aβ(1-42) and to a lesser extent of Aβ(1-40) [2, 10]. They are closely associated with axonal and dendritic degeneration (neuritic dystrophy) as well as with activated inflammatory cells – microglia and astrocytes [2, 5, 10]. The diffuse plaques are amorphous depositions comprising mostly Aβ (1-42), usually nonneuritic and free of glial responses. They are considered immature lesions and are often present in the brain of non-demented elderly persons [2, 10]. Although the amyloid burden does not seem to correlate either with the severity of the clinical manifestations or the duration since the onset of symptoms [22, 23], results from a longitudinal amyloid positron emission tomography (PET)-imaging study [24] suggest a gradual increase of aggregated Aβ as the disease progresses.
The second pathological feature of AD is represented by neurofibrillary tangles (Figure 2), which are intraneuronal aggregates of abnormally hyperphosphorylated microtubule-associated protein tau [7]. Microscopic investigations showed that neurofibrillary tangles are mainly composed of paired helical filaments [25]. Microtubules have essential biological functions, providing support and shape to cells, as well as transport routes for various molecules and cellular components (e.g., vesicles, mitochondria). Therefore, their progressive collapse caused by accumulation of misfolded tau impairs the transport of nutrients and the transmission of neuronal signals [7, 26, 27].

AD is a complex disease with still largely unknown causes. It may have an early (<60-65 years) or late (>60-65 years) onset, however with common pathologies [28, 29]. The early onset form affects only 6-7% of all AD patients [30] and is mostly an autosomal dominant (familial) disorder [31]. Familial AD has been mainly attributed to mutations occurring in APP or in presenilin 1 and presenilin 2, which are homologous genes encoding the catalytic components of the γ-secretase [32]. APP mutations at the β-secretase cleavage site result in an increased production of all Aβ species, mutations around the γ-secretase cleavage
site and presenilin mutations lead to an increased $\text{A}\beta(1-42)/\text{A}\beta(1-40)$ ratio [6, 33], while mutations within $\text{A}\beta$ sequence may alter its aggregation properties [3, 6]. Among familial AD-related mutations of APP, V642 mutations to I, F and G, located near the γ-secretase cleavage-site are the most frequent, whereas A617G, L648P, K595N and M596L are present in fewer cases [34] (Figure 3).

Figure 3 Diagram of APP-695 isoform, with focus on the cleavage sites of the secretases involved in APP processing and on the most common sequence mutations of APP encountered in familial AD [34]. The primary sequence of the predominantly 40/42 amino acids long $\text{A}\beta$ peptide, released as a result of cleavages by β- and γ-secretases in the amyloidogenic pathway of APP metabolism, is highlighted in red.

The late-onset form of AD, encompassing 93-94% of total cases [30], is also characterized by abnormal $\text{A}\beta$ accumulation, albeit presumably due to the defective clearance of the peptide [35]. As a main genetic component, the presence of $\varepsilon4$ allele of apolipoprotein E (ApoE) has been shown to increase the risk of AD and reduce the average age at the onset of symptoms, with homozygous subjects more exposed than heterozygous ones [36]. Variants of genes regulating the immune response involved in $\text{A}\beta$ clearance or the cholesterol metabolism, essential for synapse formation, could also contribute to AD development [37]. However, the majority of late-onset AD patients have a normal genetic background, supporting the hypothesis that a combination of environmental and lifestyle factors are generally at the origin of the sporadic AD cases [38]. Thus, the risk of developing AD was shown to increase exponentially
with age in the 65-85 years old group, with no gender differences [39]. High blood pressure and levels of serum cholesterol during adulthood, with associated vascular changes and atherosclerosis, could also contribute to the occurrence of AD dementia by accelerating the degeneration of the aging brain [40]. Hypercholesterolemia was also shown to cause an increased production of \( \text{A}\beta(1-42) \) from APP and to affect the metabolic pathways of the peptide [41]. With regard to lifestyle factors, the intensity of social and physical activities were found to inversely correlate with the risk of AD in the elderly [42]. Apparently the sustained stimulation assures a more effective use of the brain networks, therefore a cognitive reserve strong enough to delay the clinical manifestation of AD [43].

1.1.2 **Antibody- and peptide-based therapeutic approaches targeting \( \beta \)-amyloid**

At present there is no curative treatment available for AD. The usually recommended neurotransmitter-based therapy, involving administration of cholinesterase inhibitors and memantine, only targets the cognitive symptoms, having no effect on neurodegeneration, which continues to progress [44]. Research has provided so far strong evidence that the imbalance in A\( \beta \) metabolism, namely the increased production and decreased clearance of the peptide represents a critical event in the development of AD [45]. Accordingly, current potential disease-modifying therapies aim to either block or decrease A\( \beta \) formation from APP (\( \beta \)- and \( \gamma \)-secretase inhibitors), improve clearance of A\( \beta \) (immunotherapy) or prevent A\( \beta \) association (aggregation inhibitors).

To prevent or diminish the production of A\( \beta \) peptides from APP, several drugs were designed to interfere with the catalytic activity of the responsible proteases, namely \( \gamma \)- and \( \beta \)-secretases. However, the \( \beta \)-secretase inhibitors and \( \gamma \)-secretase cleavage site modulators that progressed to clinical trials have failed so far to fulfill the required efficacy or safety profiles [46].
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To date, immunotherapy has been the most extensively investigated approach for the removal of Aβ peptides, in monomeric, oligomeric or aggregated form. Passive immunotherapy usually consists in regular administration of monoclonal antibodies raised against specific Aβ epitopes. On the other hand, active immunotherapy requires less frequent administration of either intact or truncated Aβ peptides, often conjugated to a protein carrier, with the purpose of generating a lasting immune response. As a result, polyclonal antibodies of various affinities to the antigen are produced, in concentrations that differ between patients and may not reach in certain cases effective values [44].

In transgenic mouse models of AD, both passive immunization with monoclonal anti-Aβ antibodies [45, 47, 48] and active immunization with full-length Aβ peptides or Aβ fragments [49-52] were able to prevent Aβ aggregation, clear amyloid plaques and improve cognitive performance. The positive effects on Aβ clearance following anti-Aβ immunotherapy could be explained by three potential mechanisms of action. In mouse brain, anti-Aβ antibodies recognizing an N-terminal epitope of Aβ, which is accessible within plaques (Figure 5), were shown to bind to amyloid deposits and promote their removal by microglia, through an Fc-mediated pathway [47, 53-55] (Figure 4c). There is also evidence for plaque removal in a microglia-independent manner [56-58] by antibodies that bind the N-terminal Aβ sequence. They were found to inhibit Aβ oligomerization and aggregation and also to dissolve pre-existing amyloid deposits through an assumed change in Aβ equilibrium from more toxic aggregated states to less toxic monomeric states [59-61] (Figure 4b). The “amyloid sink hypothesis” represents the third possible mechanism and states that antibodies may additionally cause dissolution of amyloid plaques by sequestering monomeric Aβ in the periphery and shifting the active transport of the peptide across the blood-brain barrier in this direction (Figure 4a). This theory was advanced by De Mattos et al., who reported clearance of Aβ plaques from mouse brain as a result of peripheral administration of a high affinity monoclonal antibody that recognizes a central epitope of Aβ and therefore cannot bind aggregates, but only monomeric Aβ [45, 62, 63].
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**Figure 4** Potential mechanisms of action of anti-Aβ antibodies. In the periphery, anti-Aβ antibodies may capture monomeric Aβ peptides and function thereby as a “sink”, causing the efflux of Aβ from the central nervous system (CNS) to plasma, across the blood-brain barrier (BBB) (a). In the brain, anti-Aβ antibodies may interact with soluble Aβ peptides (monomers, oligomers) and prevent their accumulation (b) or bind Aβ fibrils within plaques and induce their removal by microglia through an Fc-mediated pathway (c). Reprinted from [64], copyright 2012, with permission from Elsevier.

Based on the promising preclinical results, immunotherapy has been proposed as a possible therapeutic approach for AD in humans [65, 66]. A phase II multicenter clinical trial of active immunization with preaggregated Aβ(1-42) (AN1792(QS-21) vaccine) showed a reduction of amyloid plaque burden and slower cognitive decline in AD patients. However, the trial was interrupted due to the occurrence of meningoencephalitis in some of the immunized participants [66, 67] and a follow-up study with yearly assessments and post-mortem neuropathological examinations indicated progression of AD-related neurodegeneration and cognitive decline, despite vaccination [68].

The molecular mechanism responsible for the observed plaque clearance in immunized AD patients was revealed upon subsequent investigation of antibodies generated in APP-transgenic mice by active immunization with Aβ(1-42) and Aβ-derived aggregates. Thus, the “plaque-specific” anti-Aβ antibodies were shown to recognize an N-terminal Aβ epitope (4FRHDSGY10) [59], which is accessible in both oligomeric and fibrillar Aβ (Figure 5) [6, 69].
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Figure 5  (a) Different epitope specificities of “plaque-specific” anti-Aβ antibodies and physiological Aβ-autoantibodies; (b) Ribbon representation of the NMR structure of Aβ (proto)fibrils (PDB file 2BEG, [69]), in which the β-strands formed by amino acid sequences (18-26) and (31-42) are depicted as arrows and the β-turn in-between is shown as a grey loop. In the process of aggregation, Aβ monomers form parallel β-sheets oriented perpendicularly to the (proto)fibril axis. The unstructured N-terminal region of Aβ is not involved in fibril-growth, being accessible for antibody binding. Reproduced from [6], copyright 2007, with permission of Karger AG.

At present, several clinical trials are carried out to further evaluate the therapeutic potential of improved protocols for Aβ-based active immunization and to assess the effect of passive immunization with anti-Aβ antibodies in AD patients [70]. Two phase 3 clinical trials designed to evaluate the efficacy and safety of a humanized N-terminal anti-Aβ monoclonal antibody, Bapineuzumab, in patients with mild to moderate AD have recently been completed (http://clinicaltrials.gov). Results presented at the 16th EFNS congress in Stockholm showed that the treatment with
Bapineuzumab did not reach clinical endpoints (no significant benefit on cognitive or functional performance in the Bapineuzumab-treated group was obtained); however, effects of Bapineuzumab on CSF biomarkers such as phosphorylated tau (p-tau) were observed [71].

Recently, physiological Aβ-specific antibodies (Aβ-autoantibodies) have been detected in serum and CSF of AD patients and healthy individuals [72-76], as well as in commercially available human intravenous immunoglobulin (IVIg) preparations, which are fractionated blood products used for the treatment of immune deficiencies and other disorders [77]. Unlike the “plaque-specific” anti-Aβ antibodies, physiological Aβ-autoantibodies were found to specifically bind the carboxy-terminal sequence (21-37) of Aβ, located within Aβ aggregation domain [78, 79] (Figure 5). Dodel et al. [80] reported that administration of Aβ-autoantibodies led to reduced plaque formation and improvement of behavior in a mouse model of AD. Considering that IVIg preparations contain Aβ-autoantibodies, they were used in small pilot trials for the treatment of AD patients [73, 75, 81], with promising effects on cognition, and have also been introduced in clinical trials as a potential AD treatment (www.clinicaltrials.gov; [81]).

Another line of research for AD treatment involves the study of small organic compounds and peptide agents able to impede Aβ accumulation and/or display neuroprotective activity against Aβ toxicity.

Humanin (HN) is a linear 24 amino acids long peptide (MAPRGFSCLLLTSEIDLPVKRRA), cloned from the intact occipital region of an AD brain [82]. HN has been shown to prevent in vitro neuronal cell death caused by Aβ and familial AD-related genetic mutations of APP and presenilins [83]. Recent in vivo studies also revealed that HN derivatives prevent Aβ-induced memory impairment in mouse models of AD [84, 85]. Using immunoblotting with anti-HN antibodies, expression of HN in different mouse organs could be shown [86]. HN immunoreactivity was also detected in intact occipital neurons of an AD brain, but only in traces in an age-matched control brain [86], which further supports the protective function of this molecule in AD. Although HN does not
affect the release of Aβ peptides from APP [82], it has been efficient against different pathways of Aβ neurotoxicity [87-89]. The first aim of this dissertation was to study the molecular interaction between HN and Aβ(1-40), which may be of particular relevance for the development of AD therapeutics. The interaction epitopes and the binding affinity of the complex between HN and Aβ(1-40) were determined using affinity-mass spectrometry, biosensor analysis and ELISA and a structural characterization by molecular docking simulation was also performed.

1.1.3 Biomarkers for diagnosis of Alzheimer's disease

Aging represents the main risk factor for dementia. One in four seniors presents symptoms of mild cognitive impairment (MCI) and 10-12% of MCI cases develop AD within one year [90]. A definite diagnosis of AD is only done post-mortem, based on pathological criteria related to the brain densities of senile plaques and neurofibrillary tangles [91-93]. However, significant progress has been made in biomarker research towards a more accurate diagnosis of AD in living individuals, which is critical not only for a suitable medical intervention, but also for correct group assignment in various studies on the disease mechanisms or in clinical trials of potential therapeutics. Also, since amyloid deposition in AD begins 10 years or more before the synapse and neuron loss becomes severe enough to generate the clinical signs of cognitive impairment [94, 95], a special emphasis has been placed on the discovery of biomarkers for asymptomatic (preclinical) AD. These would enable a timely intervention, before the irreversible neurodegeneration is too advanced, thus increasing the chances of success for potential disease-modifying therapies.

Aβ peptides, as key molecules in AD-related neuropathology, have been the focus of most studies in the field. These revealed that, while CSF levels of Aβ(1-40) do not significantly differ between AD patients and controls [96, 97], CSF levels of Aβ(1-42) are lower in the AD group [98], reflecting the higher propensity of Aβ(1-42) to aggregate into plaques [99, 100]. Although sensitive, this molecular marker does not discriminate well AD from other types of dementia [101-103] and does
not correlate with the duration since the onset of symptoms or with the severity of
cognitive decline of AD patients [104], probably due to the relative stabilization of
the amyloid burden by the time the clinical manifestations occur [105].
Nevertheless, CSF $A\beta(1-42)$ levels were shown to inversely correlate with the in
vivo amyloid burden determined by Pittsburgh compound B (PIB)-PET, regardless
of the cognitive status [106, 107] and were able to predict the onset of
symptomatic AD in longitudinal studies on initially non-demented individuals [107].
Due to the high overlapping of CSF $A\beta(1-42)$ levels between AD and control
groups, cut-off values are yet to be defined before this biomarker could be used to
detect preclinical AD [108].

CSF tau levels were also investigated and found to be increased in AD patients
compared to controls [96, 103, 109]. Enhanced values of this biomarker are due to
neuronal injury, which is not specific to AD, but also encountered in other
neurodegenerative disorders [109]. However, CSF tau levels positively correlate
with the degree of cognitive impairment in AD [110]. Other studies focused on the
determination of p-tau species in CSF, which were shown to improve the
specificity of AD diagnosis [111, 112], while assuring similar discrimination power
between AD patients and controls as obtained for CSF tau levels [112]. More
recently, the ratios CSF tau/A$\beta(1-42)$ and CSF p-tau/A$\beta(1-42)$ were found to
predict conversion from an intact cognitive state to MCI or AD [113] and represent
therefore other potential markers of asymptomatic AD, in addition to CSF A$\beta(1-42)$
levels.

Although CSF molecular markers indicate with higher sensitivity biochemical
alterations within the brain, blood-derived biomarkers would be preferred in clinical
routine, owing to their less invasive character. Reports on potential serum-
biomarkers for AD diagnosis provided so far contradictory results and it is still
unclear whether changes in the periphery sufficiently reflect pathologies within the
brain [114-116]. Thus, studies investigating the $A\beta(1-42)$ levels in serum showed
reduced [117, 118] or increased values [119, 120] in AD patients compared to
control subjects. Others indicated no difference between groups [121, 122].
Furthermore, no correlation was found between the serum $A\beta(1-42)$ levels and the
Aβ(1-42) levels in CSF of AD patients and healthy individuals [123, 124], the accumulation of Aβ peptides in AD brain [106] or the progression of cognitive deterioration in AD [125, 126]. Another line of research focused on blood protein signatures of various growth factors, cytokines, chemokines and related signaling proteins [127, 128] and revealed a combination of 18 proteins that might discriminate between AD and control subjects. However, a recent validation study on samples from a larger, independent cohort showed that only the epidermal growth factor (EGF), the platelet-derived growth factor (PDG-BB) and the macrophage inflammatory protein 1α (MIP-1α) differentiated AD from control subjects, but not from patients with other types of dementia [129].

The biomarker value of the recently discovered physiological Aβ-autoantibodies, in addition to their potential therapeutic application for AD (see chapter 1.1.2), was also investigated, however with hitherto inconsistent results. In AD patients compared to controls, the serum levels of free, non-antigen-bound Aβ-autoantibodies were found to be reduced [130-132], enhanced [133] or unchanged [134, 135] by indirect ELISA. Other studies reported increased levels of Aβ-autoantibodies after acidic dissociation of preformed Aβ-immune complexes in serum of AD patients [136, 137] or no difference between AD and control subjects [138].

One of the goals of this dissertation consisted in the development of new sandwich and indirect ELISA protocols for the determination of antigen-bound, total and free Aβ-autoantibodies levels, using the recent finding that they bind the Aβ(21-37) epitope [78, 79]. The novel ELISA designs were first employed to investigate whether the levels of Aβ-autoantibodies in serum of healthy adults correlate with age and cognitive status and may therefore represent an early indicator of age-associated cognitive decline. Analysis of serum and CSF samples from AD patients and age-matched control subjects was also performed, with the purpose of evaluating the biomarker value of Aβ-autoantibodies and to obtain molecular insights into their contribution to Aβ clearance.
Imaging biomarkers for AD diagnosis and prognosis, such as volumetric measurements of cerebral atrophy by structural magnetic resonance imaging (MRI) or live PET detection and quantification of amyloid plaques using tracers with high affinity and specificity for fibrillar Aβ are also available [108]. However, the large-scale applicability of these methods is currently limited by the associated high costs and radiation exposure.

The ideal biomarker for clinical routine must be sufficiently sensitive, specific and reproducible, but also inexpensive and easily accessible. Additionally, it must have prognostic and diagnostic value and deliver useful information on the efficacy of treatment. A panel of complementary biomarkers, preferably blood-derived, represents the most realistic and probable solution to this long list of requirements for future applicability in the AD field.

1.2 Mass spectrometric methods for structural characterization of proteins

Mass spectrometry (MS) is a fast and sensitive technique for protein and peptide characterization that delivers accurate molecular mass information, thus enabling analyte identification, evaluation of sample homogeneity, analysis of protein-protein and protein-ligand interactions, detection of post-translational modifications (e.g., glycosylation, phosphorylation, lipidation) or amino acid exchanges [139-141].

In mass spectrometry ionized molecules are separated in a vacuum on the basis of differences in their mass (m) and charge number (z), using electrical and magnetic fields, process which occurs in the mass analyzer (e.g., TOF, Ion Trap). In the next step, the ions reach the detector, which is connected to a data acquisition system, where the abundance of ions at any given m/z is recorded [140-142]. The application of mass spectrometry to large complex molecules like proteins has been made possible by the development of new ionization techniques able to convert the polar, non-volatile biopolymer macromolecules into intact charged molecules in the gas-phase [141, 143]. Two of them, electrospray
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ionization (ESI) [144, 145] and matrix-assisted laser desorption/ionization (MALDI) [146-148] are best suited for use with macromolecules and macromolecular complexes [141].

In MALDI-MS a low amount of analyte is incorporated in a large excess of non-volatile matrix material by co-crystallization on the metal sample support. A pulsed laser is then used to irradiate the UV- or IR-absorbing matrix, which transfers the received energy into excitation energy of the crystallized analyte-matrix mixture [141, 143, 149]. As a result, desorption (vaporization) of both matrix and target molecules takes place, forming a dense gas cloud that expands supersonically into the vacuum. According to proposed mechanistic models, analyte ionization occurs in the expanding plume as a result of collisions between neutral analyte molecules, photoionized matrix ions, protons and cations [141, 147, 150] (Figure 6).

MALDI is a ionization method that generates predominantly singly charged ions, which makes the interpretation of spectra straightforward [141, 143]. Due to the high sensitivity of MALDI-MS, the usual amount of analyte required for analysis is ~1 pmol. However, sub-femtomole amounts of protein have been shown to be detectable [142, 143].

**Figure 6** Schematic representation of the MALDI process. Laser irradiation causes the desorption of the matrix-analyte mixture from the stainless steel sample support. Analyte ionization occurs in the resulting gas cloud. Adapted from [141], copyright 2005, with permission from John Wiley and Sons.
ESI is a process that produces intact ionized molecules from an analyte solution, at atmospheric pressure. The sample is dissolved in an acidified (with formic or acetic acid) mixture of an organic solvent (usually methanol or acetonitrile) and water, and introduced into the ESI source by means of infusion syringes, separation devices (for high performance liquid chromatography, capillary electrophoresis) or other interfaces, which provide nL-µL/min flow rates [140, 141, 144].

The ESI source consists of a metal capillary kept at elevated potential relative to the interface plate presenting the entrance to the mass spectrometer. In presence of the strong electric field, the liquid flow is dispersed into a fine spray of charged droplets, which are attracted to the inlet orifice of the mass spectrometer due to the difference in potential. As the solvent evaporates, the charge density on the surface of the droplets increases until the Rayleigh limit is reached (when Coulomb repulsion equals the surface tension). At this point the droplets break into smaller charged droplets that undergo the same process of “Coulomb explosion” until ion desorption into the gas phase finally takes place [140, 141]. It is assumed that most of the ions already desorb at the solution/air interface at the tip of the metal capillary, the so-called “Taylor cone” [140, 144] (Figure 7). To accelerate solvent evaporation in ESI, a counterflow of drying gas (N₂) is employed, while a coaxial flow of nebulizer gas (N₂) applied around the metal capillary facilitates the formation of fine droplets [140, 142, 144].
The ESI process generates multiply charged ions ([M+nH]^{n+} in positive ion mode and [M-nH]^{-n} in negative ion mode), which enables the analysis of large ionized molecules by mass spectrometers with limited m/z ranges [142, 149]. For proteins sprayed from acidic solutions, protonation of basic amino acid residues (Arg, Lys, His) and of the unmodified N-terminal amino group is observed in mass spectra recorded in positive ion mode. Negative ion mass spectra show deprotonation of acidic amino acid residues (Asp, Glu, also Tyr) and of the free carboxy-terminus [140]. The accessibility of these sites is influenced by pH, temperature and presence of denaturing agents in solution, property used to investigate conformational changes in proteins [140, 141, 151].

ESI produces stable singly or multiply charged ions that can be fragmented within the mass analyzer, e.g., by collision with an inert gas, to obtain additional structural information on the analyte (tandem mass spectrometry – MS/MS or MS^n) [144, 149]. This approach is widely used for sequence determinations and
identification of post-translational modifications, since it delivers not only molecular weight information, but also fragmentation patterns, thus providing a much more accurate structural fingerprint [139, 141].

ESI-MS is a highly sensitive method, which allows the analysis of peptides and proteins in the low picomole to femtomole concentration range. It is a fast approach for checking the structure and purity of synthetic peptides and determination of by-products [140, 144] and, due to the “soft” ionization technique, has been applied in the last years in many studies for the characterization of non-covalent biopolymer complexes [141, 151, 152]. A prerequisite for the successful application, however, is the careful optimization of “native” solution conditions and mass spectrometric parameters, to minimize dissociation of the complex and allow its ionization and transfer into the analyzer cell [151-153].

1.3 Analytical approaches for elucidation of interaction structures in protein complexes and quantification of binding affinities

Several analytical techniques are currently available for the characterization of non-covalent protein interactions, which represents an essential step towards the in-depth understanding of the biological functions of proteins. Due to its relevance for clinical diagnosis and therapy, the study of antigen-antibody complex formation is of particularly high interest [154]. An antigen is defined as any molecule that is recognized by an antibody. The region on the surface of the antigen to which a specific antibody binds is known as an epitope or antigenic determinant, while the corresponding antibody domain, complementary in shape and structure to the epitope, is called paratope [155].

For elucidation of protein epitopes, screening methods such as Pepscan or alanine-scanning may be applied in combination with binding assays (e.g. ELISA). Pepscan method involves the synthesis of overlapping peptides covalently bound to a solid support, which are further used in antigenicity studies by ELISA [156-158]. Alanine scanning is a similar technique, consisting in the production of epitope peptides in which all amino acid residues are individually replaced with
alanine. Subsequent tests for binding to the target antibody by ELISA allows the identification of the amino acid residues essential for the interaction with the paratope, which form the so-called functional epitope [159-162].

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are also used for the characterization of protein complexes and are able to deliver detailed information on their three-dimensional structure and binding residues [163-165]. However, these techniques have a number of drawbacks that limit their application. X-ray crystallographic measurements require large amounts of highly purified analyte and extensive optimization of experimental parameters (temperature, pH, protein concentration) [166, 167]. Moreover, flexible loops and post-translational modifications (e.g., glycan chains) must be removed prior to analysis, which can modify the physiological properties of the proteins [164, 166]. For NMR measurements, high amounts of pure sample must also be available and data collection and analysis are time-consuming [168, 169].

For the identification of interaction structures in protein complexes, affinity-mass spectrometry in combination with selective proteolytic digestion (epitope-excision) and ligand-affinity selection of the fragments (epitope-extraction) has been developed and successfully applied in several studies [170-175]. In mass spectrometric epitope-excision (Figure 8a), the protein or peptide ligand (e.g., antibody) is immobilized on a matrix and the analyte (e.g., antigen) is added to the microcolumn. Proteolytic digestion with various proteases is then carried out, the unbound fragments are washed away and the epitope-containing peptides are eluted and analyzed by mass spectrometry. In the mass spectrometric epitope-extraction procedure (Figure 8b), the protein or peptide analyte is first digested in solution with various proteases and the resulted peptide fragment mixture subjected to interaction with the immobilized ligand. After dissociation, the bound epitope peptides are collected and analyzed by mass spectrometry.
Introduction

Figure 8 Illustration of mass spectrometric epitope-excision (a) and -extraction (b) methodologies. (a) In epitope-excision, the analyte is allowed to bind the immobilized ligand and the complex is subsequently digested with various proteases; (b) In epitope-extraction, the peptide mixture resulted from in-solution digestion of the analyte is presented to the immobilized ligand. At the end of both procedures, the epitope-containing fragments are dissociated (usually with 0.1% aqueous TFA) and analyzed by mass spectrometry.

Surface plasmon resonance (SPR) has long been a method of choice for the determination of affinity and equilibrium dissociation constants of various biopolymer complexes. It involves covalent attachment of the ligand on the surface of a gold chip and measurement of changes in the refractive index at the chip surface, as a result of analyte binding [176-179]. The recently emerged surface acoustic wave (SAW) technology [180] is an alternative method of increasing popularity, due its high sensitivity, capability of simultaneous detection of mass and viscosity changes triggered by biomolecular interactions and its higher tolerance to various conditions of temperature, ion strength and viscosity compared to SPR [181, 182]. SAW biosensors (e.g., S-sens K5 Biosensor from SAW-Instruments, Bonn, Germany) are chip-based systems for detection and
quantification of affinity interactions, based on the conversion of a high frequency signal into a surface acoustic wave through an inverse piezoelectric effect. The velocity of the SAW is affected by changes in mass loading and viscosity caused by molecular interactions on the chip surface, which are analyzed as shifts in signal phase and amplitude, respectively (Figure 9). Various materials may be used for coating the quartz chips used with the SAW-biosensors, but gold and other noble metals are most often employed [182-184].

Figure 9  SAW biosensor setup and functioning principle. The SAW biosensor is operated by the driving electronics (7), which generates and detects the acoustic wave (4) by means of IDTs (interdigital transducers) (3) on the surface of a piezoelectric crystal (2). Analyte molecules (6) from the liquid sample (1) (flow direction is indicated by arrows) bind to the ligands (5) immobilized on the chip surface (e.g., antibodies). Changes in mass loading and viscosity due to the molecular interactions affect the SAW velocity and are rendered as shifts in phase (Δφ) and amplitude (ΔA) between the input and output (8) electrical signals. Reprinted from [180], copyright 2008, with kind permission from Springer Science and Business Media.

1.4  Scientific goals of the dissertation

Alzheimer’s disease is a fatal neurodegenerative disorder with a higher incidence rate among the elderly than all other types of dementia and no treatment available at present. Although the molecular mechanisms responsible for the onset and progress of AD have not been fully elucidated yet, there is a consensus regarding the essential role played by Aβ in the pathological processes underlying AD. Therefore, promising disease-modifying therapies consist in the administration of molecular agents able to inhibit Aβ fibrillization or/and disrupt preformed Aβ aggregates. Intrinsic factors that naturally contribute to Aβ clearance and to the
Introduction

Line of defense against Aβ neurotoxicity are of special interest, since their study may provide a better understanding of the yet unclear mechanisms triggering AD and also treatments with presumably minimal side effects.

This dissertation focuses on the molecular characterization of Humanin (HN) peptides and physiological Aβ-autoantibodies, both endogenous molecules with neuroprotective properties against Aβ toxicity, in view of their potential application in therapy or diagnosis of AD. The main goals of the dissertation can be summarized as follows:

- **Characterization of the Aβ-binding properties of Humanin peptides**
  The affinity of synthetic HN to Sepharose-immobilized or soluble Aβ(1-40) was shown by affinity-mass spectrometry and direct mass spectrometric measurements of the HN-Aβ(1-40) complex, respectively. Furthermore, ELISA and kinetic determinations using a SAW-biosensor were performed to assess the strength of the HN-Aβ(1-40) interaction and the influence of various HN mutations and modifications upon its binding to Aβ(1-40).

- **Identification of the binding epitopes between Humanin and Aβ(1-40)**
  For this purpose, mass spectrometric epitope-excision and -extraction approaches were employed, using affinity columns with Sepharose-coupled Aβ(1-40) or HN and the corresponding binding partner as analyte. Additionally, a molecular modeling study of the interaction structure between HN and Aβ(1-40) was performed.

- **Structural characterization of Humanin and Aβ epitope peptides and measurement of their binding affinities**
  The specificity and affinity of the interaction between Humanin epitope peptides and Aβ(1-40) or Aβ epitope sequence were assessed by affinity-mass spectrometry and SAW-biosensor measurements.
Introduction

- **Development of novel sandwich and indirect ELISA protocols for the determination of antigen-bound, free and total physiological Aβ-autoantibodies in human serum and CSF**
  The new ELISAs were designed on the basis of the epitope specificity of Aβ-autoantibodies. Optimization procedures were carried out regarding, e.g., concentration of the coating antibody, sample preparation protocol, composition of washing buffer, number of washing steps after analyte addition, concentration of the detection antibody. The optimization ELISAs were performed using a commercially available human IgG preparation and serum/CSF samples from healthy individuals.

- **Application of the new ELISAs for the determination of antigen-bound, free and total Aβ-autoantibodies in serum from healthy adults of various ages**
  ELISA measurements were carried out on 39 serum samples from healthy adults and the performance of the assays was assessed. To determine whether the levels of Aβ-autoantibodies represent an early indicator of an age-associated cognitive decline in healthy individuals, statistical analysis of the ELISA data for possible correlations with age and cognitive status of the study participants was subsequently performed and evaluated.

- **Determination of antigen-bound, free and total Aβ-autoantibodies in serum and CSF from AD patients and control subjects, using the newly designed ELISAs**
  The levels of antigen-bound Aβ-autoantibodies were measured in serum and CSF of a total number of 112 AD patients and age- and gender-matched control subjects. A smaller sample size was available for the determination of total Aβ-autoantibodies. The ELISA results were analyzed for correlations with the neuropsychological performance and age of the study participants and the diagnostic power of the Aβ-autoantibodies was assessed, in an effort to better understand their role and potential applications in AD.
2 RESULTS AND DISCUSSION

2.1 Synthesis and analytical characterization of neuroprotective peptide Humanin and its derivatives

2.1.1 Structure and biological function of Humanin peptides

Humanin is a linear 24 amino acids long peptide discovered during a functional expression screening of a cDNA library constructed from the intact occipital region of an AD brain. The study aimed to identify encoded neuroprotective factors able to inhibit neurotoxicity by V642I-APP [83]. HN has been shown to prevent the neuronal cell death caused in vitro by Aβ and familial AD-related genetic mutations of APP and presenilins [34, 82, 83]. Using immunoblotting with anti-HN antibodies, expression of HN in different mouse organs could be shown [86]. HN immunoreactivity was also detected in human AD brain, but only in traces in an age-matched control brain [86].

The structure-function relationship for the neuroprotective action of HN has been investigated in vitro on primary neuronal cultures. N- and C-terminal deletion studies and Ala-scan mutations indicated HN(3-19) as the minimal region required for HN activity, in which seven amino acid residues were found to be essential (Figure 10). Moreover, substitution of Cys-8 with Lys or Arg did not affect the neuroprotective properties of HN, while Ser14Gly-mutant (HNG) displayed a 1000-fold increase in neuroprotective efficacy compared to wild-type HN [34, 83, 185]. Presently, the most active HN derivative is Colivelin, composed of activity-dependent neurotrophic factor (ADNF) fused to the N-terminus of a HN(3-19) mutant [186].

Recent in vivo studies revealed that HNG treatment is able to decrease Aβ levels in the brain, reduce plaque burden and attenuate cognitive deficits of transgenic mouse models of AD [84, 85]. Colivelin was also found to prevent memory impairment and hippocampal neuronal loss caused by administration of Aβ in mice [186].
HN has been shown to be a secretory peptide with a putative binding site on the neuronal cell surface, as suggested by cross-linking experiments with radiolabeled HN [83]. A number of studies focused on identifying the HN receptor, as well as other possible HN-binding partners that mediate its anti-apoptotic activity. Thus, evidence has been obtained that HN protects neurons by interacting with a tripartite cytokine-like receptor complex [187] and participating in STAT3 transcription factor regulation through a tyrosine kinase pathway [188]. Other studies indicated intracellular binding partners such as apoptosis-inducing protein Bax [189] and insulin-like growth factor-binding protein-3 (IGFBP-3) [190]. Although HN does not affect the release of A\beta peptides from APP [34], it has been efficient against different pathways of A\beta neurotoxicity. Zou et al. [89] showed that HNG changes A\beta morphology from fibrillar to amorphous and reduces thereby A\beta-induced calcium influx in cultured rat hippocampal neurons, while Ying et al. [87] reported that HN reduces A\beta aggregation by suppressing its effect on mononuclear phagocytes. More recently, HNG was shown to inhibit A\beta fibrillization and disrupt preformed A\beta fibrils into amorphous aggregates, in a study employing Thioflavin T fluorescence assay and transmission electron microscopy [88].

This first part of the thesis is focused on the molecular characterization of HN peptides and the study of their interaction with A\beta(1-40), which may be of relevance for the development of AD-specific therapeutics. The results should contribute to clarify the role of HN in its neuroprotective function against A\beta neurotoxicity.
Results and discussion

Figure 10 Amino acid sequence and ribbon representation of the HN structure. The essential residues for the in vitro neuroprotection by HN according to Ala-scan data [34] are marked in red. The figure is based on solution NMR data (PDB file 1Y32, [191]) and was prepared using the program BallView 1.3.2 [192].

2.1.2 Synthesis and mass spectrometric structural characterization of Humanin peptides

HN peptides shown to protect neurons in vitro against toxic effects of Aβ (HN 1, HNG 3) or found inactive under the same experimental setting (HNA 2) [34], as well as new HN derivatives with either blocked (BG₅HNₐcm 8) or modified Cys (BG₅HNS 9) were synthesized as carboxyamides, using Fmoc/tert-butyl chemistry. Biotinylated sequences with an N-terminal penta-Gly spacer (see Table 1) were produced for use in ELISA and biosensor experiments.

MAPRG⁵FSCLL⁷LTTSE¹⁵IDLVP²⁰KRRA
Since HN peptides contain a hydrophobic middle domain that confers them a tendency to aggregate during chain elongation in solid-phase peptide synthesis (SPPS), resins with reduced loading capacities were chosen for their preparation (NovaSyn® TGR and Rink Amide MBHA). Also, protocols including double Fmoc-deprotection and coupling steps, as well as acetylation of unreacted amino-groups (capping) were applied, as described in the Experimental part.

The crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C₈ semipreparative column and characterized by MALDI-TOF or ESI-Ion Trap mass spectrometry. Analytical data of all synthesized HN peptides are included in Table 1. Analytical chromatograms and ESI-Ion Trap mass spectra of pure HN 1 and BG₅HN 5 are shown for exemplification in Figure 11.

Table 1 Amino acid sequences and structural characterization of synthetic HN peptides

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Code</th>
<th>Sequence</th>
<th>HPLC Rt (min)ᵃ</th>
<th>[M+H]⁺ exp./calc.ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HN</td>
<td>MAPRGFSC₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>23.32</td>
<td>2687.05/2687.28</td>
</tr>
<tr>
<td>2</td>
<td>HNA</td>
<td>MAPRGFSA₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>23.73</td>
<td>2655.1/2655.2</td>
</tr>
<tr>
<td>3</td>
<td>HNG</td>
<td>MAPRGFSC₈LLLLTG¹⁴EIDLPVKRRA</td>
<td>23.46</td>
<td>2657.5/2657.3</td>
</tr>
<tr>
<td>4</td>
<td>G₂HN</td>
<td>(G)₂MAPRGFSC₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>24.73</td>
<td>2972.1/2972.5</td>
</tr>
<tr>
<td>5</td>
<td>BG₂HN</td>
<td>Biotin-(G)₂MAPRGFSC₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>25.83</td>
<td>3198.68/3198.84</td>
</tr>
<tr>
<td>6</td>
<td>BG₂HNA</td>
<td>Biotin-(G)₂MAPRGFSA₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>26.24</td>
<td>3166.7/3166.8</td>
</tr>
<tr>
<td>7</td>
<td>BG₂HNG</td>
<td>Biotin-(G)₂MAPRGFSC₈LLLLTG¹⁴EIDLPVKRRA</td>
<td>25.97</td>
<td>3168.8/3168.8</td>
</tr>
<tr>
<td>8</td>
<td>BG₂HNAcm</td>
<td>Biotin-(G)₂MAPRGFSC₈(Acm)LLLLTS¹⁴EIDLPVKRRA</td>
<td>24.98</td>
<td>3269.6/3269.9</td>
</tr>
<tr>
<td>9</td>
<td>BG₂HNS</td>
<td>Biotin-(G)₂MAPRGFSS₈LLLLTS¹⁴EIDLPVKRRA</td>
<td>23.98</td>
<td>3182.7/3182.8</td>
</tr>
</tbody>
</table>

ᵃ RP-HPLC column: Vydac C₈; Flow rate: 1mL/min; Gradient: 30%-60% eluent B in 30 min;ᵇ MALDI-TOF MS; c ESI-Ion Trap MS; Acm – Acetamidomethyl
2.1.3 Analysis of Humanin binding to neuronal cell surface

There is evidence indicating that Aβ toxicity may be mediated by its interaction with neuronal membrane proteins, notably APP [193]. In the light of HN affinity for Aβ peptide(s) (which will be discussed in detail in the following chapters), APP could represent another membrane receptor of HN, which competes with Aβ for the APP binding site and inhibits Aβ-related toxic effects.
Results and discussion

First studies have been performed on the possible interaction of HN with the extracellular juxtamembrane Aβ(17-28) sequence of the membrane-anchored amyloid precursor protein in neuronal cells [192]. Fluorescence-staining indicated a concentration-dependent membrane attachment of synthetic BG₅HNG ⁷, correlated with neuronal maturation (Figure 12). Immunofluorescence staining of endogenous APP provided the visualization of the protein expression at an earlier date than the observed HN-membrane binding (Figure A2a, Appendix 2), while first FRET studies indicated no close proximity between BG₅HNG ⁷ and fluorescence-tagged APP (Figure A2b, Appendix 2).

![Image of neuronal cells with fluorescent staining](image)

![Bar chart showing colocalization of BG₅HNG with MAP2](chart)
Figure 12  (a) Fluorescence staining of microtubule-associated protein 2 (MAP2) in mouse hippocampal neurons (DIV 23), using chicken anti-MAP2 antibody and Alexa-647-conjugated anti-chicken antibody; (b) Fluorescence staining of BG\textsubscript{5HNG} 7 in mouse hippocampal neurons (DIV 23) with Alexa-488-conjugated streptavidin; (c) (a+b) merged images, with additional use of 4',6-diamidino-2-phenylindole (DAPI); (d) \textit{In vitro} colocalization of BG\textsubscript{5HNG} 7 with MAP2 in mouse hippocampal neurons of increasing age (DIV); (e) Concentration-dependent \textit{in vitro} colocalization of BG\textsubscript{5HNG} 7 with MAP2 in mouse hippocampal neurons (DIV 23); DIV – days \textit{in vitro} [192]
2.2 Elucidation of the interaction structure between Humanin and β-amyloid

2.2.1 Specificity and affinity of the Humanin-Aβ interaction

2.2.1.1 Study of Humanin-Aβ(1-40) binding by affinity-mass spectrometry

In order to evaluate the specificity of the HN interaction with Aβ, Aβ(1-40) was immobilized on an NHS-activated Sepharose matrix and exposed to an equimolar mixture of HN and neurotensin (control peptide). The MALDI-TOF mass spectrum of the supernatant from the column showed only molecular ions of the control peptide (Figure 13a). No ions were detectable in the final washing fraction, while the spectrum of the elution fraction contained exclusively the protonated and sodiated singly-charged ions of HN (Figure 13b), thus confirming the specificity of the HN-Aβ(1-40) interaction [192].

Figure 13 MALDI-TOF affinity-mass spectra of HN binding to Sepharose-immobilized Aβ(1-40), using neurotensin as control. (a) The supernatant contains only the control peptide, m/z 1673.9; (b) HN binds specifically to the Aβ(1-40)-affinity column, being the only peptide present in the elution fraction (m/z 2686.8) [192].

![MALDI-TOF spectra](image-url)
2.2.1.2 Direct analysis of the Humanin-Aβ(1-40) complex using high resolution mass spectrometry

To ascertain the assumed stoichiometry and presence of a single interaction site, high resolution ESI-FTICR mass spectrometry was employed for direct molecular characterization of the HN-Aβ(1-40) complex (Figure 14) [192]. ESI-mass spectrometry as a “soft” ionization technique has been applied in the last years in many studies for the characterization of non-covalent biopolymer complexes [141, 151, 152]. A prerequisite for the successful application is the careful optimization of “native” solution conditions and mass spectrometric parameters, to minimize dissociation of the complex and allow its ionization and transfer into the analyzer cell [151-153]. The Ala-mutant peptide BG5HNA 6 was used instead of wild-type HN, because the Cys8Ala replacement provides higher binding affinity to Aβ(1-40) (see Figure 17) and prevents possible disulfide bridge formation, which may compete with the formation of the HN-Aβ(1-40) complex. A small extent of disulfide dimerization was observed in wild-type HN upon incubation in PBS (see Figure A3 in Appendix 3).

Analysis of a dilution series of BG5HNA 6 in 0.5 mM ammonium acetate (pH 6) provided 25 µM as the minimal peptide concentration yielding a good signal to noise ratio in positive ion nano-ESI-FTICR mass spectrometry. To determine the highest relative amount of the complex, a range of peptide 6:Aβ(1-40) concentration ratios was evaluated and revealed a 2-fold molar excess of the more acidic Aβ to be best suited; in contrast, an excess of the more basic HN peptide may suppress ionization of the less basic Aβ-HN complex.
Results and discussion

Figure 14  Nano-ESI-FTICR mass spectrum of the 1:1 complex of Aβ(1-40) (50 µM) and BG5HNA 6 (25 µM) in 0.5 mM ammonium acetate (pH 6). The isotopic distribution of the (5+) molecular ion of the Aβ(1-40)-HN complex is shown in the insert [192].

2.2.1.3 Binding affinities of Humanin peptides to Aβ(1-40) measured with a surface acoustic wave biosensor

A quantitative determination of the HN-Aβ(1-40) interaction was performed by SAW-bioaffinity analysis [194], which has been recently emerging as an effective biosensor system and alternative to surface plasmon resonance. Aβ(1-40) was immobilized on an NHS-activated gold chip, as described in the Experimental part, and increasing concentrations of wild-type BG5HN 5 in PBS were injected. The shifts in the signal’s phase corresponding to different analyte dilutions were fitted according to a 1:1-Langmuir binding model and the calculated observed rate constants \( k_{obs} \) were plotted as a function of the applied concentrations, which provided an equilibrium dissociation constant (\( K_D \)) of 0.61 µM (Figure 15) [192]. The biosensor quantification results were in good agreement with the ELISA determinations (see Figure 17).
2.2.1.4 Affinity characterization of the Humanin-Aβ(1-40) complex by ELISA

The interaction affinities of wild-type HN and HN variants to Aβ(1-40) were further characterized by indirect ELISA, using HN sequences elongated with a penta-Gly spacer and biotinylated at the N-terminus, to ensure optimal exposure of the HN epitope to Aβ(1-40) during incubation and detection with horseradish peroxidase (HRP)-conjugated anti-biotin antibody (Figure 16).

Figure 16 Schematic representation of the indirect ELISA protocol applied for testing the affinities of wild-type HN and HN variants to Aβ(1-40); OPD – o-phenylenediamine dihydrochloride; PBST – 0.1% Tween-20 in PBS; BSAT – 5% BSA in PBST
Results and discussion

Based on the ELISA data, the effect of HN sequence mutations on the Aβ(1-40) recognition could be assessed (Figure 17). The Cys8Ala-variant BG₅HNA 6 exhibited the highest affinity to Aβ(1-40). The wild-type peptide BG₅HN 5 and the Ser14Gly-mutant (BG₅HNG 7) had similar binding levels to Aβ(1-40) as 6 at low concentrations, but showed binding saturation at concentrations > 0.33 µM. The two HN derivatives in which Cys-8 was alkylated by Acm (BG₅HN_Acm 8) or substituted with Ser (BG₅HNS 9) revealed significantly lower affinity to Aβ(1-40) than the wild-type peptide 5. The results are consistent with Cys-8 and adjacent residues being part of the HN epitope, in agreement with the mass spectrometric results presented in the following chapters, since replacement of Cys-8 with the more polar Ser residue (peptide 9) or introduction of the Acm-group in 8 may disturb the mainly hydrophobic interaction structure between HN and Aβ [192].

![Figure 17](image)

**Figure 17** Comparison between the binding affinities of HN and HN mutants to Aβ(1-40) by ELISA; OD – optical density [192]

### 2.2.2 Identification of the Humanin epitope recognized by Aβ

#### 2.2.2.1 Mass spectrometric epitope-extraction and -excision of Humanin using various proteases

To identify the epitope sequence of HN involved in Aβ(1-40) binding, mass spectrometric epitope-extraction and -excision were employed, using an Aβ(1-40)-
Results and discussion

affinity column and Glu-C, trypsin and chymotrypsin proteases [192]. In a first epitope-extraction experiment, HN was digested with Glu-C in solution and the resulting peptide fragment mixture subjected to interaction with the Sepharose-immobilized Aβ(1-40). The mass spectrometric analysis revealed the non-binding C-terminal fragment HN(16-24) in the supernatant fraction (Figure 18a), while in the elution fraction only the peptide HN(1-15) was found (Figure 18b), indicating the epitope localization within this N-terminal sequence.

![Figure 18](image)

**Figure 18** MALDI-FTICR mass spectrum of (a) the supernatant fraction obtained after digestion of HN with Glu-C protease and addition of the fragment mixture on the Aβ(1-40)-affinity column (proteolytic extraction), showing the non-binding peptide HN(16-24). The Glu-C cleavage site is indicated by an arrow; n.i. – not identified; (b) the elution fraction after proteolytic extraction of HN with Glu-C, showing the binding sequence HN(1-15) (underlined) [192]

In a following proteolytic excision experiment, HN was first bound to the Aβ(1-40) column and then subjected to tryptic cleavage. MALDI-FTICR mass spectrometry
Results and discussion

of the elution fraction (Figure 19a) provided molecular ions [M+H]$^+$ of the HN peptides (5-21), (5-22) and (1-21), covering the amino acid sequence (5-21). This result was confirmed by epitope-extraction of HN with trypsin (Figure 19b), which showed in the elution fraction the overlapping partial tryptic fragments (5-21), (1-21), (1-22) and (1-23). Combining the results of the proteolytic extraction and excision experiments, the HN epitope could be assigned to HN(5-15).

Figure 19 MALDI-FTICR mass spectrum of (a) the elution fraction obtained after proteolytic excision of HN with trypsin. The trypsin cleavage sites are indicated by arrows and the HN(5-21) epitope sequence is underlined; (b) the elution fraction after proteolytic extraction of HN with trypsin. The HN(5-21) epitope is underlined [192].

Additional confirmation was provided by an extraction experiment with chymotrypsin, which produced only the supernatant, non-binding HN fragments (1-
Results and discussion

6), (13-22), (12-22), (13-24), (12-24), (11-24), (10-24), by cleavages at Leu and Phe residues within the epitope (Figure 20).

![Figure 20 MALDI-FTICR mass spectrum of (a) the supernatant fraction obtained after digestion of HN with chymotrypsin and addition of the fragment mixture on the Aβ(1-40)-affinity column (proteolytic extraction), showing non-binding peptides. The chymotrypsin cleavage sites are indicated by arrows; (b) the elution fraction obtained after proteolytic extraction of HN with chymotrypsin. The protease cleaved inside the HN(5-15) epitope at Phe-6, Leu-9, Leu-10, Leu-11 and Leu-12, therefore no Aβ-bound chymotryptic fragments of HN are observed in the elution fraction [192].

A summary of the HN epitope fragments found in the elution fractions from the proteolytic experiments with Glu-C protease, trypsin and chymotrypsin is shown in Table 2. In conclusion, the interacting region of HN with Aβ(1-40) was determined by proteolytic extraction- and excision-mass spectrometry to be HN(5-15), within the (3-19) “core” domain comprising the essential residues for in vitro neuroprotective effect [34].
Results and discussion

Table 2  Mass spectrometric epitope-extraction/-excision data for HN [192]

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protease</th>
<th>Eluted fragments</th>
<th>[M+H]^+ exp. a</th>
<th>[M+H]^+ calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Trypsin</td>
<td>5-21</td>
<td>1847.9354</td>
<td>1848.0192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-21</td>
<td>2303.2608</td>
<td>2303.2507</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-22</td>
<td>2459.3343</td>
<td>2459.3518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-23</td>
<td>2615.3926</td>
<td>2615.4529</td>
</tr>
<tr>
<td>Extraction</td>
<td>Glu-C</td>
<td>1-15</td>
<td>1637.8341</td>
<td>1637.8395</td>
</tr>
<tr>
<td>Extraction</td>
<td>Chymotrypsin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Excision</td>
<td>Trypsin</td>
<td>5-21</td>
<td>1848.0098</td>
<td>1848.0192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-22</td>
<td>2004.1062</td>
<td>2004.1203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-21</td>
<td>2303.2691</td>
<td>2303.2507</td>
</tr>
</tbody>
</table>

a MALDI-FTICR MS

2.2.2.2 Synthesis and mass spectrometric characterization of Humanin epitope peptides

Two elongated variants of the HN(5-15) epitope were chosen for synthesis and use in subsequent affinity studies (see Table 3). The longer epitope peptide HN(3-19) was advantageous to avoid solubility problems due to the strong hydrophobicity of HN(5-15). To further increase the hydrophilicity of the epitope sequence, a HN(3-19) mutant having Cys-8 replaced by Lys (HN(3-19)C8K) was also prepared. The neuroprotective efficacy of both peptide sequences in neuronal cell lines has been previously described to be similar to that observed for wild-type HN [34].

The peptides were obtained as carboxamides by SPPS according to Fmoc/tert-butyl strategy, using a low-capacity Rink Amide MBHA resin to prevent chain aggregation during the process. The same protocol applied for the synthesis of wild-type HN and HN mutants was employed, which included double Fmoc-deprotection steps, double coupling for each amino acid and capping of unreacted amino groups. The peptide sequences to be used for kinetic determinations (13
and 14) were prepared from 11 and 12 by elongation of the N-terminus with a penta-Gly spacer and subsequent biotinylation.

All crude peptides were subjected to RP-HPLC purification on a semipreparative C\textsubscript{8} column and the identity of the desired sequences was confirmed by mass spectrometry. The structural characteristics of the HN epitope sequences are summarized in Table 3.

**Table 3** Amino acid sequences and structural characterization of synthetic HN epitope peptides

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Code</th>
<th>Sequence</th>
<th>HPLC Rt (min)</th>
<th>[M+H]\textsuperscript{+} exp./calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>HN(3-19)</td>
<td>H-PRGFSC\textsubscript{8}LLLLTSEIDLP-NH\textsubscript{2}</td>
<td>17.74</td>
<td>1873.0498/1873.0252 \textsuperscript{b}</td>
</tr>
<tr>
<td>12</td>
<td>HN(3-19)C8K</td>
<td>H-PRGFSC\textsubscript{8}LLLLTSEIDLP-NH\textsubscript{2}</td>
<td>18.00</td>
<td>1897.5922/1898.1110 \textsuperscript{b}</td>
</tr>
<tr>
<td>13</td>
<td>BG\textsubscript{3}HN(3-19)</td>
<td>Biotin-(G)\textsubscript{5}PRGFSC\textsubscript{8}LLLLTSEIDLP-NH\textsubscript{2}</td>
<td>20.14</td>
<td>2385.7/2385.8 \textsuperscript{c}</td>
</tr>
<tr>
<td>14</td>
<td>BG\textsubscript{3}HN(3-19)C8K</td>
<td>Biotin-(G)\textsubscript{5}PRGFSC\textsubscript{8}LLLLTSEIDLP-NH\textsubscript{2}</td>
<td>20.51</td>
<td>2410.8/2410.8 \textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} RP-HPLC column: Vydac C\textsubscript{8}; Flow rate: 1mL/min; Gradient: 30%-60% eluent B in 30 min; \textsuperscript{b} ESI-FTICR MS; \textsuperscript{c} ESI-Ion Trap MS

The ESI-FTICR MS spectra acquired for the pure epitope peptides 11 and 12 are shown as examples in Figure 21. Double and triple-charged molecular ions could be identified for both peptides and the m/z values corresponding to the highest signals were used to calculate with high accuracy the molecular weights of the compounds.
2.2.2.3 Binding studies of Humanin epitope peptides to Aβ(1-40)

To ascertain the binding of the elongated HN(3-19) \textsuperscript{11} epitope sequence to Aβ, an affinity-MS experiment was performed. 10 µg of synthetic peptide \textsuperscript{11} was dissolved in PBS and incubated with Sepharose-immobilized Aβ(1-40) for 2 h. The affinity column was then extensively washed with PBS and the non-covalently bound molecules released by addition of aqueous 0.1% TFA. The last washing fraction, as well as the elution fraction from this experiment were lyophilized and measured by MALDI-TOF mass spectrometry.
While the mass spectrum of the final washing fraction contained only background ions (Figure 22a), analysis of the elution fraction revealed a signal at m/z 1874.3, corresponding to the singly-charged molecular ion \([\text{M+H}]^+\) of HN(3-19) 11 (Figure 22b). Thus, the affinity of HN(3-19) 11 to A\(\beta\)(1-40) was confirmed, supporting the epitope determination results.

Figure 22  HN(3-19) 11 affinity binding to Sepharose-immobilized A\(\beta\)(1-40). (a) MALDI-TOF MS of the last washing fraction; (b) MALDI-TOF MS of the elution fraction

A similar affinity-MS experiment was carried out using HN(3-19)C8K 12 as analyte. The resulting mass spectrometric data also indicated specific binding of peptide 12 to the A\(\beta\)(1-40)-column (Figure A4 in Appendix 4).

To allow a quantitative assessment of the influence that Cys-8 replacement by Lys within HN(3-19) sequence has on the interaction with A\(\beta\)(1-40), as well as a comparison of binding affinities to A\(\beta\)(1-40) between HN epitope peptides and the parent HN molecule, kinetic measurements were performed using the SAW-biosensor. For this purpose, A\(\beta\)(1-40) was covalently attached to the biosensor chip surface, according to the protocol detailed in the Experimental part. In brief, a self-assembled monolayer (SAM) composed of 16-mercaptophexadecanoic acid
chains was allowed to form on the gold surface of the chip and the carboxyl groups were activated to ester form by treatment with a mixture of NHS and EDC in PBS. Next, Aβ(1-40) was immobilized via available primary amino groups and the unreacted ester groups of the linker were blocked with ethanolamine.

To measure the equilibrium dissociation constants (K_D), increasing concentrations of biotinylated HN epitopes 13 and 14 in PBS were injected over the Aβ(1-40) molecules covalently attached to the biosensor chip surface. After each binding, regeneration of the surface was performed with ACN:0.1% aqueous TFA (2:1). For the determination of the association kinetics between the peptide analytes and the Aβ(1-40) ligand, data were extracted from the sensor signals for all concentrations using a monomolecular growth model. To determine the corresponding dissociation kinetics, an exponential decay model was employed. Finally, the calculated rate constants (k_obs) were plotted as a function of the concentrations employed and linear regression was applied, providing a K_D of 2.17 µM for BG5HN(3-19) 13 and 2.59 µM for BG5HN(3-19)C8K 14 (Figure 23).

In conclusion, the epitope peptides BG5HN(3-19) 13 and BG5HN(3-19)C8K 14 showed similar binding affinities to Aβ(1-40), but lower compared to the complete sequence BG5HN 5, for which a K_D of 0.61 µM has been determined (see Figure 15). This finding might indicate a contribution to HN-Aβ interaction of amino acid residues outside HN(3-19) region, which are not essential for the binding, but could help stabilize the peptide complex. Another possibility is that synthetic HN epitope peptides adopt in PBS conformations less favorable for Aβ-binding than the conformation of the parent HN molecule.
2.2.3 Identification of the Aβ epitope recognized by Humanin

2.2.3.1 Mass spectrometric epitope-extraction and -excision of Aβ(1-40) using various proteases

For the identification of the Aβ epitope recognized by HN, proteolytic extraction and excision in combination with MS were applied, using a HN-affinity column and trypsin, Glu-C, Asp-N and chymotrypsin as proteases [192]. The mass spectrum of
Results and discussion

the supernatant fraction upon tryptic digestion of Aβ(1-40) in solution and subsequent affinity selection of the proteolytic peptides on Sepharose-immobilized HN revealed the non-binding fragments Aβ(1-16) and Aβ(6-16), thus excluding the N-terminal region as part of the epitope (Figure 24a). The corresponding elution fraction contained four overlapping peptides covering the sequence Aβ(17-28), which was assigned as the binding region to HN (Figure 24b).

![Supernatant and Elution Mass Spectra](image)

Figure 24 MALDI-FTICR mass spectrum of (a) the supernatant fraction after addition of trypsin-digested Aβ(1-40) on the HN-affinity column (proteolytic extraction). The trypsin cleavage sites are indicated by arrows; (b) the elution fraction after addition of trypsin-digested Aβ(1-40) on the HN-affinity column (proteolytic extraction). The trypsin cleavage sites are indicated by arrows and the minimal epitope sequence Aβ(17-28) is underlined; n.i. – not identified [192]
This epitope was confirmed by proteolytic excision of HN-bound Aβ(1-40) with trypsin, which provided only Aβ(6-40) and intact Aβ(1-40) in the elution fraction, indicating that residues Lys-16 and Lys-28, although accessible for cleavage in solution, were shielded in the HN-Aβ(1-40) complex against proteolysis (Figure 25).

**Figure 25** MALDI-TOF mass spectrum of (a) the fragment peptides obtained after tryptic digestion of free Aβ(1-40) in solution. The trypsin cleavage sites are indicated by arrows; (b) the elution fraction obtained after proteolytic excision of Aβ(1-40) with trypsin. The Lys-16 and Lys-28 residues, shielded from tryptic digestion in the Aβ(1-40)-HN complex, are indicated by crossed arrows and the Aβ(6-40) epitope sequence is underlined [192].
Studies using additional proteases produced complementary results that ascertained the Aβ epitope. An extraction experiment with Glu-C provided the non-binding fragments Aβ(4-11) and Aβ(12-22) in the supernatant fraction, thus excluding the epitope location within these N-terminal sequences (Figure 26a). The corresponding elution fraction contained peptides overlapping Aβ(12-40) (Figure 26b).

![Figure 26](image.png)

**Figure 26** MALDI-TOF mass spectrum of (a) the supernatant fraction obtained after digestion of Aβ(1-40) with Glu-C protease and addition of the fragment mixture on the HN-affinity column (proteolytic extraction), showing the non-binding peptides Aβ(4-11) and Aβ(12-22). The Glu C-cleavage sites are indicated by arrows. The signals below 800 m/z are produced by matrix ions; (b) the elution fraction obtained after proteolytic extraction of Aβ(1-40) with Glu-C. The Aβ(12-40) epitope is underlined. The signals below 1700 m/z are produced by matrix ions [192].
Proteolytic excision of HN-bound Aβ with Asp-N protease showed Aβ(7-40) as the only epitope fragment in the elution (Figure 27b). Shielding from cleavage at Asp-23 was observed, although the residue was accessible for proteolysis in solution (Figure 27a).

Further, epitope-excision with chymotryptsin revealed shielding of Leu-17, Phe-19 and Phe-20 upon HN binding, while Phe-4, Tyr-10, His-13 and Ala-30 were amenable to digestion (Figure 28).
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Figure 28 MALDI-TOF mass spectrum of (a) the fragment peptides obtained after in solution digestion of free Aβ(1-40) with chymotrypsin. The chymotrypsin cleavage sites are indicated by arrows; (b) the elution fraction obtained after proteolytic excision of Aβ(1-40) with chymotrypsin. Leu-17, Phe-19 and Phe-20, shielded from chymotryptic digestion in the Aβ(1-40)-HN complex, are indicated by crossed arrows [192].

The molecular ions found in the elution fractions from all the described proteolytic experiments are summarized in Table 4. The conclusion consistent with all proteolytic results is that HN interacts with the Aβ(17-28) sequence. Furthermore, residues Lys-16, Leu-17, Phe-19, Phe-20, Asp-23 and Lys-28 are all protected by HN binding against proteolytic cleavage, which suggests their involvement in the HN-Aβ(1-40) complex.
Results and discussion

Table 4 Mass spectrometric epitope-extraction/-excision data for Aβ(1-40) [192]

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protease</th>
<th>Eluted fragments</th>
<th>[M+H]^+ exp. a,b</th>
<th>[M+H]^+ calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Trypsin</td>
<td>17-28</td>
<td>1325.6851 a</td>
<td>1325.6741</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-28</td>
<td>2643.3048</td>
<td>2643.2592</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-40</td>
<td>3708.8933</td>
<td>3708.8960</td>
</tr>
<tr>
<td>Extraction</td>
<td>Glu-C</td>
<td>4-40</td>
<td>4014.6 b</td>
<td>4014.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12-40</td>
<td>3022.6</td>
<td>3022.6</td>
</tr>
<tr>
<td>Excision</td>
<td>Trypsin</td>
<td>6-40</td>
<td>3711.2 b</td>
<td>3711.2</td>
</tr>
<tr>
<td>Excision</td>
<td>Asp-N</td>
<td>7-40</td>
<td>3573.8 b</td>
<td>3574.1</td>
</tr>
<tr>
<td>Excision</td>
<td>Chymotrypsin</td>
<td>14-30</td>
<td>1848.8 b</td>
<td>1848.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-40</td>
<td>3152.1</td>
<td>3151.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-40</td>
<td>3867.1</td>
<td>3867.4</td>
</tr>
</tbody>
</table>

a MALDI-FTICR MS; b MALDI-TOF MS

Aβ region (17-28) was shown to be critically important for the aggregation of Aβ. Thus, the Aβ(12-24) sequence is involved in parallel β-sheet formation in fibrils [195, 196], while residues (17-21) generate side-chain interactions and dimerization of Aβ [197]. The importance of the Aβ(16-22) sequence for assembly has been shown by single site mutations [32, 198, 199]. Moreover, the sequence Aβ(25-35) is essential for oligomerization and fibril formation [200]. Therefore, due to its interaction with residues (17-28) of monomeric Aβ(1-40), HN could prevent Aβ oligomerization, fibrillization and subsequent toxicity [192].

2.2.3.2 Synthesis and mass spectrometric characterization of Aβ epitope peptides

After the mass spectrometric identification of the Aβ(17-28) epitope, a further goal of this dissertation was to characterize the binding specificity and strength of synthetic Aβ(1-28) to HN using affinity-mass spectrometry, ELISA and SAW-bioaffinity determinations. For this purpose, Aβ(1-28) and other Aβ sequences of
various lengths (see Table 5) were prepared as carboxamides on NovaSyn® TGR resin by SPPS, according to Fmoc/tert-butyl chemistry. Since Aβ regions (17-21) and (30-40) are highly hydrophobic, for the peptides spanning this domains a synthesis protocol optimized to contain double Fmoc-deprotection and amino acid coupling steps, as well as capping of unreacted amino groups with acetic anhydride was employed. For the preparation of BG5Aβ(1-16) 18 and BG5Aβ(20-30) 21, single Fmoc-deprotection and double coupling steps were considered adequate.

After completion of the synthesis, the Aβ peptides were cleaved from the resin and precipitated in diethyl ether, as described in the Experimental part. Following resolubilization and liophylization of the peptides, RP-HPLC was carried out on a C4 or a C18 semipreparative column, depending on the sequence hydrophobicity. Purity of the final compounds was assessed by analytical RP-HPLC and mass spectrometry. The structural characteristics of the synthetic Aβ peptides are given in Table 5. Analytical chromatograms together with ESI-Ion Trap and MALDI-FTICR mass spectra of pure BG5Aβ(17-28) 19 and BG5Aβ(12-40) 17 are shown for exemplification in Figure 29.

Table 5 Amino acid sequences and structural characterization of synthetic Aβ peptides

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Code</th>
<th>Sequence</th>
<th>HPLC R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt; exp./calc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Aβ(17-28)</td>
<td>LVFFAEDVGSNK</td>
<td>28.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1325.4/1325.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>BG5Aβ(1-40)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;DAEFRHDSGYEVHHQKLVFFAEDVGSNK</td>
<td>33.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4841.2/4841.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>BG5Aβ(12-40)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;VHHQKLVFFAEDVGSNKGAIIGLMVGGVV</td>
<td>33.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3531.85/3531.83&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>BG5Aβ(1-16)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;DAEFRHDSGYEVHHQK</td>
<td>21.9&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2466.2/2466.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>19</td>
<td>BG5Aβ(17-28)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;LVFFAEDVGSNK</td>
<td>30.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1835.79/1835.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>BG5Aβ(17-36)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;LVFFAEDVGSNKGAIIGLMV</td>
<td>33.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2592.9/2592.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>BG5Aβ(20-30)</td>
<td>Biotin-(G)&lt;sub&gt;5&lt;/sub&gt;FAEDVGSNKGA</td>
<td>25.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1605.0/1605.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flow rate: 1mL/min; Gradient: 0%-90% eluent B in 45 min; * RP-HPLC column: Vydac C4; ** RP-HPLC column: Vydac C18; † MALDI-TOF MS; ‡ ESI-Ion Trap MS; ‡ MALDI-FTICR MS
2.2.3.3 Analysis of Aβ(17-28) epitope binding to Humanin and Humanin epitope peptides

To confirm the specific binding of synthetic epitope peptide Aβ(17-28) 15 to HN, an affinity-MS experiment was performed first. 10 µg of Aβ(17-28) were dissolved in PBS and allowed to interact with Sepharose-linked HN for 2 h. The affinity column was then washed with 60 mL PBS, bound molecules were eluted with 0.1% aqueous TFA and the collected samples subjected to mass spectrometric analysis.
No peptide ions could be detected in the last washing fraction (Figure 30a), while the mass spectrum of the elution showed a signal at m/z 1325.0, corresponding to the molecular ion [M+H]^+ of Aβ(17-28) (Figure 30b).

![MALDI-TOF mass spectrum](image.png)

**Figure 30** Binding of Aβ(17-28) 15 to Sepharose-immobilized HN, studied by affinity-mass spectrometry. (a) MALDI-TOF mass spectrum of the last washing fraction, in which only matrix ions are detectable; (b) MALDI-TOF mass spectrum of the elution fraction. Unlabeled signals are produced by matrix ions.

An indirect ELISA was performed next, to assess the binding affinities of Aβ sequences encompassing the (17-28) region to HN [192]. A similar protocol to the one previously applied to characterize the interaction of wild-type HN and HN variants with Aβ(1-40) was employed. In brief, G5HN 4 was adsorbed on 96-well microtiter plates, followed by blocking of non-specific binding sites with 5% BSA in
PBST (BSAT) and addition of 8-dilutions series of biotinylated Aβ peptides. For detection, a HRP-conjugated goat anti-biotin antibody was used. The ELISA curves obtained by plotting optical density values (OD) vs. peptide concentrations is shown in Figure 31.

![Figure 31](image_url)

**Figure 31** Comparison between the binding affinities of Aβ(1-40) and Aβ partial sequences to HN by indirect ELISA [192]

All Aβ peptides comprising the (17-28) epitope bound to HN, consistent with the proteolytic-MS data. BG5Aβ(1-16) 18 and BG5Aβ(20-30) 21, which lack the epitope sequence, showed no affinity to HN.

SAW-bioaffinity analyses were subsequently performed for quantitative determination of the interactions between HN epitope peptides BG5HN(3-19) 13 and BG5HN(3-19)C8K 14 and the Aβ(17-28) epitope. For this purpose, Aβ(17-28) 15 was covalently attached to the biosensor chip surface, according to the protocol described in the Experimental part and increasing concentrations of biotinylated HN epitopes 13 and 14 in PBS were injected. After each binding, regeneration of the surface was performed with ACN:0.1% aqueous TFA (2:1).

The shifts in the signal’s phase corresponding to different analyte dilutions were fitted according to a 1:1-Langmuir binding model and the calculated rate constants $k_{obs}$ were plotted as a function of the applied concentrations, which provided an
equilibrium dissociation constant ($K_D$) of 1.86 µM for BG$_5$HN(3-19) 13 and 1.95 µM for BG$_5$HN(3-19)C8K 14 (Figure 32). These $K_D$ values are similar to the ones previously obtained for the binding of HN epitope peptides 13 and 14 to Aβ(1-40) (2.17 µM for BG$_5$HN(3-19) 13 and 2.59 µM for BG$_5$HN(3-19)C8K 14; see Figure 23).

\[ K_D = \frac{k_{off}}{k_{on}} = 1.86 \pm 0.93 \text{ µM} \]

\[ R^2 = 0.97 \]

\[ K_D = 1.95 \text{ µM} \]

**Figure 32** Binding and dissociation curves and $K_D$ determination of the complex between immobilized Aβ(17-28) and (a) epitope peptide BG$_5$HN(3-19) 13; (b) epitope peptide BG$_5$HN(3-19)C8K 14
2.2.4 Structure modeling of the Humanin-\(\text{A}\beta(1-40)\) complex

Theoretical methods based on the Rosetta Protein Structure Prediction and Design Suite [201, 202] were used to obtain structural information on the interaction between HN and \(\text{A}\beta(1-40)\) [192]. Using the available NMR structures of both peptides (HN – PDB entry 1Y32; \(\text{A}\beta(1-40)\) – PDB entry 1AML), the RosettaDock tool [203] was employed in a first approach for rigid body positioning of the docking partners and simultaneous optimization of side-chain conformations. Of the 10,000 structures thus generated, none showed a reasonable binding energy upon evaluation with Rosetta energy function. A possible explanation is that HN and \(\text{A}\beta(1-40)\), possessing highly flexible structures, adapt to each other upon complex formation; therefore, a prediction model for their association by rigid docking seems unlikely to be successful.

In a second approach, the best structures from the 5 lowest-energy clusters of the Rosetta calculations were selected for molecular dynamics (MD) simulations. These HN-A\(\text{A}\beta(1-40)\) complexes are illustrated in Figure 33 and corresponding binding energies are shown in Table 6.

<table>
<thead>
<tr>
<th>Energy Function</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosetta</td>
<td>-111.29</td>
<td>-110.55</td>
<td>-110.29</td>
<td>-108.74</td>
<td>-108.28</td>
</tr>
<tr>
<td>MM-GBSA</td>
<td>-30.88</td>
<td>-27.98</td>
<td>-29.07</td>
<td>-58.59</td>
<td>-38.11</td>
</tr>
</tbody>
</table>

Table 6 Binding energies of the best structures from the 5 lowest-energy clusters according to the Rosetta scoring function (arbitrary units) and the MM-GBSA approach (kcal/mol) [192]
Results and discussion

Figure 33  Structure representations of the *ab initio* Rosetta simulations of HN (top left, in red) and Aβ(1-40) (top left, in blue), together with the 5 selected complexes (a) before MD and (b) after MD. The side-chains of the amino acids (5-15) from HN and (17-28) from Aβ are shown [192].
According to the MD results, hydrogen bonding, hydrophobic and electrostatic interactions take place upon HN-Aβ(1-40) association. The most stable complex (C4, Figure 34) showed the Phe-6 residue of HN involved in hydrogen bonding with Phe-19 and in a parallel π-overlap with Phe-20 of Aβ(1-40); Van der Waals contacts are present between Phe-6 of HN and Val-18 of Aβ(1-40), as well as between Ser-7 of HN and Leu-17 and Phe-19 residues of Aβ(1-40). These interactions are consistent with the shielding effects observed in the proteolytic excision-mass spectrometric experiments at residues Lys-16, Leu-17, Phe-19 and Phe-20 of Aβ(1-40). They also support the observed differences in Aβ(1-40)-binding affinities of the HN mutants containing a modified Cys-8 residue (Figure 17). The structure model further suggests the formation of a salt bridge between Asp-17 of HN and Lys-28 of Aβ(1-40), in agreement with the observed inaccessibility of Lys-28 residue to proteolytic cleavage. In conclusion, the molecular modeling study of the HN-Aβ(1-40) complex provided useful complementary information on the relative orientations of the peptides and their interacting structures [192].

Figure 34 Interaction structure of the HN-Aβ(1-40) complex revealed by MD simulation. The binding residues Phe-6 and Ser-7 of the HN peptide are shown in red, while the interacting sequence 17LVFF20 of Aβ(1-40) is coloured in blue. The figure was prepared using the program BallView 1.3.2 [192].
2.3 Molecular characterization of β-amyloid autoantibodies from healthy adults and Alzheimer’s disease patients

2.3.1 Discovery and neuroprotective potential of human Aβ-autoantibodies

A first study performed by Du et al. revealed the presence of IgG-type Aβ-autoantibodies in human CSF by indirect ELISA and indicated significantly lower levels of Aβ-autoantibodies in AD patients compared to healthy controls. Also, preincubation of the CSF samples with Aβ(1-40), Aβ(1-42) and Aβ(25-35) resulted in significant reduction of the corresponding ELISA responses. This effect was most pronounced when Aβ(25-35) was used [204].

Evidence for the presumed neuroprotective function of Aβ-autoantibodies was obtained from experiments performed both in vitro and in vivo. Thus, Aβ-autoantibodies isolated from IVIg were shown to block β-amyloid fibril formation and to inhibit β-amyloid-induced neurotoxicity in cultured rat hippocampal neurons [74]. In a mouse model of AD, plaque formation was reduced after passive immunization with Aβ-autoantibodies and clearance of Aβ led to an improvement of mice behavior [80]. In AD patients, promising effects on cognition were observed in small pilot trials involving administration of IVIg [73, 75, 81]. Also, significant increase in serum/plasma Aβ and decrease in CSF Aβ were reported [73, 75]. IVIg preparations, which are pooled immunoglobulins obtained from the blood of thousands of healthy donors [75], have been recently introduced into clinical trials as a potential AD treatment (www.clinicaltrials.gov; [81]).

These findings suggest that Aβ-autoantibodies might be protective against AD and raise the question whether decreased levels of Aβ-autoantibodies are characteristic to AD pathology. However, to date there is no clear conclusion regarding the applicability of Aβ-autoantibodies as biomarkers for AD, since the results published so far of their determination by ELISA in serum or plasma from AD patients and control subjects are inconsistent [130, 131, 133-135, 205] (see discussion in chapter 2.3.4).
2.3.2 Elucidation of the epitope recognized by human Aβ-autoantibodies

Knowledge of the epitope specificity of human Aβ-autoantibodies is important for understanding their mechanism of action in vivo. To identify the Aβ domain recognized by physiological Aβ-autoantibodies, mass spectrometric epitope-excision and -extraction methodologies were employed in previous work performed in our laboratory [78, 206].

In a first step, Aβ-autoantibodies were isolated from a commercial IVIg preparation (Octagam® from Octapharma), using an affinity column with Cys-Aβ(1-40) covalently linked to a polymeric matrix (UltraLink® Iodoacetyl, Thermo Scientific Pierce) through a thioether bond. Purified Aβ-autoantibodies were next coupled to NHS-activated Sepharose and used for mass spectrometric epitope mapping experiments, in which Glu-C, trypsin, α-chymotrypsin and pronase were employed as proteolytic enzymes.

Glu-C digestion of Aβ(1-40) in PBS generated peptide fragments of various lengths, due to specific cleavages after all Glu and Asp residues, namely Glu-3, Glu-11, Glu-22, Asp-7 and Asp-23. However, epitope-extraction carried out by adding the digestion mixture on the Aβ-autoantibodies affinity column yielded in the elution fraction only the N-terminally truncated fragment Aβ(12-40) and intact Aβ(1-40). An epitope-excision experiment was subsequently performed by first incubating Aβ(1-40) with Sepharose-immobilized Aβ-autoantibodies and then subjecting the immune complex to proteolytic digestion by Glu-C. The mass spectrum of the elution fraction contained singly charged molecular ions [M+H]⁺ of the peptide fragment Aβ(12-40) and full Aβ(1-40), consistent with the epitope-extraction result. Additionally, Glu-22 and Asp-23 residues were found protected against proteolysis in the immune complex, which suggested they were part of the Aβ epitope sequence.

A following proteolytic excision with trypsin delivered the overlapping Aβ fragments (6-40) and (17-40) in the elution, indicating the epitope localization within the shorter Aβ(17-40) sequence. Furthermore, shielding from cleavage at Lys-28 was
observed. Finally, Pronase (a mixture of various endo- and exoproteases) was employed in an epitope-excision experiment and was able to cleave the antibody-bound Aβ mainly in the N-terminal part. The mass spectrometric analysis of the elution fraction revealed seven overlapping fragments that covered the Aβ amino acid sequence (21-37) (see Table 7).

Considering all the proteolytic results and the observed shielding effects, the carboxy-terminal Aβ(21-37) fragment (\textsuperscript{21}AEDVGSNKGAIIGLMV\textsuperscript{37}) was assigned as the minimal epitope still showing affinity to the Aβ-autoantibodies. A summary of the Aβ fragments identified by mass spectrometry in the elution fractions from all the proteolytic epitope mapping experiments is presented in Table 7.

Table 7  
Summary of the mass spectrometric epitope-extraction and -excision results for the identification of the Aβ sequence binding to physiological Aβ-autoantibodies from IVIg

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protease</th>
<th>Eluted fragments</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Glu-C</td>
<td>12-40</td>
<td>\textsuperscript{12}YE\textsuperscript{V}H\textsuperscript{H}K\textsuperscript{L}V\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td>Excision *</td>
<td>Glu-C</td>
<td>12-40</td>
<td>\textsuperscript{12}YEV\textsuperscript{HH}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td>Excision *</td>
<td>Trypsin</td>
<td>17-40</td>
<td>\textsuperscript{17}LV\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-40</td>
<td>\textsuperscript{6}HD\textsuperscript{S}G\textsuperscript{Y}E\textsuperscript{V}H\textsuperscript{H}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td>Excision</td>
<td>Pronase</td>
<td>5-40</td>
<td>\textsuperscript{5}RH\textsuperscript{D}S\textsuperscript{G}Y\textsuperscript{E}\textsuperscript{V}H\textsuperscript{H}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-40</td>
<td>\textsuperscript{6}HD\textsuperscript{S}G\textsuperscript{Y}E\textsuperscript{V}H\textsuperscript{H}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-40</td>
<td>\textsuperscript{7}DS\textsuperscript{G}Y\textsuperscript{E}\textsuperscript{V}H\textsuperscript{H}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-37</td>
<td>\textsuperscript{11}EV\textsuperscript{H}\textsuperscript{H}Q\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{37}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14-40</td>
<td>\textsuperscript{14}HQ\textsuperscript{K}L\textsuperscript{V}\textsuperscript{F}A\textsuperscript{E}\textsuperscript{D}V\textsuperscript{G}S\textsuperscript{N}K\textsuperscript{G}A\textsuperscript{I}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21-40</td>
<td>\textsuperscript{21}AED\textsuperscript{V}G\textsuperscript{S}N\textsuperscript{K}G\textsuperscript{A}I\textsuperscript{G}L\textsuperscript{M}V\textsuperscript{G}V\textsuperscript{V}\textsuperscript{40}</td>
</tr>
</tbody>
</table>

* The amino acid residues found shielded are marked in bold in the fragment sequences.

For comparison, Aβ-autoantibodies from a lyophilized IgG preparation (product of Calbiochem) were also affinity-purified on the Cys-Aβ(1-40) column, immobilized on Sepharose and used for Aβ epitope-extraction and -excision with Glu-C. The elution fractions from both experiments contained N-terminally truncated
fragments overlapping Aβ sequence (12-40). This result indicated that the Aβ-autoantibodies present in the Calbiochem IgG preparation also recognize the C-terminal region of Aβ, as previously shown for the IVIg-derived Aβ-autoantibodies.

The molecular information of the Aβ(21-37) epitope represented the starting point for the development of ELISA protocols for the determination of free, bound and total Aβ-autoantibodies in healthy individuals and AD patients, which was one of the major goals of this dissertation.

2.3.3 Determination of Aβ-autoantibodies in serum from healthy adults of various ages

During healthy aging, a modest decline of fluid cognitive abilities (e.g., psychomotor speed, attention, short-term storage, verbal and visual episodic memory, visuospatial abilities and verbal fluency) can be observed, while crystallized cognitive functions such as semantic and procedural knowledge remain unimpaired [207, 208]. Cognitive changes may start around the age of 20-30 years and progress until late adulthood, with increasing interindividual variability [209, 210]. Healthy aging is further associated with biological changes, among which a decline in the specific immune response to antigenic stimuli was reported [211]. Age-related changes of the immune system are involved in the decreased response to vaccination, as well as in the susceptibility of elderly persons to infectious diseases and cancer [212, 213].

As presented in chapter 2.3.1, several studies suggest that physiological Aβ-autoantibodies have a neuroprotective function [74, 75, 80, 81, 214], which raises the question whether cognitive changes occurring during healthy aging are associated with a possible decrease in the levels of Aβ-autoantibodies.

Forty-seven healthy adults (21 males, 26 females) aged 18 to 89 years ($M = 51.7$, $SD = 20.54$) took part in this study. Educational level ranged from 10 to 21 years
(M = 14.9, SD = 3.19) and was not associated with age (r = -0.16, p = 0.29). Sample details are depicted in Table 8.

Neuropsychological examination included the following tests and test batteries: first, the Consortium to Establish a Registry for Alzheimer’s disease (CERAD-NP-plus) test battery [215] was used, namely the subtests Mini Mental State Examination (MMSE), Boston naming test, Semantic and Phonemic fluency, Word list learning, Word list delayed recall, Word recognition, Figure copy, Figure recall and Trail making test (TMT) A and B; in addition, the German Wechsler Adult Intelligence Scale (HAWIE-R) was conducted, namely the subtests Digit-symbol substitution test, Mosaic test and Digit span test; finally, the German version of the revised Benton visual retention test [216] was applied. The participants in this study showed non-pathological, age-related cognitive decline, especially in speed-related tasks and tasks of executive function (e.g., TMT, Digit-symbol test), as well as in visual memory (e.g., Benton test; see Table A6 in Appendix 6). As expected in a cognitively healthy group, almost no variance in the scores of the following tests was observed: MMSE, Boston naming test, Word recognition and Figure copy [71].

Table 8 Means (M) and standard deviations (SD) of demographic data, MMSE scores and levels of Aβ-IgG immune complexes and free Aβ-autoantibodies (n = 47) [71]

<table>
<thead>
<tr>
<th>Sample characteristics</th>
<th>M</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51.7</td>
<td>20.54</td>
<td>18-89</td>
</tr>
<tr>
<td>Education (years)</td>
<td>14.9</td>
<td>3.19</td>
<td>10-21</td>
</tr>
<tr>
<td>MMSE</td>
<td>29.5</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Aβ-IgG levels (OD) a</td>
<td>0.596</td>
<td>0.24</td>
<td>0.09-0.99</td>
</tr>
<tr>
<td>Free Aβ-autoantibodies levels (OD) a</td>
<td>0.175</td>
<td>0.06</td>
<td>0.09-0.34</td>
</tr>
</tbody>
</table>

OD – optical density (450 nm); MMSE – Mini Mental State Examination (CERAD-NP-plus; range 0-30)
a n = 39

The main goals of this part of the dissertation were (1) to establish novel ELISAs for the determination of intact Aβ-IgG immune complexes, free and total Aβ-autoantibodies and (2) to investigate whether their serum levels correlate with age
and cognitive status and thus could serve as an early indicator of an age-associating cognitive decline.

2.3.3.1 Development of ELISA methods for the determination of antigen-bound and free Aβ-autoantibodies

To evaluate the biomarker potential of Aβ-autoantibodies and to better understand their mechanism of action, various research groups applied indirect ELISA protocols to determine the levels of Aβ-autoantibodies in serum or plasma of patients with AD and control subjects. These previous studies have provided controversial results, since some groups reported lower levels of Aβ-autoantibodies in AD patients than in healthy individuals [130-132], while other groups found either increased levels [133] or no differences [134, 135]. Recently, Gustaw et al. [136] suggested that the detection of Aβ-autoantibodies in biological fluids was affected by the presence of Aβ peptides and consequently of preformed Aβ-immune complexes. Using acidic dissociation of Aβ-IgG immune complexes and antigen removal prior to ELISA measurements, this group reported higher levels of Aβ-autoantibodies in serum of AD patients compared to healthy controls [136, 137]. However, using a similar procedure, Klaver et al. [138] found no significant differences between AD and control groups.

In the light of these conflicting results, an alternative approach would be the direct analysis of intact antigen-antibody immune complexes, which have been shown to be reliable biomarkers in various infectious diseases [217] and types of cancer [218, 219]. The analysis of intact immune complexes as an alternative to acidic dissociation may also provide valuable additional information on possible problems related to antibody avidity and clearance of immune complexes.

The novel sandwich ELISA for the determination of Aβ-IgG immune complexes in serum was established based on the differential epitope specificities of Aβ-autoantibodies, which recognize Aβ(21-37) and of a mouse monoclonal 6E10 antibody (mAb 6E10), which binds to Aβ(3-8) (Figure 35).
Results and discussion

**Figure 35** Epitope specificities of the coating antibody (mAb 6E10) and the Aβ-autoantibodies [71]

The principle of sandwich ELISA for the determination of Aβ-IgG immune complexes is schematically shown in Figure 36a. In brief, mAb 6E10, which was used to capture Aβ-bound autoantibodies, was first coated on the ELISA plates. After blocking with BSA, human serum, which contains Aβ-IgG immune complexes, was added. For detection, a horseradish peroxidase (HRP)-labeled IgG that recognizes human IgG and has no cross-reactivity with mouse IgG was employed.

**Figure 36** ELISA determination of β-amyloid immune complexes (Aβ-IgG) and free Aβ-autoantibodies in human serum. (a) Sandwich ELISA to determine Aβ-IgG immune complexes; (b) Indirect ELISA to determine free Aβ-autoantibodies. In both cases detection was performed with a HRP-labeled anti-IgG antibody [71].
To establish the concentrations of capture and detection antibodies giving the highest optical density (OD) response in sandwich ELISA, a simultaneous two-dimensional serial dilution (chessboard titration) of the antibodies was performed, while the concentration of the test sample (human serum IgG preparation from Calbiochem) was kept constant (1:100 dilution from a 7 µg/µL stock solution). The highest OD value after subtracting the non-specific sample binding (NSB) was obtained using 1 µg/mL mAb 6E10 and 0.2 µg/mL HRP-conjugated goat anti-human IgG (Figure 37).

The composition of washing buffer and the number of washing steps after sample addition were also varied to establish the assay conditions providing the highest OD after NSB subtraction (see Figure A5 in Appendix 5).

Finally, various preincubation conditions for the IgG preparation (Calbiochem) prior to its addition to the mAb 6E10 antibody-coated plates in sandwich ELISA were tested (Figure 38) and led to almost identical results (coefficient of variation between the OD readings for the IgG samples diluted 1:33.3: CV\textsubscript{33.3} = 7.49%, below the maximal intra-assay CV of 10%). An 1 h incubation time at room temperature (RT) was chosen for further experiments.

Figure 37 Chessboard titration in sandwich ELISA for determining the optimal concentrations of capture and detection antibodies. The OD readings were performed at 450 nm [71].
Results and discussion

**Figure 38** Influence of different preincubation conditions for the IgG preparation (Calbiochem) on the sandwich ELISA response. The OD readings were performed at 450 nm [71].

Using the optimized sandwich ELISA protocol described in detail in the Experimental part, the levels of β-amyloid immune complexes were investigated in two IgG preparations: (1) human serum IgG from Calbiochem, commercialized for research purposes only and (2) IVIg (Gamunex® 10%) from Talecris Biotherapeutics, in use for treatment of different infectious, inflammatory and autoimmune disorders. Aβ-IgG immune complexes were detected in both preparations, slightly higher levels being observed in the product from Calbiochem (Figure 39). The latter was applied as reference on each plate in subsequent ELISAs for the analysis of serum samples, to allow data normalization between different plates and experiments.

Considering that Aβ-autoantibodies recognize the Aβ(21-37) epitope, an indirect ELISA for the determination of free Aβ-autoantibodies in serum was also developed. The principle of the assay is schematically shown in Figure 36b. To prevent conformational changes that may occur during direct adsorption on the ELISA plates, biotin-(G)5-Aβ(12-40) epitope peptide (17 in Table 5) was employed as capture antigen on streptavidin-coated plates. Washing and blocking steps, addition of the detection antibody and OD readings were performed as in sandwich ELISA for the determination of Aβ-IgG immune complexes. Also, human serum IgG preparation (Calbiochem) was used as reference. Since there is no
unique method for expressing ELISA responses and arbitrary units are derived from absorbance readings, it was considered adequate to present the results of Aβ-autoantibodies determinations as OD values.

![Graph showing comparison of Aβ-IgG levels detected in the IgG preparation from Calbiochem and in the IVlg (Gamunex® 10%) preparation from Talecris Biotherapeutics by sandwich ELISA. The OD readings were performed at 450 nm [71].](image)

**Figure 39** Comparison of Aβ-IgG levels detected in the IgG preparation from Calbiochem and in the IVlg (Gamunex® 10%) preparation from Talecris Biotherapeutics by sandwich ELISA. The OD readings were performed at 450 nm [71].

### 2.3.3.2 Levels of antigen-bound and free Aβ-autoantibodies in serum from healthy adults of various ages

The newly developed sandwich ELISA protocol was employed for the analysis of serum samples from 39 healthy individuals aged 18 to 89 years ($M = 48.8, SD = 19.87$), generating OD values between 0.09-0.99 ($M = 0.596, SD = 0.24$; Figure 41).

The ELISA determinations of Aβ-IgG levels in both reference and serum samples showed intra-assay coefficients of variation (CVs) < 10% and inter-assay CVs < 15%. A sigmoidal (5-parameters logistic) mathematical model was applied for reference curve fitting, providing good fits ($R^2 > 0.98$; Figure 40a). Since changes in OD units reflect equal changes in analyte levels only in the linear region of the assay response, the reference curves were evaluated for linearity, which was observed between 0.068-1.109 OD units ($R^2 > 0.97$ for linear regression). All
serum samples, which were diluted 1:100, gave absorbance readings within this interval and were included in the statistical analysis. The lower limit of detection (LLOD) of the assay, defined as 3 standard deviation (SD) units above the mean OD readings of blank samples (blocking buffer without serum or IgG reference) was 0.064, slightly below the minimal OD cut-off value for the linearity constraint.

\[\text{OD} = \frac{\text{Absorbance}}{\text{Sample Volume}}\]

\[\text{Dilution factor} = 100, 1000, 10000\]

Figure 40  Example of IgG (Calbiochem) reference curves in (a) sandwich ELISA for the determination of A\(\beta\)-IgG immune complexes; (b) indirect ELISA for the determination of free A\(\beta\)-autoantibodies. In both cases, the IgG dilution factors are plotted on a logarithmic scale and the corresponding OD readings (at 450 nm) fitted to a sigmoidal (5-parameters logistic) mathematical model using the WorkOut 2.0 software. The triplicate OD readings for each IgG dilution are represented by red crosses. The linear range of each curve is highlighted in a blue box [71].
The new indirect ELISA was applied to determine the levels of free Aβ-autoantibodies in 47 serum samples, which were also diluted 1:100, as for the determination of Aβ-IgG immune complexes by sandwich ELISA. The OD readings of free Aβ-autoantibodies in both reference and serum samples showed similar CVs to those obtained for the determination of Aβ-IgG immune complexes. The linear range of the reference curve, fitted to a 5-parameters mathematical model ($R^2 > 0.98$), was between 0.084-1.059 OD units, above the calculated LLOD of 0.072 OD units (Figure 40b). Eight samples provided OD values beneath the established linear interval of the assay response and were excluded from the statistical evaluation, leaving 39 sera (OD values between 0.09-0.34; $M = 0.175$, $SD = 0.06$; Figure 41) from healthy individuals aged 18 to 89 years ($M = 53.4$, $SD = 20.51$) to be further analyzed.

In both young and older healthy subjects, low but detectable levels of free Aβ-autoantibodies, significantly lower than those of Aβ-IgG immune complexes ($t_{(35)} = 10.12$, $p < 0.0001$; Figure 41) were observed.

![Figure 41](image.png)  
**Figure 41** Comparison between the levels of Aβ-IgG immune complexes and free Aβ-autoantibodies (OD at 450 nm) in serum of healthy adults. The mean level of Aβ-IgG immune complexes is significantly higher than the one of free Aβ-autoantibodies ($p < 0.0001$) [71].

The levels of Aβ-IgG immune complexes in serum did not correlate with the age of the investigated healthy individuals ($r = -0.08$, $p = 0.63$; Figure 42a), their cognitive test scores (Table 9) or years of education ($r = 0.01$, $p = 0.94$). Also, no correlation
was found between the levels of free Aβ-autoantibodies and age ($r = -0.28$, $p = 0.09$; Figure 42b), cognitive test scores (Table 9) or years of education ($r = 0.22$, $p = 0.18$).

![Figure 42](image1.png)

**Figure 42** Correlation analysis between the age of healthy adults and serum levels of (a) Aβ-IgG immune complexes (OD at 450 nm; $r = -0.08$, $p = 0.63$) and (b) free Aβ-autoantibodies (OD at 450 nm; $r = -0.28$, $p = 0.09$) [71]

Finally, the ratio of serum levels of Aβ-IgG immune complexes to free Aβ-autoantibodies was calculated and showed no correlation with age ($r = 0.15$, $p = 0.42$; Figure 43), cognitive performance (Table A7, Appendix 7) or years of education ($r = -0.01$, $p = 0.94$).

![Figure 43](image2.png)

**Figure 43** Correlation analysis between the age of healthy individuals and the ratio of serum levels of Aβ-IgG immune complexes to free Aβ-autoantibodies ($r = 0.15$, $p = 0.42$) [71]
### Results and discussion

**Table 9** Pearson’s $r$ correlations between the levels of Aβ-IgG immune complexes ($n = 39$) versus free Aβ-autoantibodies ($n = 39$) and cognitive performance [71]

<table>
<thead>
<tr>
<th>Cognitive test</th>
<th>Aβ-IgG immune complexes (OD)</th>
<th>Free Aβ-autoantibodies (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$p$-value</td>
</tr>
<tr>
<td>Semantic fluency *</td>
<td>0.004</td>
<td>0.98</td>
</tr>
<tr>
<td>Phonemic fluency</td>
<td>0.23</td>
<td>0.17</td>
</tr>
<tr>
<td>Word list learning **</td>
<td>-0.24</td>
<td>0.15</td>
</tr>
<tr>
<td>Word recall **</td>
<td>-0.02</td>
<td>0.89</td>
</tr>
<tr>
<td>Figure recall **</td>
<td>-0.003</td>
<td>0.99</td>
</tr>
<tr>
<td>TMT-A **</td>
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<td>0.69</td>
</tr>
<tr>
<td>TMT-B **</td>
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</tr>
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<td>Digit span test</td>
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<td>0.79</td>
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<tr>
<td>Digit-symbol test **</td>
<td>-0.13</td>
<td>0.42</td>
</tr>
<tr>
<td>Mosaic test **</td>
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<td>0.66</td>
</tr>
<tr>
<td>Benton test (correct) **</td>
<td>0.008</td>
<td>0.96</td>
</tr>
<tr>
<td>Benton test (error) **</td>
<td>-0.05</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Benton test (correct answers; range 0-20); Benton test (errors; range 0-30); Digit span test (HAWIE-R; range 0-28); Digit-symbol substitution test (HAWIE-R; range 0-93); Figure recall (CERAD-NP-plus; range 0-14); Mosaic test (HAWIE-R; range 0-51); Phonemic/Semantic fluency (CERAD-NP-plus); TMT-A/B – Trail making test part A/B (CERAD-NP-plus; A: range 0-180 sec.; B: range 0-300 sec.); Word list learning (CERAD-NP-plus; range 0-30); Word recall (CERAD-NP-plus; range 0-10); Word recognition (CERAD-NP-plus; range 0-10 true positives)

* Significant correlation between cognitive test performance and age
* Significant correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm

The variation with time of Aβ-IgG levels in serum of ten subjects (five males and five females), aged 26 to 86 years ($M = 52.1$, $SD = 18.48$) was also investigated and stable values for the Aβ-IgG immune complexes during a time interval of four weeks (week 0, week 1, week 4; $F_{(2,18)} = 0.23$, $p = 0.80$; Figure 44) were observed.
Results and discussion

**Figure 44** Comparison between the levels of Aβ-IgG immune complexes (OD at 450 nm) in serum provided by ten healthy individuals at three different time-points over a four weeks period ($F_{(2,18)} = 0.23, p = 0.80$). Each of the ten curves represents one individual subject [71].

Overall, these data show that in serum of healthy adults aged 18-89 years, most of the Aβ-autoantibodies are bound to Aβ-peptides, forming Aβ-IgG immune complexes and only a small amount is circulating in free form (Figure 41). These results are in agreement with the publications reporting a significant increase of detectable levels of Aβ-autoantibodies upon acidic treatment of serum [136, 137]. The identification of circulating Aβ-IgG immune complexes in serum also provides a direct proof for the role of Aβ-autoantibodies in the binding and subsequent clearance of Aβ in vivo.

The participants in the present study showed non-pathological, age-related cognitive decline, revealed especially in tests of visual memory and executive function, as well as in speed related-tasks (Table A6, Appendix 6; see also [208]). Independent of age, participants were in a good health condition (only 17 participants took medication and only four of them more than one type of medication). No correlation was found between age or cognitive performances of healthy adults and the serum levels of Aβ-IgG immune complexes, free Aβ-autoantibodies or their ratio.
In conclusion, these data indicate that healthy aging per se is not associated either with an altered production of Aβ-autoantibodies or with an altered antigen-binding avidity, as reported in the case of AD patients [220]. The balanced formation and removal of the immune complexes in healthy individuals is also supported by the observed stability of Aβ-IgG immune complexes in serum over the investigated period of four weeks. According to these results, serum levels of antigen-bound and free Aβ-autoantibodies are not associated with age or cognitive functions of healthy adults [71].

2.3.3.3 Development of an indirect ELISA for the determination of total Aβ-autoantibodies

Indirect ELISA determinations of free Aβ-autoantibodies in serum or plasma of healthy individuals and AD patients showed so far low detectable levels (e.g., [134, 135]) and provided inconsistent results regarding their biomarker value [130-133, 204] (see chapter 2.3.3.1). These effects were mainly attributed to the partial blocking of Aβ-autoantibodies by antigen binding [136]. Indeed, acidic dissociation (pH 2.5) of the preformed immune complexes from a human IVIg preparation generated a significant increase of the Aβ-autoantibodies response in ELISA with coated Aβ compared to the untreated sample [221]. Procedures involving acidification (pH 2.5 or 3.5) and subsequent removal of the antigen (Aβ) by ultrafiltration [136, 137] were also reported to enhance the levels of Aβ-autoantibodies detectable by indirect ELISA in human plasma. The drawbacks of these methods are (i) the necessity of pH readjustment to the physiological value of 7.4 prior to ELISA measurements, with potentially associated errors and (ii) the introduction of an additional sample preparation step for antigen removal, which may also cause variability of the assay results. To avoid this, an alternative ELISA for the determination of “total” Aβ-autoantibodies was established, using dimethyl sulfoxide (DMSO) to dissociate preformed Aβ-IgG immune complexes.

DMSO is an excellent organic solvent, able to dissolve both polar and nonpolar compounds and to penetrate biological membranes without exerting toxic effects.
Due to these properties, it is often employed to dissolve various analytes for NMR studies, as an extractant of target components from biological lysates or as a drug delivery solvent for *in vivo* or *in vitro* test experiments on cells. The usually applied DMSO concentrations vary between 0.1 % and 5% [222].

However, the use of DMSO in biological assays may cause protein denaturation, with potential exposure of normally buried hydrophobic domains and artifactual increase of the experimental results [223]. Therefore, optimization of DMSO concentration in the sample dilution buffer, as well as an evaluation of the possible effects that DMSO treatment has on antibodies conformation and antigen recognition were performed in the process of developing a new ELISA for the determination of total Aβ-autoantibodies in human serum. The experiments were carried out according to the ELISA protocol applied to determine the free Aβ-autoantibodies in serum (see Figure 36b and Experimental part for details), in which only the sample preparation procedure was modified.

First, the influence of different DMSO concentrations on the binding responses of Aβ-autoantibodies from human IgG (Calbiochem) was assessed. For this purpose, equal volumes of IgG stock solution (12 µg/µL in BSAT) were treated with increasing volumes of DMSO and then subjected to three-fold serial dilutions in DMSO-containing BSAT. Consequently, the final concentrations of DMSO in the sample buffer ranged from 0% to 5% (Figure 45). Analysis of the ELISA curves obtained after NSB subtraction from the assay responses (Figure 45a) revealed that increasing the DMSO concentration resulted in higher OD values.

This effect was attributed to the dissociation of preformed Aβ-IgG immune complexes by DMSO and a subsequent shift in binding of the released Aβ-autoantibodies to the immobilized biotin-(G)₅-Aβ(12-40) antigen (peptide 17 in Table 5), available at concentrations exceeding those of Aβ in the IgG dilutions. In agreement with the proposed mechanism, the highest variations of the OD responses between the five ELISA curves were observed for the most concentrated samples (diluted 1:100, 1:300 and 1:900), which provided more Aβ-autoantibodies for detection upon DMSO treatment than the more diluted samples.
Considering the possible denaturation of the Aβ-autoantibodies in presence of DMSO, analysis of the corresponding NSB curves (Figure 45b) was also performed. Thus, similar OD values were found for the 0-1% DMSO range, while significantly higher NSB responses were observed for 2% and 5% DMSO in the sample buffer. These results suggest that concentrations of DMSO higher than 1% cause unfolding of the IgG molecules and consequently enhance their non-specific binding to the ELISA plates.

**Figure 45** Binding of Aβ-autoantibodies from human IgG (Calbiochem) to biotin-(G)₅-Aβ(12-40) immobilized on streptavidin-coated plates in indirect ELISA. (a) Influence of DMSO concentration in the sample buffer on the binding responses of Aβ-autoantibodies after NSB subtraction; (b) Influence of DMSO concentration in the sample buffer on the non-specific sample binding (NSB). The OD readings were performed at 450 nm.
Next, the levels of Aβ-autoantibodies detected by indirect ELISA in DMSO- vs. acid-treated IgG samples (incubation at pH 3.5 for 20 min) were compared. The OD results suggested that 1% DMSO had the greatest dissociation power of preformed Aβ-IgG complexes, higher than the one of acidification (Figure 46). This effect may be due to the mainly hydrophobic interaction between Aβ and Aβ-autoantibodies (see the epitope identification in chapter 2.3.2), which is more affected by the addition of an organic solvent than by the decrease in pH.

![Figure 46](image)

**Figure 46** Influence of different preincubation conditions for human IgG on the binding responses of Aβ-autoantibodies to biotin-(G)₅-Aβ(12-40) in indirect ELISA.

The binding of the IgG preparation to ELISA plates coated with the non-epitope peptides biotin-(G)₅-Aβ(1-16), biotin-(G)₅-Aβ(17-28) and biotin-(G)₅-Aβ(20-30) (peptides 18, 19 and 21 in Table 5) was also investigated and gave no affinity response, suggesting that DMSO does not induce changes in the interaction specificity of Aβ-autoantibodies.

**2.3.3.4 Levels of total Aβ-autoantibodies in serum from healthy adults of various ages**

The new ELISA protocol for the determination of total Aβ-autoantibodies (see Experimental part for details) was employed to analyze serum samples from 39...
healthy individuals aged 18 to 89 years ($M = 48.8$, $SD = 19.87$). The samples were diluted 1:100, as for the analysis of Aβ-IgG immune complexes and free Aβ-autoantibodies, to enable comparisons. The OD readings of total Aβ-autoantibodies in both IgG reference and serum samples showed similar CVs as previously observed for the determination of antigen-bound and free Aβ-autoantibodies (intra-assay CVs < 10% and inter-assay CVs < 15%).

Statistical evaluation of the ELISA data indicated, as expected, a significant increase of detectable Aβ-autoantibodies by indirect ELISA upon DMSO treatment, which led to levels of total Aβ-autoantibodies exceeding the ones determined for free and antigen-bound Aβ-autoantibodies ($F_{(2,114)} = 76.0$, $p < 0.0001$ in 3-way ANOVA with Tukey’s post test; Figure 47).

![Figure 47](image.png)

**Figure 47** Comparison between the levels of free, antigen-bound and total Aβ-autoantibodies (OD at 450 nm) in serum of healthy adults. The mean level of total Aβ-autoantibodies is significantly higher than the mean levels of free Aβ-autoantibodies ($t_{(76)} = -11.7$, $p < 0.0001$) and Aβ-IgG immune complexes ($t_{(76)} = -2.5$, $p = 0.04$), respectively.

However, no correlations were found between the levels of total Aβ-autoantibodies and age ($r = -0.09$, $p = 0.59$; Figure 48) or cognitive test scores.
According to these results, serum levels of total Aβ-autoantibodies are not associated with age or cognitive functions of healthy adults. These findings support the previous conclusion derived from the ELISA determinations of free and antigen-bound Aβ-autoantibodies, that healthy aging per se is not accompanied by an altered production of Aβ-autoantibodies.

2.3.4 Determination of Aβ-autoantibodies in serum and cerebrospinal fluid of Alzheimer's disease patients

Since AD-related pathological processes start well before the onset of clinical manifestations [224-227], the identification of biochemical markers that would allow an early diagnosis and therapeutic intervention is of great importance. CSF levels of Aβ(1-42) and tau are currently the only reliable biomarkers for the diagnosis of AD, with sufficiently high sensitivity and specificity [228], while efforts to establish less invasive blood-derived biomarkers have remained so far unsuccessful.
The recently discovered physiological Aβ-autoantibodies, detected both in serum and CSF [72-76, 81], generated a high research interest as another potential biomarker for AD. Currently available data on the serum levels of Aβ-autoantibodies in AD patients compared to healthy individuals are however controversial. Several groups found that the serum levels of free, non-antigen bound Aβ-autoantibodies were lower in AD patients than in controls [130-132], while others reported either higher values [133] or no difference [134, 135]. So far, there is only one reported study on the CSF levels of free Aβ-autoantibodies, showing decreased values in AD patients compared to control subjects [204]. Gustaw et al. [136] suggested that the presence of Aβ-autoantibodies not only as free, non-antigen bound, but also as preformed immune complexes with Aβ-peptides, is a potential cause of these controversial results. Subsequent serum determinations of Aβ-autoantibodies after acidic dissociation of the Aβ-immune complexes indicated higher levels of Aβ-autoantibodies in AD patients compared to controls [136, 137]. Nevertheless, these results could not be reproduced by Klaver et al. [138], who found no difference in the levels of total Aβ-autoantibodies between AD and control subjects.

Based on the finding that Aβ-autoantibodies recognize the Aβ(21-37) epitope [78, 79], unlike the antibodies produced by immunization, which bind the Aβ(4-10) epitope [59], a novel sandwich ELISA for the determination of intact Aβ-IgG immune complexes was developed and first applied for the analysis of serum samples from healthy individuals aged 18 to 89 years (see chapter 2.3.3). The methodology represents an alternative or complementary approach to the previously described ELISA for the determination of total Aβ-autoantibodies levels [136-138]. It does not require additional sample preparation steps such as acidic dissociation and may provide valuable information on possible problems related to antibody avidity and clearance of the immune complexes. Another important aspect consists in the subtraction of the NSB from the OD response of each sample, procedure previously reported only in a few ELISA-based studies of Aβ-autoantibodies [138].
In this part of the dissertation, the experimental procedure was also optimized for CSF analysis. Two washing buffers, PBS-Tween (0.05% Tween-20 in PBS, v/v) and PBS-Triton (0.1% Triton X-100 in PBS, v/v) and various CSF dilutions (1:300, 1:100, 1:30, 1:10, 1:3 and 1:1) were tested. The highest OD response was obtained using PBS-Tween for washing and 1:1 CSF dilution.

The final sandwich ELISA protocols were applied to determine the levels of $\text{A}\beta$-IgG immune complexes in serum and CSF samples of a total number of 112 AD patients and age- and gender-matched control subjects. The purpose of the study was to evaluate the diagnostic power of $\text{A}\beta$-IgG levels, as well as their correlations with the neuropsychological performance and age of the participants.

Demographic data is depicted in Table 10. Altogether, 58 AD patients were recruited at the Memory Clinic of the Hospital for Neurology of the University of Ulm, Germany. Patients underwent a comprehensive clinical neurological examination, a routine blood analysis, morphological imaging (MRI or CT), ApoE genotyping and a detailed neuropsychological assessment, including the Mini Mental State Examination test (MMSE, range 0-30 points; [229]) and the Alzheimer’s Disease Assessment Scale – Cognitive subscale (ADAS-Cog, range 0-70 errors). Probable AD was diagnosed according to NINCDS-ADRDA [230] and DSM-IV-TR criteria of the American Psychiatric Association. Furthermore, 54 unrelated age- and gender-matched control subjects were recruited at the same site and did not display any cognitive or neurological deficits following thorough clinical and neuropsychological examination [231].

The statistical evaluation of the demographic and clinical data indicated a similar distribution of age ($t_{(85)} = 0.79, p = 0.43$ for serum donors; $t_{(64)} = -1.12, p = 0.27$ for CSF donors) and gender ($\chi^2_{(1)} = 0.05, p = 0.82$ for serum donors; $\chi^2_{(1)} = 0.43, p = 0.51$ for CSF donors) in the AD and the control group. As expected, AD patients received fewer points in the MMSE ($t_{(47)} = -14.00, p < 0.0001$ for serum donors; $t_{(44)} = -10.16, p < 0.0001$ for CSF donors) and committed more errors in the ADAS-Cog neuropsychological test battery ($t_{(37)} = 11.30, p < 0.0001$ for serum donors; $t_{(26)} = 6.12, p < 0.0001$ for CSF donors) than the control subjects. They
Results and discussion

also presented significantly lower levels of \( A\beta(1-42) \) \((t_{(69)} = -9.39, p < 0.0001)\) and higher levels of T-tau \((t_{(69)} = 8.88, p < 0.0001)\) in CSF. Furthermore, an increased incidence of ApoE \( \varepsilon 4 \) allele was observed in the AD cases \((\chi^2_{(1)} = 15.26, p < 0.0001; \text{Table 10})\).

Table 10  Demographic and clinical characteristics of Alzheimer’s disease patients (AD) and controls (C) [231]

<table>
<thead>
<tr>
<th></th>
<th>Serum donors database</th>
<th>CSF donors database</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD ((n = 45))</td>
<td>C ((n = 42))</td>
</tr>
<tr>
<td>Age (years)</td>
<td>70.0 ± 7.5</td>
<td>68.7 ± 7.4</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>33.3</td>
<td>33.3</td>
</tr>
<tr>
<td>ApoE (% ( \varepsilon 4 ))</td>
<td>58.5</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>((n = 41))</td>
<td>((n = 33))</td>
</tr>
<tr>
<td>MMSE</td>
<td>19.7 ± 4.4</td>
<td>29.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>((n = 44))</td>
<td>((n = 37))</td>
</tr>
<tr>
<td>ADAS-Cog</td>
<td>27.0 ± 8.3</td>
<td>8.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>((n = 30))</td>
<td>((n = 25))</td>
</tr>
<tr>
<td>CSF A(\beta)(1-42) (pg/mL)</td>
<td>499 ± 177</td>
<td>999 ± 322</td>
</tr>
<tr>
<td></td>
<td>((n = 44))</td>
<td>((n = 36))</td>
</tr>
<tr>
<td>CSF T-tau (pg/mL)</td>
<td>786 ± 381</td>
<td>288 ± 132</td>
</tr>
<tr>
<td></td>
<td>((n = 44))</td>
<td>((n = 36))</td>
</tr>
<tr>
<td>Serum A(\beta)-IgG (OD) (^a)</td>
<td>0.569 ± 0.2</td>
<td>0.463 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>((n = 45))</td>
<td>((n = 42))</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. For gender and ApoE status, percentages per group are given. ADAS-Cog – Alzheimer Disease Assessment Scale-Cognitive Subscale (range 0-70 errors); MMSE – Mini Mental Status Examination (range 0-30 points); OD – optical density

\(^a\) Diluted 1:100
\(^b\) Diluted 1:1
2.3.4.1 Levels of antigen-bound Aβ-autoantibodies in serum of AD patients

The serum determinations of Aβ-IgG immune complexes showed intra-assay CVs <10% and inter-assay CVs <15%. Two samples (from one AD patient and one control subject) were excluded from the statistical analysis, since the Aβ-IgG levels exceeded the ELISA cut-off values.

Higher levels of Aβ-IgG immune complexes were determined in serum of AD patients compared to the controls ($F_{(1,84)} = 4.94, p = 0.03$; Table 10, Figure 49a). According to ROC (receiver operating characteristic) curve analyses, the serum Aβ-IgG levels discriminate the AD patients from the control subjects with 81% specificity and 44% sensitivity ($AUC = 0.63$, 95% CI: 0.75-0.51; Figure 49b). When the assay sensitivity was set to 80%, specificity reached a maximum of 33%.

The serum levels of Aβ-IgG immune complexes increased with advancing age in the AD patients ($r = 0.37, p = 0.01$) but not in the controls ($r = -0.14, p = 0.38$; Figure 50a). Furthermore, they were negatively correlated with the MMSE scores ($r = -0.23, p = 0.04$; Figure 50b) and positively with the ADAS-Cog scores across

![Figure 49](image-url)
groups \((r = 0.32, \ p = 0.02; \text{ Figure } 50c)\), i.e., reaching higher values with decreasing cognitive test performance.

Since old age and ApoE \(\varepsilon4\) status are considered to be associated with an increased risk of AD pathology [232], age was included as covariate into the group comparison and potential differences between the levels of A\(\beta\)-IgG immune complexes were further investigated with respect to ApoE genotype. However, there was no difference between the serum levels of A\(\beta\)-IgG immune complexes in the case of ApoE \(\varepsilon4\) (homo- and heterozygotes) and non-ApoE \(\varepsilon4\) carriers, either in the AD or the control group.
Results and discussion

2.3.4.2 Levels of antigen-bound Aβ-autoantibodies in CSF of AD patients

The CSF determinations of Aβ-IgG immune complexes showed intra-assay CVs <10% and inter-assay CVs <15%. The levels of Aβ-IgG immune complexes were higher in AD patients compared to the controls ($F_{(1,63)} = 4.98$, $p = 0.03$; Table 10, Figure 51a). ROC curve analyses indicated 59% specificity and 70% sensitivity.
Results and discussion

(AUC = 0.65, 95% CI: 0.79-0.52; Figure 51b) for the diagnostic discrimination of the assay between AD cases and controls. When specificity of the Aβ-IgG determinations was set to 80%, sensitivity reached a maximum of 33%. When sensitivity was set to 80%, specificity reached a maximum of 31%.

The ratio of the CSF to serum levels of Aβ-IgG immune complexes showed 82% specificity and 50% sensitivity in ROC curve analysis (AUC = 0.67, 95% CI: 0.83-0.50). When sensitivity was set to 80%, specificity was only 35%. In comparison, ROC analysis of the CSF T-tau/Aβ(1-42) concentration ratio showed 91% specificity and 93% sensitivity (AUC = 0.97, 95% CI: 0.10-0.94).

Figure 51 Aβ-IgG immune complexes in CSF. (a) Comparison between the levels of Aβ-IgG immune complexes (OD at 450 nm) in CSF of AD patients and controls (* p ≤ 0.05); (b) ROC curve analysis (FPR: false positive rate; TPR: true positive rate; AUC: area under the curve) [231]

The CSF levels of Aβ-IgG immune complexes across all subjects were negatively correlated with the MMSE scores (r = -0.30, p = 0.02) and positively correlated with the ADAS-Cog test scores (r = 0.48, p = 0.006), increasing with the decline of cognitive performance (Figure 52a, b). Furthermore, they were negatively correlated with the Aβ(1-42) concentration in CSF of AD patients (r = -0.35, p = 0.04; Figure 53a), but not of control subjects.
Results and discussion

Figure 52 Correlation analysis between CSF levels of Aβ-IgG immune complexes (OD at 450 nm) and (a) MMSE score (range 0-30 points) across all subjects; (b) ADAS-Cog score (range 0-70 errors) across all subjects [231]

A highly significant positive correlation was observed across groups between the levels of Aβ-IgG immune complexes in CSF and serum ($r = 0.54, p = 0.0002$; Figure 53b). Considering the dilution factors applied in ELISA, the Aβ-IgG levels were approximately 100 fold lower in CSF than in serum, suggesting that the Aβ-autoantibodies are produced and bind to Aβ mainly in the periphery.

There was no effect of age or ApoE ε4 (homo- and heterozygote) genotype on the Aβ-IgG levels in the CSF of either AD patients or control subjects.
Results and discussion

Figure 53  (a) Correlation analysis between the levels of Aβ-IgG immune complexes (OD at 450 nm) and Aβ(1-42) in CSF of AD patients; (b) Correlation analysis between the levels of Aβ-IgG immune complexes (OD at 450 nm) in serum and CSF across all subjects [231]

This is a first time report on the determination of intact Aβ-IgG immune complexes in serum and CSF of AD patients and age- and gender-matched control subjects, using a sandwich ELISA approach. In summary, Aβ-IgG immune complexes were detected in all analyzed serum and CSF samples, suggesting a contribution of IgG-type Aβ-autoantibodies to Aβ clearance in vivo. Higher Aβ-IgG levels were found in both serum and CSF of AD patients compared to controls, in agreement with two previous studies [136, 137] that revealed increased total levels of Aβ-autoantibodies in AD patients. An elevated antibody production would be expected in response to Aβ accumulation, which is either due to deficient clearance mechanisms [233, 234] or to an increased formation of Aβ peptides. The latter is mainly the case in familial AD, owing to genetic mutations of amyloid precursor protein (APP) and presenilin 1 and 2 [6, 33], but it can also occur in sporadic AD, where it was suggested to be partially caused by the enhanced expression and activity of APP cleaving enzyme 1 (BACE 1) [235]. However, due to the variability within groups leading to overlapping values, the Aβ-IgG levels displayed only moderate discrimination powers in ROC analyses. A possible application of serum Aβ-IgG immune complexes for AD diagnosis in a panel of blood-derived biomarkers remains to be further tested.
The progression of the disease, despite significantly higher levels of immune complexes in serum and CSF of AD patients compared to controls, could indicate defective clearance mechanisms in AD, leading to the accumulation of Aβ-immune complexes. In healthy individuals, antigen-bound antibodies are captured by macrophages through Fc receptor-mediated recognition and transferred to mastocytes in liver or spleen for degradation during the process of “immune adhesion”, which is regulated by antibody avidity [47, 236]. A possible explanation for the apparent clearance deficiency of Aβ-IgG immune complexes is provided by the observations of Jianping et al. [220], who found the avidity of Aβ-autoantibodies to be lower in AD patients than in healthy controls and suggested that this could impair the removal of Aβ-IgG immune complexes by macrophages.

The ELISA results further revealed that serum and CSF levels of Aβ-IgG immune complexes were negatively correlated with the cognitive performance of the study participants. Thus, subjects with higher Aβ-IgG levels had weaker performances during MMSE screening and ADAS-Cog neuropsychological testing. The increased levels of Aβ-IgG immune complexes and their inverse correlation with the cognitive status would point to a pathological process, potentially associated with defective clearance mechanisms, as discussed above. Thereby, the reported cognitive improvements of AD patients treated with IVIg [73, 75, 81] might be partially attributed to the replacement of deficient Aβ-autoantibodies by passive immunization. However, more research is needed to gain a deeper understanding of the underlying mechanisms that cause the apparent accumulation of Aβ-IgG immune complexes, potentially revealing a new approach for diagnosis and targeted treatment of AD.

The correlation of both serum and CSF Aβ-IgG levels with the cognitive status across groups represents a valuable characteristic and it would be interesting to assess their potential use for predicting conversion to AD or evaluating the efficacy of therapeutic interventions in AD (e.g., passive immunization with IVIg preparations containing Aβ-autoantibodies).

In agreement with previous work [71], the serum levels of Aβ-IgG immune complexes...
complexes were not correlated with the age of control subjects. In the AD group, however, increased age was associated with higher levels of Aβ-IgG immune complexes in serum and might therefore represent a factor for reduced Aβ clearance in AD. A positive correlation with age was also reported by Gustaw-Rothenberg et al. [137] for the difference values between the Aβ-autoantibody levels before and after acidic dissociation of the Aβ-IgG immune complexes, which might be comparable with the levels of intact Aβ-IgG immune complexes.

2.3.4.3 Levels of total Aβ-autoantibodies in serum of AD patients

A small sample size, consisting of sera from 11 AD patients and 11 age- and gender-matched control subjects, was available for the determination of total Aβ-autoantibodies, using the newly developed DMSO-based indirect ELISA protocol. The samples were diluted 1:100, as for the measurements of Aβ-IgG immune complexes, to enable comparisons. The intra-assay CVs were <10% and the inter-assay CVs <15%.

Higher levels of total Aβ-autoantibodies were determined in serum of AD patients compared to the controls (p = 0.038, Figure 54a), in agreement with two previous studies [136, 137] that revealed increased levels of total Aβ-autoantibodies in AD patients after acidic dissociation of preformed Aβ-immune complexes. Also, the serum levels of total Aβ-autoantibodies were negatively correlated with the MMSE scores across groups (r = -0.45, p = 0.036; Figure 54b), i.e., reaching higher values with decreasing cognitive test performance.

These results support the conclusion derived from the ELISA determinations of Aβ-IgG immune complexes (see discussion in chapter 2.3.4.2) and the literature data, regarding an apparently increased production of Aβ-autoantibodies in AD [136, 137].
Figure 54 (a) Comparison between the levels of total Aβ-autoantibodies (OD at 450 nm) in serum of AD patients and control subjects. The mean level of total Aβ-autoantibodies is significantly higher in the AD group than in controls ($p = 0.038$); (b) Correlation analysis between the serum levels of total Aβ-autoantibodies (OD at 450 nm) and MMSE score (range 0-30 points) across all subjects ($r = -0.45, p = 0.036$)
3 EXPERIMENTAL PART

3.1 Materials and reagents

All amino acid derivatives and benzotriazol-1-yl-oxytrispyrrolidinophosphonium-hexafluoro-phosphate (PyBOP) were purchased from Novabiochem (Läufelfingen, Switzerland) and GL Biochem Shanghai Ltd (Shanghai, China). NovaSyn® TGR and Rink Amide MBHA were from Novabiochem. Coupling agents, cleavage reagents and scavengers (N-methylmorpholine (NMM), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), trifluoroacetic acid, triisopropylsilane) were obtained from Sigma-Aldrich, Germany. N,N-dimethylformamide (DMF), ethanol, diethyl ether and acetonitrile (ACN) were from VWR (Darmstadt, Germany). 16-mercaptotetradecanoic acid and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich, while N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) was obtained from Merck (Darmstadt, Germany). Sequencing grade modified trypsin, Asp-N and Glu-C were from Promega (Mannheim, Germany), TLCK-treated α-chymotrypsin was purchased from Sigma-Aldrich. Dihydroxy benzoic acid (DHB) was from Fluka, α-cyano-4-hydroxicinnamic acid (CHCA) was obtained from Sigma-Aldrich. Mouse monoclonal 6E10 antibody (mAb 6E10) was purchased from Covance (Emeryville, CA, USA). Streptavidin, hydrogen peroxide and o-phenylenediamine dihydrochloride (OPD) were Merck products, while horseradish peroxidase (HRP)-labeled goat anti-human IgG (H+L) antibody showing no cross-reactivity with mouse IgG was purchased from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA) was a PAA Laboratories GmbH product (Pasching, Austria) and Tween-20 and Triton X-100 were obtained from Sigma-Aldrich. All reagents and solvents were of analytical grade or highest available purity.

3.2 Synthesis and purification of Humanin and Aβ peptides

All peptides were synthesized as carboxamides on NovaSyn® TGR resin (0.29 mmol/g coupling capacity) or Rink Amide MBHA resin (0.4 mmol/g coupling
capacity), manually or using a semi-automated peptide synthesizer (EPS-221 from Abimed, Langenfeld, Germany). Fmoc/tert-butyl chemistry was applied in all cases. Benzotriazole-1-yl-oxy-trispyrrolidino-phosphonium-hexafluorophosphate (PyBOP) and N-methylmorpholine (NMM) were used for amino acid activation. In each synthesis cycle, the N-terminal 9-fluorenlymethoxycarbonyl (Fmoc) protecting group of the previously attached amino acid to the growing peptide chain on the resin was removed with 2% piperidine, 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in N,N-dimethylformamide (DMF) (2 × 10 min). After washing with DMF, the mixture of 5 equiv of the following Fmoc-amino acid derivative:PyBOP:NMM in DMF was added over the resin twice (50 min/coupling). In the case of HN peptides, the remaining free amino groups after each second coupling were acetylated using acetic anhydride:NMM:DMF (1.4:1:1.6, v/v/v, 30 min). At the end of the synthesis, the resin was washed with DMF and ethanol and dried under vacuum.

Cleavage of the peptides from the resin and simultaneous side-chain deprotection (except for Cys-acetamidomethyl; Acm) was performed with a mixture of 95% TFA, 2.5% triisopropylsilane and 2.5% H₂O for 3 h (2.5 mL solution for 100 mg resin). The resin was then removed by filtration and the collected cleavage solution was added over cold diethyl ether (10 mL diethyl ether/mL cleavage cocktail). Following precipitation at -20 °C, peptides were separated by vacuum filtration, washed with cold diethyl ether, dissolved in 60 % ACN, 0.1 % TFA in water and lyophilized.

RP-HPLC was performed on an UltiMate 3000 system (Dionex, Germering, Germany) using different columns, depending on the peptide hydrophobicity: (1) Grace-Vydac semipreparative C₈ column (250 × 10 mm I.D., 10 µm silica, 300 Å pore size) and analytical C₈ column (250 × 4.6 mm I.D., 5 µm silica, 300 Å pore size) were used for HN peptides and Aβ(1-16); (2) Grace-Vydac semipreparative C₄ column (250 × 10 mm I.D., 10 µm silica, 300 Å pore size) and analytical C₄ column (250 × 4.6 mm I.D., 5 µm silica, 300 Å pore size) were employed for other Aβ sequences. Flow rates were 3 mL/min for semipreparative HPLC and 1 mL/min for analytical HPLC.
Binary gradients of 0.1% aqueous TFA (eluent A) and 0.1% TFA 80% ACN in H₂O (eluent B) were applied and chromatograms were recorded by UV detection at 220 nm.

3.3 Preparation of affinity columns and affinity-mass spectrometry

An aliquot of 100 µL HN in H₂O (1µg/µL) was mixed with 200 µL coupling buffer (100 mM HEPES, 0.5 M NaCl, pH 7.4) and added to 66.6 mg activated CH-Sepharose 4B (Sigma-Aldrich, Germany). After 2 h reaction at 20 °C under vigorous shaking, the mixture was transferred into a microcolumn (MoBiTec, Goettingen, Germany) and washed with 10 mL blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3), 10 mL washing buffer (0.2 M CH₃COONa, 0.5 M NaCl, pH 4) and again with 10 mL blocking buffer. To block unreacted carboxyl groups, the affinity gel was kept in blocking buffer for 1 h at 20 °C. The washing steps with alternating pH were then repeated. Hydrophobically retained molecules were removed by passing through the column 2 mL 6 M guanidine hydrochloride in H₂O, 5 mL PBS (6.46 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.68 mM KCl, pH 7.4), 2 mL 70% ethanol in H₂O and finally 10 mL PBS. The affinity column was stored in 20% ethanol in H₂O at 4 °C.

In a similar manner, 100 µL Aβ(1-40) in TFE (1µg/µL) were mixed with 200 µL coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8) and allowed to react with 66.6 mg activated CH-Sepharose 4B for 2 h at 20 °C. Blocking and washing of the affinity gel were performed as described above.

For affinity-mass spectrometry, an equimolar mixture of 9 µg HN and 5.6 µg neurotensin (Bachem) was dissolved in PBS (pH 7.4) and added to the Aβ(1-40)-affinity column. After 2 h incubation at 20 °C under gentle shaking, the unbound material was removed by washing with 60 mL PBS and elution of the affinity-bound peptide was performed with 2 × 500 µL 0.1% aqueous TFA (15 min each). After lyophilization and Zip-Tip® (Millipore, USA) desalting, supernatant and elution fractions were analyzed by MALDI-mass spectrometry. A similar protocol was
applied to investigate the affinity of HN epitope peptides to Sepharose-immobilized Aβ(1-40) and of Aβ(17-28) to Sepharose-coupled HN. For this purpose, 10 µg of synthetic analyte peptide were applied in each experiment on the corresponding affinity column.

3.4 Bioaffinity determinations using a surface acoustic wave biosensor

The S-sens K5 Biosensor instrument (SAW-Instruments, Bonn, Germany) is a chip-based system for detection of affinity interactions based on the conversion of a high frequency signal into a surface acoustic wave (SAW), through an inverse piezoelectric effect [181, 237]. The velocity of the SAW is affected by changes in mass loading and viscosity caused by molecular interactions on the gold chip surface, which are analyzed as shifts in signal phase and amplitude, respectively.

The gold chip surface was covered with a self-assembled monolayer (SAM) by incubation with 10 mM 16-mercaptohexadecanoic acid in chloroform for 12-16 h at 20 °C. Next, the chip was washed with chloroform and ethanol and inserted into the biosensor instrument, where activation of the SAM-carboxyl groups was carried out using 50 mM NHS and 200 mM EDC (1:1 v/v) in PBS (5 mM Na₂HPO₄, 150 mM NaCl, pH 7.4). Two injections of 10 µM Aβ(1-40) or Aβ(17-28) in PBS (150 µL each, prepared from stock solutions of 0.5 µg/µL peptide in TFE) were performed to immobilize the peptide; unreacted active groups were deactivated with 1 M ethanolamine, 0.5 M NaCl (pH 8.3) and unspecific binding sites blocked with a 150 µL injection of 10 µM neurotensin (Bachem) in PBS. The dissociation constants (KD) of the peptide complexes were determined by injecting solutions of increasing concentrations (0.3-10 µM, prepared from stock solutions of 225 µg/µL peptide in H₂O) of biotinylated HN peptides in PBS (150 µL for each concentration), with regeneration of the surface after each binding with 150 µL ACN:0.1% aqueous TFA (2:1). The flow rate was 20 µL/min for all biosensor operations.

The binding and dissociation curves generated at different ligand concentrations were fitted according to the “1:1- Binding & Residue” model using the program
OriginPro-7.5 (OriginLab Corp., Northampton, USA) and FitMaster addin for Origin (SAW-Instruments, Bonn, Germany). The calculated (observed) rate constants $k_{obs}$ for the 5 channels were averaged for each dilution, plotted against the analyte concentration and linear regression was applied. The equilibrium dissociation constant ($K_D$) was obtained according to the equation:

$$K_D = k_{off} \times k_{on}^{-1}$$

where $k_{off}$ [sec$^{-1}$] is the dissociation rate constant representing the y-intercept and $k_{on}$ [conc$^{-1}$ sec$^{-1}$] the association rate constant representing the slope of the linear best fit.

After completion of the experiments, the gold surface of the chip was completely regenerated using a 1:1 mixture of 96 % H$_2$SO$_4$ and 30 % H$_2$O$_2$ (Piranha solution) for 45-60 min. Next, the chips were washed with ethanol, dried and kept at 4 °C.

### 3.5 Proteolytic epitope-extraction and -excision-mass spectrometry

For proteolytic excision experiments, 10 µg Aβ(1-40) in PBS (6.46 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 137 mM NaCl, 2.68 mM KCl, pH 7.4) were added on the HN-affinity column and allowed to bind for 2 h at 20 °C. Excess peptide was washed away with 60 mL PBS and digestion of the immobilized complex was performed in 250 µL PBS for 2 h at 37 °C, using a protease:peptide (E:S) ratio of 1:20 for trypsin, 1:40 for Asp-N and 1:50 for chymotrypsin. Non-binding peptide fragments were removed with 60 mL PBS until no MS signal was detectable in the last washing fraction (1 mL). The remaining affinity-bound peptides were eluted with 2 × 500 µL 0.1% aqueous TFA for 15 min, respectively. In all excision experiments, Sepharose-immobilized HN and Aβ(1-40) were found protected from proteolytic cleavage at the applied experimental conditions.

In the proteolytic extraction experiments, 10 µg HN/Aβ(1-40) were first digested with trypsin (E:S ratio, 1:20) in 250 µL 50 mM NH$_4$HCO$_3$ solution (pH 7) for 3h/4h
Experimental part

at 37 °C or with Glu-C protease (E:S ratio, 1:20) in 250 µL 100 mM NH₄HCO₃ (pH 7) for 36h/12 h at 37 °C and the resulting peptide mixture was applied onto the HN or Aβ(1-40)-affinity column, respectively. To protect the affinity gel from enzymatic digestion, 6 µg/mL aprotinin and 15 µg/mL leupeptin were simultaneously added to the column. After removal of unbound fragments with 60 mL PBS, the peptides retained on the column were eluted with 0.1% aqueous TFA.

In all experiments, the supernatant, the final washing fraction and the elution fraction were lyophilized, desalted using Zip-Tip® (Millipore, USA) and analyzed by MALDI-FTICR or MALDI-TOF mass spectrometry.

3.6 Mass spectrometry

MALDI-TOF mass spectrometry was performed with a Bruker Biflex linear TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen UV laser (337 nm), a 26-sample SCOUT source and a dual channel plate detector. Acquisition of spectra was carried out at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV. Bruker XMASS Software was used for calibration, acquisition and processing of spectra. Some measurements were also performed on a Waters Micromass TOF Spec 2E (Waters, Milford, Massachusetts, USA), equipped with a nitrogen UV laser (337 nm) and Mass Lynx data system for spectra acquisition and instrument control. The potentials applied were: source 20 kV, focusing 16 kV, extraction 19.5 kV, detector 3.5 kV. A saturated solution of α-cyano-4-hydroxy-cinnamic acid (CHCA) in ACN:0.1% aqueous TFA (2:1) was used as matrix for all MALDI-TOF MS measurements. Prior to the MS analysis, 1 µL matrix solution was mixed with 1 µL analyte solution on the stainless steel sample support and allowed to dry.

High resolution FTICR-mass spectrometry was performed with a Bruker Daltonics Apex II instrument equipped with a 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell and a Scout-100 MALDI (with a 337 nm UV laser) or a nano-ESI ion source. The ions were accumulated at 30 V and extracted at 15 V.
The ICR cell trapping potentials were set at 1.2 V. For MALDI-FTICR mass spectrometry, 0.5 µL from a 50 mg/mL matrix solution of 2,4-dihydroxy-benzoic acid (DHB) in ACN:0.1% aqueous TFA (2:1) were mixed with 0.5 µL analyte solution on the steel sample support and allowed to dry. For nano-ESI-FTICR mass spectrometry of the HN-Aβ(1-40) complex, 25 µL Aβ(1-40) in H2O (100 µM) were mixed with 12.5 µL BG5HNA 6 in H2O (100 µM) and 12.5 µL of 2 mM ammonium acetate (pH 6). The resulting solution (50 µL) contained 2.5 nmol Aβ(1-40) (50 µM final concentration) and 1.25 nmol BG5HNA 6 (25 µM final concentration) in 0.5 mM ammonium acetate (pH 6) and was incubated for 2 h at 20 °C. Nano-ESI-FTICR mass spectra were obtained by accumulation of 15 single scans, with the capillary exit potential set to 20 V and the skimmer 1 set to 10 V, while the capillary potential was adjusted between -1100 V and -1200 V until a stable spray was obtained. The ions were accumulated in the RF-only hexapole for 0.15 s before being transferred into the ICR cell. Calibration, acquisition and processing of spectra were carried out with the XMASS Software (Bruker Daltonics, Bremen, Germany).

ESI-Ion Trap MS measurements were carried out on an Esquire 3000+ Ion Trap mass spectrometer from Bruker Daltonics. Peptides were dissolved at 10-50 µM in 50 % methanol, 2 % acetic acid in water and infused to MS from a programmable syringe pump (Harvard Apparatus, Kent, UK), set at a flow rate of 5-10 µL/min. The ion source parameters were: nebulizing gas (N2) pressure 15-20 psi, drying gas (N2) flow rate 6 L/min, drying gas temperature 200-250 °C, capillary voltage -3.5 kV, end plate offset 500 V, capillary exit 80-120 V.

3.7 ELISA of Humanin and Aβ peptides

Costar flat bottom 96-well microtiter plates (Biorad, München, Germany) were coated overnight at 4 °C with 100 µL/well Aβ(1-40) or (G)3HN in PBS (6.46 mM Na2HPO4, 1.47 mM KH2PO4, 137 mM NaCl, 2.68 mM KCl, pH 7.4). The peptides were initially dissolved at 0.5 µg/µL in TFE and the necessary amounts diluted with PBS to the coating concentration of 5 µg/mL. After 4 × washing with 0.1% Tween-
20 in PBS (PBST), non-specific binding sites were blocked with 5% BSA in PBST (BSAT) for 2 h at 20 °C. The plates were then washed once and 1:3 dilutions (100 µL/well) of the analyte peptides in BSAT were added and allowed to interact with the coated peptides for 2 h. Analyte peptides were diluted to the concentrations employed in ELISA from stock solutions of 450 µM in H₂O (biotinylated HN peptides) and 100 µM in TFE (biotinylated Aβ peptides). The plates were washed 5 × with Cova Buffer (2 M NaCl, 40 mM MgSO₄ in PBST) and then incubated for 1 h with HRP-conjugated anti-biotin antibody (Dianova, Germany), diluted 1:10000 in BSAT. Following 3 washing steps with PBST and one with citrate-phosphate buffer (pH 5), 100 µL 0.1% o-phenylenediamine dihydrochloride (OPD) substrate in citrate-phosphate buffer, containing 0.02 µL 30% H₂O₂, was added to each well. Optical density (OD) was determined at 450 nm on a Wallac 1420 Victor² ELISA Plate Counter (Perkin Elmer, Rodgau, Germany). The non-specific binding (NSB) of analyte samples was assessed from triplicate wells containing all components except the coating peptide and was subtracted from the corresponding ELISA response.

3.8 Molecular modeling *

Theoretical methods based on the Rosetta Protein Structure Prediction and Design Suite [201, 202] were applied for molecular modeling. Using the Rosetta ab initio folding protocol, 10,000 structures of the individual peptides were generated and those with an energy score < -70 units for Aβ(1-40) and < -33 units for HN were selected. The structures were then clustered by the Rosetta cluster application and the 100 most populated clusters used in the docking studies (297 structures of Aβ(1-40) and 513 structures of HN). Docking was carried out with the RosettaDock tool [203]. First, a low-resolution search for each combination of an Aβ(1-40) and a HN structure was performed and 100 predictions generated for each pair, resulting in a total of 15,236,100 complexes. Most of these were removed by an energy cutoff of -15 units for the binding energy. The remaining 912 complexes were submitted to the high-resolution search, using a full-atom representation as previously proposed [238] and 100 predictions per input low-
resolution structure were obtained. These complexes were further filtered by an energy cutoff of -50 units and clustered. The best structures from the 5 lowest-energy clusters were selected for subsequent MD calculations. Simulations were carried out with the AMBER 10 program suite (University of California, San Francisco), using the Cornell et al. force-field [239] (parm99SB) and a periodic water box in the form of a truncated octahedron. After equilibration, production runs of 20 ns were performed in the canonical (NVT) ensemble and the last 5 were chosen for analysis. The binding energies of the complexes were calculated with the molecular mechanics-generalized Born/surface area (MM-GBSA) method, following the protocol proposed by Gohlke et al. [240, 241]. To identify the major interactions, the MM-GBSA energy was decomposed into contributions of amino acid pairs [240].

* Collaboration with Dr. Thomas E. Exner, Laboratory of Computational and Theoretical Chemistry, University of Konstanz, Germany [192]

3.9 Confocal laser scanning microscopy and FRET analysis *

Primary hippocampal neurons were dissected from E18 mice, dissociated, plated on glass cover slides in 24-well plates and maintained in Neurobasal medium (Invitrogen, UK) with 1 × B-27 serum-free supplement (Invitrogen, UK) and 0.5 mM GlutaMAX (Invitrogen, UK). Neurons (DIV as indicated) were treated with BG3HNG 7 for 1 h and then fixed with 4% paraformaldehyde/sucrose (Sigma-Aldrich, Germany) for 15 min, permeabilized with 0.1% Saponin (Sigma-Aldrich, Germany) and blocked with 1 × Immunoblock (Carl Roth, Germany) for 1 h. For dendritic visualization neurons were stained overnight with chicken anti-MAP2 antibody (EnCor Biotechnology, FL, USA) (1 µg/mL), at 4 °C. Cells were labeled with Alexa-647-conjugated anti-chicken antibody and Alexa-488-conjugated streptavidin (Invitrogen, UK) (1 µg/mL) and imaged by Zeiss LSM 710 with an EC Plan-Neofluar 40x/1.30 Oil objective (Zeiss MicroImaging, Germany). Co-occurrence between 7 and the cell surface was correlated by dendritic MAP2 staining with ImageJ (NIH, Bethesda, USA). Intergroup comparisons of colocalization quotient between staining of 7 and dendritic MAP2 staining were performed using Kruskal-
Wallis One Way Analysis of Variance on Ranks. To address the problem of multiple comparisons, the test was followed by a post-hoc Student-Newman-Keuls test. Data were analyzed using SigmaStat for Windows (Systat Software, IL, USA). Significance was defined as $p < 0.05$ (*).

To analyze the proximity of 7 with APP, Neuro-2A mouse neuroblastoma cells (DSMZ #ACC148) were transfected with APP695 (GenBank #A33292) N-terminally tagged by mRFP (GenBank #AF506027), both having been subcloned by PCR in a CMV-driven empty vector backbone of pEGFP-C1 (GenBank #U55763). The cells were then treated and fixed as described above, and were analyzed by FRET, which is observed when two fluorophores are in close proximity ($< 10$ nm). The donor fluorophore was excited with the Argon laser line 488 nm and the emissions recorded with a 34-channel QUASAR Detection Unit (Carl Zeiss MicroImaging, Germany).

* Collaboration with Daniel Schwanzar from the group of Prof. Dr. Christine A. F. von Arnim, Department of Experimental Neurology, University of Ulm, Germany [192]

### 3.10 Participants, neuropsychological examination and serum samples *

Subjects for the study of antigen-bound and free Aβ-autoantibodies levels in healthy adults were recruited in Konstanz, Germany, by notifications at the University of Konstanz, in public clubs, in senior citizen centers and in residential homes for the elderly, as well as in local newspapers and radio stations. The study was approved by the ethics committee of the University of Konstanz and conducted according to the guidelines outlined in the Declaration of Helsinki. Prior to participation, written informed consent was obtained. All participants were cognitively healthy and able to consent (Mini Mental State Examination $> 25$). Each subject received 30 Euro compensation for participation.

Exclusion criteria comprised: psychiatric disorders, a history of psychopharmacological medication, a history of severe head injuries or neurological problems (including epilepsy, stroke and brain tumors), dementia
(according to DSM-IV-TR of the American Psychiatric Association) or mild cognitive impairment in old age [242, 243]. Psychiatric disorders were assessed using the Mini International Neuropsychiatric Interview (M.I.N.I., German version 5.0.0 for DSM-IV). During assessment, only 17 out of 47 participants (aged 18-89, $M = 54.7$, $SD = 25.19$) took at least one of the following types of medications: antihypertensive drugs ($n = 5$), thyroid hormones ($n = 3$), anti-inflammatory and analgesics ($n = 5$), antirheumatic medication ($n = 1$), cortisol ($n = 1$), cholesterol-lowering medication ($n = 3$), antihistamines ($n = 2$), prostate medication ($n = 2$) and hormones or contraceptives ($n = 4$). Thirteen of these participants took only one type of medication; four participants (2 males, 2 females) took three or four types of medication (aged 73, 75, 82 and 87 years).

Blood samples were taken between 8:30 and 11:00 o’clock in the morning. Serum was obtained by centrifugation of the blood samples for 4 min at 2700 g. In order to investigate whether the level of Aβ-IgG immune complexes changes with time, ten participants (five males, five females) aged 26 to 86 years ($M = 52.1$, $SD = 18.48$) donated blood three more times after the initial baseline assessment (time = 0, 1 and 4 weeks), each time between 8:30 and 10:00 o’clock in the morning. From each individual, blood samples were collected exactly at the same time and the same day of the week.

* Collaboration with the group of Prof. Dr. Iris-Tatjana Kolassa, Department of Psychology, University of Konstanz and Institute of Psychology and Education, University of Ulm, Germany [71]

3.11 ELISA determination of Aβ-IgG immune complexes in human serum and cerebrospinal fluid

Costar 96-well ELISA plates (BioRad Laboratories, Munich, Germany) were coated with 100 µL/well of mouse monoclonal antibody (mAb 6E10) solution (c = 1 µg/mL in PBS, pH 7.4) and incubated overnight at 4 °C, followed by 30 min incubation at room temperature (RT). The wells were washed four times with 200 µL/well washing buffer (0.05% Tween-20 v/v in PBS, pH 7.4), and then blocked with 5% BSA (w/v), 0.1% Tween-20 (v/v) in PBS for 2 h at RT. Following
blocking, the plates were washed once with washing buffer and human serum samples were applied in triplicate (100 µL/well, 1:100 dilution in blocking buffer) and incubated for 2 h at RT. After washing the plates five times with washing buffer, 100 µL of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H+L) antibody diluted 1:5000 in blocking buffer were added to each well. After incubation for 1 h at RT, followed by three times washing with washing buffer and once with citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na₂HPO₄, pH 5.0), 100 µL/well of a mixture of o-phenylenediamine dihydrochloride in citrate-phosphate buffer (c = 1 mg/mL) and hydrogen peroxide was added (2 µL of 30% hydrogen peroxide were used per 10 mL of substrate solution). The optical density (OD) at 450 nm was measured on a Wallac 1420 Victor² ELISA Plate Counter (Perkin Elmer, Rodgau, Germany).

The described sandwich ELISA was also optimized for the analysis of CSF samples. Two washing buffers, PBS-Tween (0.05% Tween-20 in PBS, v/v) and PBS-Triton (0.1% Triton X-100 in PBS, v/v) and various CSF dilutions (1:300, 1:100, 1:30, 1:10, 1:3 and 1:1) were tested. The highest OD response was obtained using PBS-Tween for washing and 1:1 CSF dilution.

For both serum and CSF determinations, human serum γ-globulin (IgG) (Calbiochem, Merck, Darmstadt, Germany) was used as reference in each experiment and it was applied in triplicate on each ELISA plate, to allow data to be normalized between plates and different experiments. A stock solution of 7 µg/µL (approximating the average IgG level in serum of healthy individuals) in blocking buffer was prepared, diluted first 33.3 times and then three-fold serially (eight dilutions in total). The non-specific binding (NSB) of the IgG preparation and serum/CSF samples was assessed from triplicate wells containing all components except the mAb 6E10. NSB subtraction as well as calculation of average OD values (from triplicates and at least 2 × repeated experiments), standard deviations (SD) and coefficients of variation (CV) were performed with the WorkOut 2.0 software (Perkin Elmer, Rodgau, Germany).
3.12 ELISA determination of free Aβ-autoantibodies in human serum

Costar 96-well ELISA plates (BioRad Laboratories, Munich, Germany) were coated with 150 µL/well of streptavidin solution (c = 2.5 µg/mL in 5 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) and incubated overnight at 4 °C, followed by 30 min incubation at room temperature (RT). After washing the plates four times with 200 µL/well of washing buffer (0.05% Tween-20 in PBS, v/v, pH 7.4), 100 µL/well of biotin-(G)₅-Aβ(12-40) peptide (c = 2.5 µg/mL in PBS, pH 7.4) were added and incubated for 2 h at RT. Next, the wells were washed four times with 200 µL/well of washing buffer and blocked with 5% BSA (w/v), 0.1% Tween-20 (v/v) in PBS for 2 h at RT. Following blocking, the plates were washed once with washing buffer and human serum samples were applied in triplicate (100 µL/well, 1:100 dilution in blocking buffer) and incubated for 2 h at RT. The next steps (washing, adding the detection antibody and the enzymatic substrate, OD readings) were performed as described in chapter 3.11. Human serum γ-globulin (IgG) (Calbiochem, Merck, Darmstadt, Germany) was used as reference in each experiment and was applied in triplicate on each ELISA plate, as detailed in chapter 3.11. NSB subtraction as well as calculation of average OD values (from triplicates and at least 2 × repeated experiments), standard deviations (SD) and coefficients of variation (CV) were performed with the WorkOut 2.0 software (Perkin Elmer, Rodgau, Germany).

3.13 ELISA determination of total Aβ-autoantibodies in human serum

The levels of total Aβ-autoantibodies (free Aβ-autoantibodies and Aβ-autoantibodies released from immune complexes) were determined by indirect ELISA, using the same experimental conditions described for the determination of free Aβ-autoantibodies (see chapter 3.12), except for the composition of the sample dilution buffer, which consisted of 5% BSA (w/v), 0.1% Tween-20 (v/v) and 1% DMSO (v/v) in PBS. Human serum γ-globulin (IgG) (Calbiochem, Merck, Darmstadt, Germany) was used as reference and applied on each ELISA plate, similarly as presented in chapter 3.11, with the only difference that a stock solution of 12 µg/µL was used. The linear range of the IgG reference curve (R² > 0.97 for
linear regression) was between 0.102-1.056 OD units. All serum samples, which were diluted 1:100, gave absorbance readings within this interval and were included in the statistical analysis. NSB subtraction as well as calculation of average OD values (from triplicates and at least 2 × repeated experiments), standard deviations (SD) and coefficients of variation (CV) were performed with the WorkOut 2.0 software (Perkin Elmer, Rodgau, Germany).

3.14 Determination of Aβ(1-42) and total tau levels in cerebrospinal fluid *

The collection of CSF samples by lumbar puncture and the pre-analytical processing were performed using a standardized protocol [244]. In brief, CSF samples were collected into polypropylene tubes, centrifuged immediately and stored within two hours at -80°C. The CSF levels of total tau (T-tau) were determined using a sandwich ELISA (INNOTEST® hTau Ag, Innogenetics, Belgium), by which both normally phosphorylated and non-phosphorylated tau were detected. The assay was performed according to the manufacturers’ instructions and the laboratory reference ranges were as follows: < 200 ng/L and < 300 ng/L for control individuals below 65 and older than 65 years, respectively. The concentrations of total tau in the analyzed CSF samples were estimated from standard curves obtained for each assay. Tau levels > 350 ng/L were regarded as indicative of a neurodegenerative process. The analytical sensitivity of the assay was 75 pg/mL, and the intra-assay and inter-assay variations were < 8%. The CSF levels of Aβ(1–42) were determined using a commercially available sandwich ELISA kit (INNOTEST® β-amyloid(1–42), Innogenetics, Belgium), according to the protocol supplied with the kit. CSF Aβ(1-42) concentrations of the samples were estimated from standard curves obtained for each assay. Aβ(1-42) levels below 550 ng/L were regarded as abnormal.

* Collaboration with the group of Prof. Dr. Christine A. F. von Arnim, Department of Neurology, University of Ulm, Germany [231]
3.15 Statistical analysis *

Data from the determinations of Aβ-autoantibodies in serum from healthy adults of various ages were analyzed using the R statistical software package of The R Foundation of Statistical Computing (www.r-project.org; version 2.11.1 for Mac OS X, GUI 1.34 Leopard) and GraphPad Prism (www.graphpad.com; version 5.01 for Windows, GraphPad Software, San Diego California USA).

Sample characteristics and the levels of Aβ-autoantibodies (level of free Aβ-autoantibodies vs. Aβ-IgG immune complexes) were calculated with Welch’s two-sample t-test (two-tailed with modified degrees of freedom). Possible correlations between Aβ-autoantibodies, age, neuropsychological test scores and years of education were computed with the Pearson’s r product moment correlation coefficient. Since there was almost no variance (ceiling effect) in the scores of MMSE, Boston naming test, Word recognition test and Figure copy test, these tests were not included into further analysis. P-values of multiple correlations were adjusted according to Holm’s sequential rejection algorithm. The variation over time of Aβ-IgG immune complexes in serum was analyzed by mixed effects repeated measurement analysis of variance model (F-statistic) with a random intercept for participants (package nlme for R). Normality of the model’s residuals was tested using the Shapiro-Wilk normality test and visually inspected by residual density plot and Q-Q plot. All tests for statistical significance were applied with a significance level of $\alpha \leq 0.05$.

Data from the determinations of Aβ-autoantibodies in serum and CSF of AD patients and controls were analyzed with the R statistical software package. Welch’s two-sample t-tests (two-tailed with modified degrees of freedom) were applied to examine differences in demographic and cognitive data between AD patients and controls. Analysis of variance with group as factor and age as covariate were computed in order to investigate differences in the levels of Aβ-IgG immune complexes between both groups. Models’ residuals were tested for normality using the Shapiro-Wilk normality test. For categorical variables, Pearson’s Chi-squared ($\chi^2$) test was computed. Pearson's r product moment
correlation coefficient was calculated in order to investigate possible associations of serum and CSF levels of Aβ-IgG immune complexes with age and neuropsychological performance (MMSE, ADAS-Cog). The diagnostic power of the Aβ-IgG immune complexes in serum and CSF was calculated using ROC curve analysis (package Daim and pROC for R [245]). All tests for statistical significance referred to a significance level with $\alpha \leq 0.05$.

* Collaboration with Franka Thurm from the group of of Prof. Dr. Iris-Tatjana Kolassa, Department of Psychology, University of Konstanz and Institute of Psychology and Education, University of Ulm, Germany [71, 231]
4 SUMMARY

Alzheimer’s disease (AD) is a fatal neurodegenerative disorder with a higher incidence rate among the elderly than all other types of dementia and with no preventive or curative therapies available at present. Although the molecular mechanisms responsible for the onset and progress of AD have not been fully elucidated yet, there is a consensus regarding the essential role played by β-amyloid (Aβ) in the pathological processes underlying AD. According to the “amyloid cascade hypothesis”, an increased production and reduced clearance of Aβ peptide trigger a pathological chain of events that include the accumulation of insoluble, β-sheet pleated amyloid fibrils and eventually synapse and neuron loss. Therefore, promising disease-modifying therapies would consist in the timely administration of molecular agents able to inhibit Aβ fibrillization or/and disrupt preformed Aβ aggregates. Intrinsic factors that naturally contribute to Aβ clearance and to the line of defense against Aβ neurotoxicity are of special interest, since their study may provide a better understanding of the yet unclear mechanisms causing AD and also lead to treatments with presumably minimal side effects. This dissertation focused on the molecular characterization of Humanin (HN) and physiological Aβ-autoantibodies, both endogenous molecules with neuroprotective properties against Aβ toxicity, in view of their potential application in therapy or clinical diagnosis of AD.

HN is a linear 24 amino acids long peptide recently detected in human AD brain. HN specifically inhibits in vitro neuronal cell death induced by Aβ peptides and by amyloid precursor protein and its gene mutations in familial AD, thereby representing a potential therapeutic lead structure for AD; however, its molecular mechanism of action is not well understood. The first part of this thesis focused on the molecular characterization of synthetic HN peptides and the study of their interaction with Aβ(1-40), which may be of particular relevance for the development of AD-specific therapeutics.

Wild-type HN and HN sequence mutations were synthesized by solid-phase peptide synthesis (SPPS) according to Fmoc/tert-butyl chemistry and the RP-
HPLC-purified peptides characterized by MALDI- and ESI-mass spectrometry. The specific binding of HN to Aβ was first shown by affinity-mass spectrometry, using a microcolumn with Sepharose-immobilized Aβ(1-40) and neurotensin as control peptide. A direct analysis of the HN-Aβ(1-40) complex in solution was also performed by high resolution ESI-FTICR mass spectrometry and ascertained the assumed 1:1 stoichiometry of the interaction. The binding affinities of wild-type HN and HN variants to Aβ were subsequently characterized by indirect ELISA, in which the Cys8Ala-mutant exhibited the highest affinity to Aβ(1-40). The wild-type HN and the Ser14Gly-variant had similar binding levels to Aβ(1-40) as the Cys8Ala-mutant at low concentrations, but showed binding saturation at concentrations higher than 0.33 µM. The two HN derivatives in which Cys-8 was alkylated by Acm or substituted with Ser revealed significantly lower affinity to Aβ(1-40) than the wild-type HN peptide. A quantitative determination of the wild-type HN-Aβ(1-40) interaction was performed next by SAW-bioaffinity analysis and provided an equilibrium dissociation constant (K_D) in the low micromolar range. The biosensor quantification results were in good agreement with the ELISA determinations.

To identify the epitope sequence of HN involved in Aβ binding, proteolytic epitope-extraction and -excision in combination with mass spectrometry (MS) were employed, using an Aβ(1-40)-affinity column and Glu-C, trypsin and chymotrypsin proteases. Combining the results from these experiments, the interacting region of HN with Aβ(1-40) was assigned to HN(5-15), within the (3-19) “core” domain previously shown to comprise the essential residues for the in vitro neuroprotective effect of HN. Two elongated variants of the HN(5-15) epitope, with reported neuroprotective efficacies similar to that of wild-type HN were subsequently prepared by SPPS, subjected to RP-HPLC purification and characterized by ESI-mass spectrometry. The binding of the HN epitope peptides to Aβ(1-40) was first ascertained by affinity-MS. Kinetic measurements with the SAW-biosensor were further performed and showed similar affinities to Aβ(1-40) for Cys8Lys-HN(3-19) epitope variant and for HN(3-19), but lower compared to wild-type HN. This finding suggests a contribution to the HN-Aβ(1-40) interaction of amino acid residues
outside HN(3-19) region, which are not essential for the binding, but probably help stabilize the peptide complex.

For the identification of the Aβ epitope recognized by HN, proteolytic epitope-extraction and -excision combined with mass spectrometry were also applied, using a HN-affinity column and trypsin, Glu-C, Asp-N and chymotrypsin proteases. The conclusion consistent with all proteolytic results is that HN interacts with the Aβ(17-28) sequence, known to be critically important for the aggregation of Aβ. Furthermore, residues Lys-16, Leu-17, Phe-19, Phe-20, Asp-23 and Lys-28 were found protected by HN binding against proteolytic cleavage, which suggests their involvement in the HN-Aβ(1-40) complex. The Aβ epitope was ascertained by ELISA with Aβ(1-40) and Aβ partial sequences as analytes added to immobilized HN. Thus, all Aβ peptides comprising the (17-28) epitope bound to HN, while the Aβ peptides lacking the epitope sequence showed no affinity to HN. The specific binding of the synthetic epitope peptide Aβ(17-28) to HN was next confirmed in an affinity-MS experiment. SAW-bioaffinity analyses were subsequently performed for the quantitative determination of the interactions between HN epitope peptides and the Aβ(17-28) epitope. The obtained KD values were similar to those obtained for the binding of HN epitope peptides to full Aβ(1-40) sequence. Finally, a molecular dynamics simulation of the HN-Aβ(1-40) complex was consistent with the binding specificity and shielding effects of the HN and Aβ interaction epitopes.

The second part of the thesis was focused on the determination of physiological Aβ-autoantibodies in serum and CSF from healthy individuals and AD patients. Aβ-autoantibodies are currently investigated as potential therapeutic and diagnostic tools for AD. However, the analysis of free and total Aβ-autoantibodies (after dissociation of preformed Aβ antigen-antibody immune complexes) in serum has yielded so far inconsistent results regarding their function and biomarker value.

A major goal of this dissertation was to establish a novel sandwich ELISA for the direct determination of antigen-bound Aβ-autoantibodies, which have not been yet investigated in previous studies. Moreover, two indirect ELISA protocols for the measurement of free and total Aβ-autoantibodies were also developed. The new
ELISAs were designed on the evidence obtained in our laboratory, indicating that “fibril-inhibiting” Aβ-autoantibodies recognize the Aβ(21-37) epitope. Optimization procedures were carried out regarding, e.g., concentration of the coating antibody, sample preparation protocol, composition of washing buffer, number of washing steps after analyte addition, concentration of the detection antibody. The optimization ELISAs were carried out using a commercially available human IgG preparation and serum samples from healthy individuals.

The new ELISA protocols were first employed to investigate whether the levels of Aβ-autoantibodies in serum of healthy individuals correlate with age and cognitive status and may therefore represent an early indicator of an age-associated cognitive decline. For this purpose, 39 serum samples from healthy individuals covering the adulthood from 18 to 89 years were analyzed by each method. Neuropsychological examination of the participants in this study indicated non-pathological, age-related cognitive decline, revealed especially by tests of visual memory and executive function, as well as by speed-related tasks. The ELISA serum determinations showed significantly higher levels of Aβ-IgG immune complexes compared to free Aβ-autoantibodies, while the levels of total Aβ-autoantibodies exceeded both, as expected. The identification of circulating Aβ-IgG immune complexes in serum provides a direct proof for the role of Aβ-autoantibodies in the binding and subsequent clearance of Aβ in vivo. However, no correlation between the levels of antigen-bound, free or total Aβ-autoantibodies and age or cognitive performance of the participants was found, suggesting that healthy aging per se is not associated either with an altered production of Aβ-autoantibodies or with an altered antigen-binding avidity, as reported in the case of AD patients. The balanced formation and removal of the immune complexes in healthy individuals is also supported by the observed stability of Aβ-IgG immune complexes in serum over the investigated period of four weeks.

In the last part of the thesis, the new sandwich ELISA was applied for the determination of antigen-bound Aβ-autoantibodies in serum and CSF from 58 AD patients and 54 age- and gender-matched control subjects. Both serum and CSF levels of Aβ-IgG immune complexes were found to be significantly higher in AD
patients compared to control subjects. Moreover, the levels of Aβ-IgG complexes were negatively correlated with the cognitive status across the groups, increasing with declining cognitive test performance of the subjects. Serum determinations of total Aβ-autoantibodies were also performed on a smaller sample size and revealed as well significantly higher levels in AD patients than in control subjects and an inverse correlation with the cognitive status across the groups. Taken together, the results suggest an increased immune response in AD, which may be associated with deficient Aβ-IgG removal. These findings may contribute to clarify the role of Aβ-autoantibodies in AD pathophysiology and the potential applicability of Aβ-autoantibodies as biomarkers for AD.
ZUSAMMENFASSUNG


HN ist ein lineares Peptid von 24 Aminosäuren, das im menschlichen AK-Gehirn nachgewiesen wurde. HN hemmt spezifisch in vitro den neuronalen Zelltod, den Aβ und das Amyloid-Vorläufer-Protein und seine Genmutationen verursachen, wodurch HN eine potentielle therapeutische Leitstruktur für AK ist; sein molekularer Wirkmechanismus ist jedoch bisher nicht aufgeklärt. Der erste Teil der Arbeit konzentrierte sich auf die molekulare Charakterisierung von synthetischen HN-Peptiden und das Studium ihrer Wechselwirkung mit Aβ(1-40), die von besonderer Bedeutung für die Entwicklung von AK-spezifischen Therapeutika sein könnten.

Zusammenfassung


bisherige Analyse der freien und gesamten Aβ-Autoantikörper (nach Dissoziation der bestehenden Aβ-Antigen-Antikörper-Immunkomplexe) in Serum und Liquor widersprüchliche Ergebnisse in Bezug auf ihre Funktion und Biomarker-Werte.


6 References


References


References


References


References


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7 APPENDIX

7.1 Appendix 1

Abbreviations:

Aβ  β-amyloid
Acm  acetamidomethyl
ACN  acetonitrile
AD  Alzheimer’s disease
ApoE  apolipoprotein E
APP  amyloid precursor protein
Bax  Bcl2-associated X protein
BSAT  5% BSA in PBST
Calc.  calculated
cDNA  complementary DNA
CMV  cytomegalovirus
CSF  cerebrospinal fluid
CV  coefficient of variation
DMSO  dimethyl sulfoxide
EDC  1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELISA  enzyme-linked immunosorbent assay
ESI  electrospray ionization
Exp.  experimental
Fc  fragment crystallizable
FRET  Förster resonance energy transfer
FTICR  Fourier transform ion cyclotron resonance
hAPP  human amyloid precursor protein
HN  humanin
HRP  horseradish peroxidase
Ig  γ-globulin
IVIg  intravenous immunoglobulin
$K_D$  equilibrium dissociation constant
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>mRFP</td>
<td>monomeric red fluorescent protein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NSB</td>
<td>non-specific binding</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>0.1% Tween-20 in PBS</td>
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<td>positron emission tomography</td>
</tr>
<tr>
<td>p-tau</td>
<td>phosphorylated tau</td>
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<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
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<tr>
<td>RP-HPLC</td>
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</tr>
<tr>
<td>SAW</td>
<td>surface acoustic wave</td>
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<td>standard deviation</td>
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<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>TOF</td>
<td>time of flight</td>
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7.2 Appendix 2

a)

Figure A2 (a) A. Fluorescence staining of microtubule-associated protein 2 (MAP2) in mouse hippocampal neurons (DIV 7), using chicken anti-MAP2 antibody and Alexa-647-conjugated anti-chicken antibody; B. Immunofluorescence staining of endogenous hAPP in mouse hippocampal neurons (DIV 7) with a rabbit anti C-terminal APP antibody (Sigma-Aldrich, Germany) and Alexa-488-conjugated secondary antibody; C. Staining of the neuronal nucleus with 4',6-diamidino-2-phenylindole (DAPI); D. (A+B) merged images, with additional use of DAPI; DIV – days in vitro;

(b) A. Fluorescence microscopy of mRFP-hAPP695 overexpressed in Neuro-2A mouse neuroblastoma cells; B. no close proximity between BG_{7} and mRFP-hAPP695 was observed in FRET; C. (A+B) merged images, with additional use of DAPI [192]
7.3 Appendix 3

a)

b)

MW_{calc.} = 5366.9
MW_{exp.} = 5366.8

MW_{calc.} = 5366.9
MW_{exp.} = 5366.8

C)
Figure A3 Humanin dimerization through disulfide bridge formation, analyzed by ESI-Ion Trap MS. (a) HN disulfide dimerization after incubation at a concentration of 50 µM in 1:1 DMSO:NH₄OAc (0.5 mM, pH = 6) for 2 h. The isotopic distribution of the (7+) molecular ion of the HN dimer is shown in the insert; (b) A small extent of disulfide dimerization was observed in HN upon incubation in PBS at a concentration of 50 µM for 8 h; (c) No disulfide dimerization was observed in HN upon incubation in PBS at a concentration of 50 µM for 8 h in presence of a 2-fold molar excess of DTT.
7.4 Appendix 4

Figure A4  MALDI-TOF MS of the elution fraction from HN(3-19)C8K 12 affinity binding to Sepharose-immobilized Aβ(1-40)
Figure A5  Optimization of washing buffer composition and number of washing steps in sandwich ELISA. (a) Influence of washing buffer composition and number of washing steps on the OD response (mean ± SD of three determinations) in sandwich ELISA, using 1/33.3 and 1/100 dilutions from an IgG stock solution (7 µg/mL) as test analyte. For each IgG dilution, the coefficient of variation (CV) between the OD values obtained under the various experimental conditions was below the accepted upper limit for the intra-assay CV (10%), indicating no significant differences. Highlighted with a red circle are the finally applied conditions for the determination of \( \beta \)-amyloid immune complexes; (b) Effect of washing buffer composition and number of washing steps on the OD response (mean ± SD of three determinations) in sandwich ELISA, using 1/33.3 and 1/100 dilutions from a serum sample as test analyte. For each serum dilution, the CV between the OD values obtained under the various experimental conditions was below the accepted upper limit for the intra-assay CV (10%), indicating no significant differences. Highlighted with a red circle are the finally applied conditions for the determination of \( \beta \)-amyloid immune complexes [71].
### 7.6 Appendix 6

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<td>b</td>
<td>44</td>
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* Significant Pearson correlation between cognitive test performance and age
** Significant Pearson correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm
### 7.7 Appendix 7

**Table A7** Pearson’s *r* correlations between the ratio of serum levels of Aβ-IgG immune complexes to free Aβ-autoantibodies and cognitive performance (*n* = 33) [71]

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<th><em>p</em>-value</th>
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Benton test (correct answers; range 0-20); Benton test (errors; range 0-30); Boston naming test (CERAD-NP-plus; range 0-15); Digit span test (HAWIE-R; range 0-28); Digit-symbol substitution test (HAWIE-R; range 0-93); Figure recall (CERAD-NP-plus; range 0-14); Mosaic test (HAWIE-R; range 0-51); Phonemic/Semantic fluency (CERAD-NP-plus); TMT-A/B – Trail making test part A/B (CERAD-NP-plus; A: range 0-180 sec.; B: range 0-300 sec.); Word list learning (CERAD-NP-plus; range 0-30); Word recall (CERAD-NP-plus; range 0-10)

* Significant correlation between cognitive test performance and age

** Significant correlation between cognitive test performance and age after correction for multiple correlation coefficients according to Holm