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Alzheimer's disease and dementia with Lewy bodies: Special focus on the role of serpins

Nielsen, Henrietta

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ALZHEIMER'S DISEASE AND DEMENTIA WITH LEWY BODIES:

SPECIAL FOCUS ON THE ROLE OF SERPINS

Henrietta Nielsen, BSc, MSc.

Lund University
Faculty of Medicine
Department of Clinical Sciences, Malmö
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DOCTORAL THESIS

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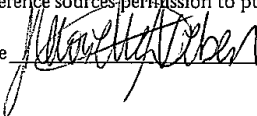
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Abstract

Serine protease inhibitors (Serpins) are involved in the pathogenesis of neurodegenerative dementia, including the two most common types, Alzheimer's disease (AD) and dementia with Lewy bodies (DLB). The pathological characteristics of AD include senile plaques, mainly composed of aggregated amyloid-beta peptide (Abeta1-42), but also serpins, and neurofibrillary tangles of hyperphosphorylated tau protein. Pathological hallmarks of DLB include aggregates of alpha-synuclein (Lewy bodies), however, co-existing AD pathology is also frequently found. In the present work, we have investigated the role of three serpins, namely alpha1-antichymotrypsin, alpha1-antitrypsin and neuroserpin, in the context of AD and DLB. We have shown that alpha1-antichymotrypsin: (i) renders the oligomer formation profile of incubated Abeta1-42, favouring dimer formation; (ii) under certain conditions appears to protect Abeta1-42 from chymotrypsin digestion and; (iii) in combination with soluble forms of Abeta1-42, significantly affects the global gene expression of primary fetal human astrocytes; (iv) can influence binding and, potentially, uptake of aggregated Abeta1-42 in primary adult human astrocytes.

In two clinical studies we have: (i) for the first time, determined cerebrospinal fluid levels of neuroserpin and established a link to AD as significantly higher levels of neuroserpin were found in AD patients than in non-demented controls and DLB patients; (ii) showed that higher levels of cerebrospinal fluid alpha1-antitrypsin and plasma alpha1-antichymotrypsin correlate to lower cognitive function in patients with DLB and AD, respectively; (iii) showed that patients with AD and DLB have higher levels of intercellular adhesion molecule-1 and platelet endothelial cell adhesion molecule-1.

Our findings support the statement that inflammatory and vascular mechanisms are involved in dementia pathogenesis and suggest that serpins most likely are involved in the processes leading to cognitive dysfunction. Further research is needed to assess the distinct actions of serpins in the mechanisms leading to neurodegeneration and dementia.

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To my family

Success is the ability to go from one failure to another,
with no loss of enthusiasm
Sir Winston Churchill

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Asplund A, Nielsen HM, Sun Y-X, Janciauskiene S, Desai UR, Wright HT.
Submitted

Paper II[#]

Effects of Alzheimer's peptide and alpha1-antichymotrypsin on astrocyte gene expression.
Baker C, Nielsen HM, Minthon L, Wright HT, Chappell S, Okyere J, May S, Morgan K, Kalsheker N, Janciauskiene SM.
Neurobiology of Aging. 2007 Jan;28(1):51-61. Epub 2005 Dec 20.

Paper III[*]

Plasma and CSF serpins in Alzheimer disease and dementia with Lewy bodies.
Nielsen HM, Minthon L, Londos E, Blennow K, Miranda E, Perez J, Crowther DC, Lomas DA, janciauskiene SM.
Neurology. 2007 Oct 16;69(16):1569-79. Epub 2007 Aug 29.

Paper IV[#]

Soluble adhesion molecules and angiotensin-converting enzyme in dementia.
Nielsen HM, Londos E, Minthon L, Janciauskiene SM.
Neurobiology of Disease. 2007 Apr;26(1):27-35. Epub 2007 Jan 31.

Paper V

Binding and uptake of aggregated Aβ1-42 by primary human astrocytes *in vitro*.
Nielsen HM, Veerhuis R, Holmqvist B, Janciauskiene SM
Manuscript

Abbreviations

Serpin	serine protease inhibitor
AD	Alzheimer's disease
DLB	dementia with Lewy bodies
DSM-IV	diagnostic and statistical manual of mental disorders, 4th edition
NINCDS-ADRDA	National institute of neurological and communicative disorders and stroke, and the Alzheimer's disease and related disorders association
MMSE	mini mental state examination
ADAS-COG	The Alzheimer's disease assessment scale
MRI	magnetic resonance imaging
PET	positron emission tomography
BPSD	behavioural and psychological symptoms in dementia
CSF	cerebrospinal fluid
Aβ	amyloid-beta peptide
T-tau	total tau
P-tau	phosphorylated tau
LOAD	late onset Alzheimer's disease
EOAD	early onset Alzheimer's disease
APP	amyloid precursor protein
ApoE	apolipoprotein E
CAA	cerebral amyloid angiopathy
NFT	neurofibrillary tangle
GFAP	glial fibrillary acidic protein
HLA	human leukocyte antigen
REM	rapid eye movement
DAT	dopamine active transporter
PINK-1	PTEN-induced putative kinase-1
LRRK2	leucine-rich repeat kinase 2
H-FABP	heart-type fatty acid-binding protein
CBG	corticosteroid-binding globulin
MENT	myeloid and erythroid nuclear termination stage-specific protein
RCL	reactive centre loop
FENIB	familial encephalopathy with neuroserpin inclusion bodies
CNS	central nervous system
tPA	tissue plasminogen activator
ADDL	Aβ-derived diffusible ligand
BBB	blood-brain-barrier
rCBF	regional cerebral blood flow
NSAID	non steroidal anti-inflammatory drug
CRMP2	collapsing response mediator protein-2
MCP-1	monocyte chemoattractant protein-1
ELISA	enzyme linked immuno-sorbent assay
ICAM-1	intercellular adhesion molecule-1
VCAM-1	vascular cell adhesion molecule-1
PECAM-1	platelet endothelial cell adhesion molecule-1
ACE	angiotensin converting enzyme

1. Introduction

The term *dementia* is derived from the Latin word *demens*, "de mens" (out of mind). Clinically, dementia is generally defined as a state of serious emotional and mental deterioration of organic or functional origin (Clarfield 2000; Lockhart and Lestage 2003). The different clinical forms of dementia such as Alzheimer's disease and dementia with Lewy bodies, Parkinson's disease dementia, frontal lobe dementia and vascular dementia are accompanied by cognitive and behavioural decline and all result from progressive neurodegeneration or vascular damage, which culminate in the development of severe dementia (Lockhart and Lestage 2003). According to the Global Burden of Disease estimates for the 2003 World Health Report, the contribution of dementia to years with disability in people aged 60 years and older was 11.2%, which is more than stroke (9.5%), musculoskeletal disorders (8.9%), cardiovascular disease (5.0 %) and all forms of cancer (2.4%) (Ferri, Prince et al. 2005). Dementia affects 7 % of the general population older than 65 years and 30 % of those older than 80 years (McKeith, Mintzer et al. 2004). The Delphi Consensus Study estimated in 2005 that 24.3 million people were afflicted by dementia, with 4.6 million new cases every year. The incidence would, according to this study (Ferri, Prince et al. 2005), double every 20 years. Considering the alarming dementia prevalence and increasing healthcare burden, it is of greatest importance to improve the knowledge of underlying disease processes in order to improve disease diagnosis and treatment.

In this thesis, we aimed to investigate the role of SERine Protease INhibitors (SERPINs) in the two major neurodegenerative dementias, namely that of Alzheimer's type and dementia with Lewy bodies, both of which still lack a cure. To date, approximately 800 members of the serpin superfamily of homologous proteins (350–500 amino acids in size) have been identified. As indicated by the family name, the majority of these proteins are serine protease inhibitors, but inhibitors of cysteine proteases and some non-inhibitory proteins are also included, due to their structural homology. Serpins are crucial in a vast variety of physiological processes such as inflammation, coagulation, fibrinolysis, apoptosis, complement activation, protein folding, neoplasia and viral pathogenesis (Irving, Pike et al. 2000; Silverman, Bird et

al. 2001). We have in this work chosen to investigate a few members of the serpin family, namely α1-antichymotrypsin, α1-antitrypsin and neuroserpin, which in earlier studies have been linked to dementia (Abraham, Shirahama et al. 1990; Gollin, Kalaria et al. 1992; Davis, Shrimpton et al. 1999). The main focus of this work is on serpins in Alzheimer's disease, but also in dementia with Lewy bodies.

2. Alzheimer's disease

Among the dementia disorders, Alzheimer's disease, first described in 1907 by Dr Alois Alzheimer (Translated by L. Jarvik and H. Greenson (1987; Alzheimer, Stelzmann et al. 1995)), is the most commonly diagnosed form of dementia, accounting for more than half of the cases (Fratiglioni, Launer et al. 2000; O'Brien, Erkinjuntti et al. 2003). The prevalence of Alzheimer's disease was estimated to 26.6 million people worldwide in 2006 and was projected to increase to 106.2 million by 2050, with the largest rates of increase in Africa (476%), Asia (497%) and Latin America and the Caribbean (534%) (Brookmeyer, Johnson et al. 2007).

2.1 Clinical picture and diagnosis

The disease is clinically diagnosed ante-mortem after ruling out other specific causes and manifests itself by progressive memory impairment according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) 2000, by the American Psychiatric Association, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) work group and the revision thereof (McKhann, Drachman et al. 1984; Dubois, Feldman et al. 2007). To fulfil the clinical criteria for Alzheimer's disease, one or more of the cognitive disturbances aphasia (language disturbance), apraxia (impaired motor activities despite intact motor function), agnosia (failure to identify or recognise objects despite intact sensory function) and disturbance in executive functions like planning, organizing, sequencing and abstracting, also has to be present. These cognitive deficits cause significant impairment in the social or occupational functioning of the patient. Neuropsychological tests are used to provide confirmatory evidence of dementia

(McKhann, Drachman et al. 1984; Dubois, Feldman et al. 2007). Common tests used to evaluate the cognitive state of the patient and to assess treatment efficacy include the Mini Mental State Examination (MMSE) (Folstein, Folstein et al. 1975) that assesses orientation in time and place, immediate recall, short-term verbal memory, calculation, language, and construct ability and grades the cognitive status of the patient on a scale of 0-30 points, where higher points indicate higher cognitive function. The Alzheimer's Disease Assessment Scale-Cognitive (ADAS-COG) is an examination that includes short neuropsychological tests, observations of the patient's behaviour by the clinician and interviews with the patient's caregiver (Mohs, Rosen et al. 1983; Pena-Casanova 1997). In addition, in the newly published proposal for the revision of the NINCDS-ADRDA criteria from 1984, imaging investigations, structural neuroimaging with magnetic resonance imaging (MRI) determining medial temporal lobe atrophy and molecular imaging with positron emission tomography (PET) determining functional deficits such as reduced glucose metabolism, have been added as supportive diagnostic features (Dubois, Feldman et al. 2007). Further, other tests are used to assess behavioural and psychological symptoms in dementia (BPSD) including non-cognitive behaviour such as wandering, agitation, sexually inappropriate behaviour and psychological symptoms such as depression, anxiety and delusions (Finkel, Costa e Silva et al. 1996).

2.1.1 Biomarkers

The NINCDS-ADRDA criteria from 1984 (McKhann, Drachman et al. 1984) stated that Alzheimer's disease can not be diagnosed by laboratory tests. Numerous markers have since then been investigated for their diagnostic utility in diagnosing Alzheimer's disease and to distinguish the disease from other dementia types. To date, three main markers have been shown to yield high sensitivity and specificity and are widely used in clinical practise. These markers, which are considered to reflect the pathological processes of Alzheimer's disease, including neuronal degeneration, hyperphosphorylation of tau protein with subsequent formation of tangles, and deposition of the amyloid-β 1-42 (Aβ1-42) peptide (see below), are measurements of the levels of total-tau (T-tau), phosphorylated tau (P-tau) and Aβ1-42 in cerebrospinal

fluid (Blennow and Hampel 2003; Hampel, Mitchell et al. 2004; Andreasen and Blennow 2005). Low levels of Aβ1-42 and higher levels of tau are significantly associated with Alzheimer's disease. Moreover, these markers are stable in longitudinal studies (Blennow, Zetterberg et al. 2007) and have also been evaluated as predictive markers of patients converting from mild cognitive impairment to Alzheimer's disease with increasing cognitive decline (Hansson, Zetterberg et al. 2006; Andersson, Blennow et al. 2007; Brys, Pirraglia et al. 2007). In the proposed revision of the NINCDS-ADRDA criteria, the past decade's extensive research on biomarkers has been taken into account and combinations of the presence of low Aβ1-42 as well high tau and phosphorylated tau levels in the cerebrospinal fluid, have been added as supportive features of Alzheimer's disease (Dubois, Feldman et al. 2007). In addition, a large variety of other markers have also been assessed for their quality as disease biomarkers. Several plaque-associated proteins have been suggested as potent markers of Alzheimer's disease, among them are the serpin and acute-phase protein α1-antichymotrypsin (Matsubara, Hirai et al. 1990) (see below). However, low sensitivity and specificity have been a problem in establishing these markers. Recent studies have, on the other hand, proposed biomarkers that yielded high accuracy. For instance, 18 signalling proteins in plasma, including chemokines and cytokines, adhesion molecules and growth factors, together have been shown to create a disease-specific phenotype that can distinguish Alzheimer's disease patients from non-demented controls with nearly 90 % accuracy. Further, this "cellular communicome" could identify and predict conversion to Alzheimer's disease in patients suffering from mild cognitive impairment, years before the actual turn-over (Ray, Britschgi et al. 2007). At the moment however, follow-up studies establishing the reference values and validity of these new biomarkers are still needed.

2.2 Genetics

The majority (90-95%) of Alzheimer's disease cases are non-familial, "sporadic" cases of late ($\geq$ 65 years) onset (LOAD), whereas the inherited forms of Alzheimer's disease, with an early disease onset (EOAD), account for as low as 5-10 % but as high as 50 % of all cases. Phenotypic analyses have shown that these two variants of

Alzheimer's disease have a strong similarity and are often indistinguishable (Selkoe 2001; Coppede, Mancuso et al. 2006).

To date, several autosomal dominantly inherited mutations are well-documented to predispose to Alzheimer's disease and to increase the production or deposition of Aβ in the brain (for significance, see below). Mutations are known within four genes encoding the amyloid precursor protein (APP), presenilin 1 and 2 (part of the γ-secretase protease complex) and apolipoprotein E (ApoE) (Selkoe 2001). Mutations on the APP and presenilin genes are associated with EOAD while the ApoE ε4 allele is the major risk factor for LOAD (Strittmatter, Saunders et al. 1993). On the other hand, the ε2 allele has been shown to have a protective role against the development of the disease (Corder, Saunders et al. 1994). Within each of the 4 genes mentioned, several mutations exist. For example, several APP mutations, altering the proteolysis of the protein, have been found in different families, including the Flemish, Dutch, Arctic, Italian and Iowa mutations. Phenotypically, these mutations include some pathological distinctions such as intracerebral haemorrhages and extensive amyloid deposition (Nilsberth, Westlind-Danielsson et al. 2001).

2.3 Pathology

Macroscopically, the Alzheimer's disease brain is affected by atrophy (cortical atrophy and widening of the ventricles) involving brain areas implicated in learning and memory processes (mainly the temporal and parietal cortex, as well as the frontal cortex, the amygdala and hippocampus), with loss of brain weight and volume. This reduction in brain volume is due to a prominent degeneration of synapses and neurons. In older patients (>80 years), cerebral atrophy has been shown to be more specific to the temporal cortex (Anderson and Hubbard 1981; Hubbard and Anderson 1981; Hubbard and Anderson 1981; Mattson 2004). In addition, vascular pathology such as cerebral amyloid angiopathy (CAA), deposition of congophilic material (Aβ) in the cerebral and mingeal blood vessels, is frequently found in Alzheimer's disease, with a prevalence of between 82-98 % (Jellinger 2002). However, the two major microscopic neuropathological characteristics (Mirra, Heyman et al. 1991; Braak, Braak et al.

1993) for the post-mortem diagnosis of definite Alzheimer's disease are extracellular amyloid deposits, called senile (neuritic) plaques, and intracellular accumulations of hyperphosphorylated tau in neurofibrillary tangles (NFT) (Hardy and Allsop 1991; Rosenberg 2000; Selkoe 2001).
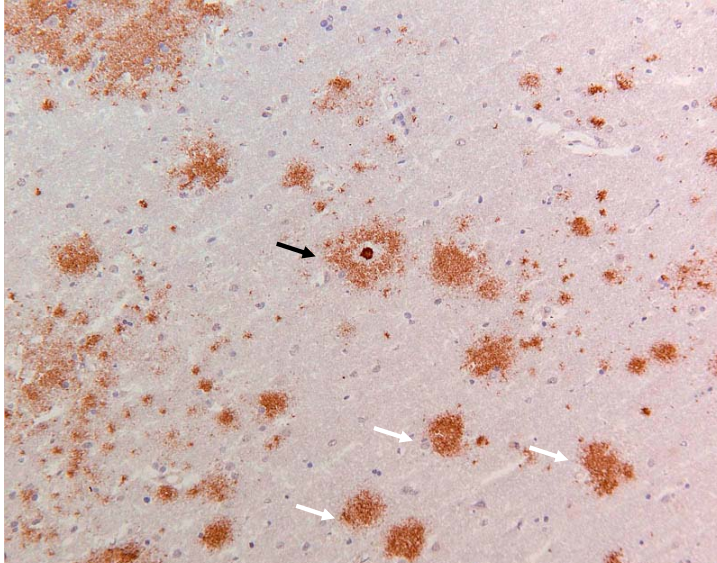
Figure 1. Amyloid plaques (brown) identified using immunohisto-chemistry (anti-Aβ plaques) (white arrows). A classical plaque with dense amyloid core is pointed out (black arrow). Unpublished results. Dr Robert Veerhuis.

2.3.1 Senile plaques and associated cells and proteins

Plaques are primarily composed of the Aβ peptide (Figure 1) , which in the senile plaques principally occurs as masses of amyloid fibrils (Selkoe 2001). Plaques can further be divided into fibrillar and non-fibrillar plaques (diffuse plaques), based on morphology, neuritic changes (degeneration of neuronal cell processes) and congophily. Non-fibrillar plaques are found also in non-demented subjects and do not correlate with intellectual impairment. However, the density of these plaques is linked to mental status. The fibrillar Aβ plaque, that is associated with mental decline, can be divided into a subset of three types; (1), the classical (senile) plaque (sized 20-60 μm) with a dense, Congo red positive core and associated degenerating neurites, (2), the primitive, round-shaped well-demarcated plaque (20-60 μm), also associated with neurite degeneration but lacking a central core, and moderately Congo-red positive,

(3), the "burnt-out", compact, small plaque (5-15 μm) with a strongly Congo-red positive core, but without neuritic changes (Delaere, Duyckaerts et al. 1991; Dickson 1997).
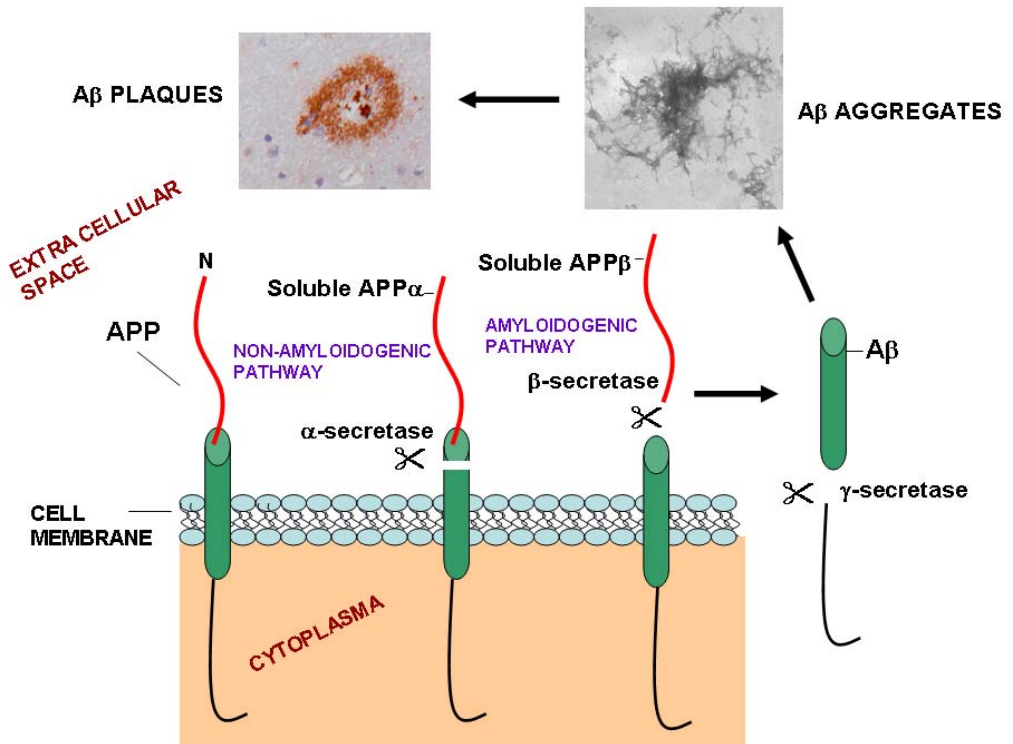
Figure 2. Generation of Aβ with the subsequent formation of Aβ aggregates and plaques

Intact Aβ is proteolytically cleaved from its precursor protein, amyloid precursor protein (APP), by proteases referred to as β- and γ-secretases (Haass 2004) and occurs as 40 and 42 amino acid peptides (Aβ40, Aβ42), of which the Aβ42 is the major component of plaques in the human brain (Citron, Diehl et al. 1996). In contrast to the amyloidogenic pathway, APP cleavage by α-secretases (the non-amyloidogenic pathway) does not generate Aβ (Figure 2). Usually co-localised with Aβ42 in the senile plaques is Aβ40, a shorter form that is less prone to aggregation (Jarrett, Berger et al. 1993). Aβ42 is produced and secreted as a soluble peptide during normal metabolism in cultured cells (Haass, Schlossmacher et al. 1992) and can be detected in brain and cerebrospinal fluid from Alzheimer's disease patients and normal individuals

(Palmert, Usiak et al. 1990; Shoji, Golde et al. 1992). Intitally, Aβ is soluble but forms aggregates under the influence of changes in the local environment, thereby contributing to the extracellular, insoluble plaques (Iversen, Mortishire-Smith et al. 1995). In addition to Aβ, more than 40 proteins have been found in the plaques. The majority of these proteins are associated with inflammation. This vast group of amyloid-associated proteins includes complement proteins, inflammatory cytokines, acute-phase reactants, adhesion molecules, lipoproteins and lipoprotein receptors and growth factors. Studies exploring the production of these amyloid associated-components revealed that almost all are produced in the brain and that activated microglia and astrocytes are the probable sources (reviewed in (McGeer and McGeer 1995)).
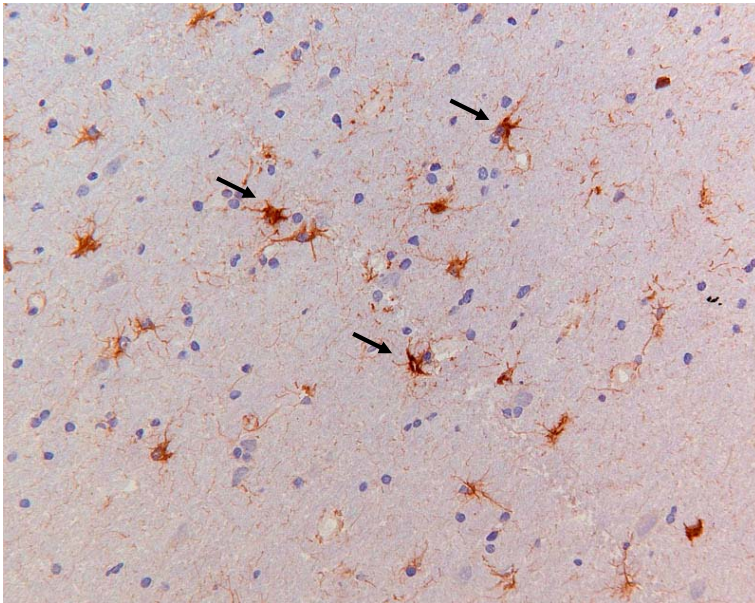
Figure 3. Reactive astrocytes (arrows), visualised using antibodies against the glial fibrillary acidic protein (GFAP), in the brain parenchyma of an Alzheimer's disease patient, are star-like cells with many cell projections. Unpublished results, Dr Robert Veerhuis.

Already in early stages of Alzheimer's disease, clusters of activated microglia, immuno-stained for human leukocyte antigens (HLA-DR), and astrocytes, immunostained for glial fibrillary acidic protein (GFAP) (Figure 3), can be found

intimately associated with the Aβ deposition in senile plaques (Schechter, Yen et al. 1981; Itagaki, McGeer et al. 1989; Rozemuller, Eikelenboom et al. 1989; Mandybur and Chuirazzi 1990; Wisniewski and Wegiel 1991; Abraham 2001; Vehmas, Kawas et al. 2003). These accumulations of activated glia cells are in turn surrounded by degenerating neurons (Smits, Rijsmus et al. 2002). Earlier studies on brain cortex biopsies from Alzheimer's disease patients suggest that astrocytes participate in the degradation of amyloid fibres (Wisniewski and Wegiel 1991) and constitute the leading factor in plaque degeneration (Wegiel, Wisniewski et al. 2000). Microglia have also been shown to be able to remove Aβ *in vitro* and *in vivo* (Ard, Cole et al. 1996; Strohmeyer, Kovelowski et al. 2005; Familian, Eikelenboom et al. 2007). However, due to their role in plaque development, as a major source of fibrillar Aβ in the brain and as inducer of secondary changes with neuronal dystrophy and astrocyte hypertrophy, they are proposed to be the driving force in fibrillar plaque formation (Wegiel, Wang et al. 2000).

2.3.2 Neurofibrillary tangles

In order to meet the post-mortem diagnostic criteria of definite Alzheimer's disease, both senile plaques and neurofibrillary tangles have to be present within specific regions of the cerebral cortex (McKhann, Drachman et al. 1984). Many neurons in the brain areas affected by Alzheimer's disease, contain bundles of abnormal fibres that fill the perinuclear cytoplasm of the cells and alter synaptic signal transmission. The main component of these highly insoluble (Selkoe, Ihara et al. 1982), aggregated, paired helical filaments, found in the neufibrillary tangles, are hyperphosphorylated, microtubule-associated tau proteins (reviewed in (Buee, Bussiere et al. 2000)). In humans, tau is found in the axons of neurons. However, it can also be found in non-neuronal cells such as glia cells that can express tau under pathological conditions (Tucker 1990; Schoenfeld and Obar 1994). In the brain, the tau protein family consists of 6 isoforms with a molecular weight range of 45-65 kDa and numerous phosphorylation sites (Blennow and Hampel 2003). Neuropil threads and neurofibrillary tangles in Alzheimer's disease develop in a set of cortical pyramidal cells. The neurodestructive processes spread from the entorhinal cortex to the

hippocampus and eventually to the isocortex. Due to the specific sequence of events, a diagnostic distinction of six stages, with a progressive increase in cortical destruction, can be made (Braak, Braak et al. 1993).

3. Dementia with Lewy bodies

Friedrich H Lewy was first to describe inclusion bodies in the dorsal motor nucleus of the vagus nerve of a patient with Parkinson's disease. However, the term "corps de Lewy" was coined by Tretiakoff who further described the same inclusion bodies in the substantia nigra (Gibb and Poewe 1986). Initially associated only with the motor manifestations of Parkinson's disease, Lewy bodies were later also linked to progressive dementia (Okazaki, Lipkin et al. 1961; Woodard 1962). Several terms were thereafter used to describe the appearance of Lewy bodies in dementia, including the diffuse type of Lewy body disease (Kosaka, Yoshimura et al. 1984), senile dementia of Lewy body type (Perry, Irving et al. 1989) and Lewy body variant of Alzheimer's disease (Hansen, Salmon et al. 1990). Today, the disease is called dementia with Lewy bodies according to the consensus guidelines (McKeith, Galasko et al. 1996; McKeith, Dickson et al. 2005). This type of dementia, with strong clinical and pathological similarity to both Parkinson's disease dementia and Alzheimer's disease, was earlier thought to be rare but is now considered to be the second most common type of degenerative dementia in older people, found by autopsy to occur in 10-15% of the cases (McKeith, Galasko et al. 1996). A community-based study of people older than 75 years found that dementia with Lewy bodies represents 22 % of all demented cases. A systematic review of population-based estimates reported a prevalence of 0-5 % in the general population, but 0-30.5 % of all dementia cases (Rahkonen, Eloniemi-Sulkava et al. 2003; McKeith, Mintzer et al. 2004; Zaccai, McCracken et al. 2005). Classical epidemiological studies are, however, still considered as lacking.

3.1 Clinical picture and diagnosis

As for other types of dementia, dementia with Lewy bodies manifests itself by a progressive cognitive decline that interferes with the affected individual's social and

occupational function. For the clinical diagnosis of dementia with Lewy bodies (possible or probable), one or more core features have to be present in combination with one or more suggestive features (probable). The core features are fluctuating cognition with pronounced variations in attention and alertness, recurrent visual hallucinations that are typically well formed and detailed, or spontaneous features of parkinsonism. The suggestive features include severe neuroleptic sensitivity, rapid eye movement (REM) sleep behaviour disorder, pre-synaptic degeneration (using imaging of the dopamine transporter (DAT) as a marker). In addition, several features have been listed as supportive, yet unspecific; repeated falls, syncope, systematised delusions, temporary loss of consciousness, hallucinations in other modalities and depression. As the clinical presentation of dementia with Lewy bodies is similar to both Parkinson's disease dementia and Alzheimer's disease, the consortium on dementia with Lewy bodies stated that many patients with dementia with Lewy bodies would also meet the criteria of Alzheimer's disease. Further, the "one year rule" was established as criterium for the distinction between dementia with Lewy bodies and Parkinson's disease dementia, stating that Parkinson's disease dementia is defined by the presentation of extrapyrimidal symptoms preceding dementia by more than one year. Dementia with Lewy bodies should be the diagnosis if dementia occurs simultaneously or within one year of the presentation of extrapyrimidal symptoms (McKeith, Galasko et al. 1996; McKeith 2006).

3.2 Genetics

As mentioned above, dementia with Lewy bodies is similar to Parkinson's disease dementia and, as will be discussed in the section below. The pathology of these diseases includes Lewy bodies with its major component fibrillar α-synuclein. Even though most cases of Parkinson's disease dementia are sporadic, mutations in the α-synuclein gene have been related to Parkinson's disease with dementia (Polymeropoulos, Lavedan et al. 1997) and later also to dementia with Lewy bodies (El-Agnaf, Curran et al. 1998). A study in 2003, including a large family with autosomal dominant Parkinson's disease, clinically ranging from dementia with Lewy bodies to typical Parkinson's disease, showed that locus triplication of α-synuclein

causes Parkinson's disease. However, this study did not distinguish between Parkinson's disease and dementia with Lewy bodies (Singleton, Farrer et al. 2003). In total, mutations in five genes have been associated with familial Parkinson's disease: parkin, PTEN-induced putative kinase 1 (PINK-1), leucine-rich repeat kinase 2 (LRRK2), α-synuclein and the oncogene DJ-1 (Farrer 2006; Thomas and Beal 2007). The genetics of dementia with Lewy bodies, however, remains elusive.

3.3 Pathology

Immunohistochemical identification of Lewy bodies is included in the consensus criteria for the diagnosis of dementia with Lewy bodies. Based on the distribution of the Lewy bodies, this type of dementia has been classified into three pathological subtypes including the limbic type, the brainstem-predominant type and the neocortical type (McKeith, Galasko et al. 1996; McKeith 2006). Further, common pathologic findings are Lewy neuritis, neuronal loss, and overlapping Alzheimer's disease pathology such as senile and diffuse plaques as well as neurofibrillary tangles (reviewed in (Buracchio, Arvanitakis et al. 2005)). Most DLB patients meet the plaque-based CERAD criteria for Alzheimer's disease (Hansen, Salmon et al. 1990) whereas few meet the tangle-based Braak stages V and VI for Alzheimer's disease (McKeith, Mintzer et al. 2004). Even though tau pathology is less frequently seen in dementia with Lewy bodies than in Alzheimer's disease, the presence of Alzheimer's tau pathology modifies the clinical presentation (lower rate of hallucinations and parkinsonism) and makes the clinical differentiation between the two dementia types more difficult (Merdes, Hansen et al. 2003). Currently, no biomarkers have been established for the diagnostic confirmation of dementia with Lewy bodies (McKeith, Burn et al. 2003). However, tau phosphorylated at different epitopes, is suggested to increase the diagnostic accuracy in distinguishing between dementia with Lewy bodies and Alzheimer's disease (Hampel, Goernitz et al. 2003; Mollenhauer, Cepek et al. 2005). Also, the pattern of Aβ peptides and tau levels in the CSF, have shown neurochemical phenotypes which differ between the two dementia types (Bibl, Mollenhauer et al. 2006; Bibl, Mollenhauer et al. 2006). In addition, oligomeric forms of α-synuclein in plasma, have been proposed as potential biomarkers for Parkinson's

disease and related diseases, but this requires more investigation. α-synuclein levels in cerebrospinal fluid have also been reported as a predictor of Parkinson's disease in comparison to neurologically healthy persons. However, investigations on patients with dementia with Lewy bodies are lacking (El-Agnaf, Salem et al. 2006; Tokuda, Salem et al. 2006). A very recent study highlighted the serum heart-type fatty acid-binding protein (H-FABP) as a biomaker candidate to distinguish dementia with Lewy bodies from Alzheimer's disease. However, this candidate did not distinguish between dementia with Lewy bodies and Parkinson's disease (Mollenhauer, Steinacker et al. 2007). In summary, there is still no consensus on a biomarker of diagnostic utility for dementia with Lewy bodies.

3.3.1 Lewy bodies

Dementia with Lewy bodies is a member of the family of neurodegenerative diseases called α-synucleinopathies. This family includes Parkinson's disease, Parkinson's disease with dementia and multiple system atrophy, since the 140 amino acid pre-synaptic protein α-synuclein, also called the non-Aβ component of Alzheimer's disease, has been identified as the major component of the Lewy bodies and Lewy neuritis, found in Parkinson's disease and dementia with Lewy bodies (Ueda, Fukushima et al. 1993; Spillantini, Schmidt et al. 1997; Arima, Ueda et al. 1998). α-synuclein is expressed in nervous tissue and can be measured in plasma as well as cerebrospinal fluid from control subjects and patients with α-synucleinopathies (Jakes, Spillantini et al. 1994; El-Agnaf, Salem et al. 2006; Tokuda, Salem et al. 2006). The physiological role of α-synuclein is not fully elucidated, but the proposed primary function in dopaminergic neurons is the regulation of dopamine content by influencing synthesis, storage into vesicles, release and re-uptake by neurons and tone at the synapse (Sidhu, Wersinger et al. 2004; Sidhu, Wersinger et al. 2004). The Lewy bodies are eosinophilic neuronal inclusions that, apart from α-synuclein, consist of numerous known components such as ubiquitin (Leigh, Probst et al. 1989; Iwatsubo, Yamaguchi et al. 1996). The known components of the Lewy bodies have been divided into four groups, structural elements postulated to be Lewy body fibrils, proteins involved in the cellular response to fibril deposition, protein which diffuse

into the Lewy bodies from the cytosol and enzymes of the phosphorylation/dephosphorylation system (Pollanen, Bergeron et al. 1993).

4. Serpins

Serpins are found predominantly in blood plasma and account for more than 10 % of its total protein content (Travis and Salvesen 1983). The serpins are variably glycosylated, which however, is not a pre-requisite for protease inhibitory activity (Guzdek, Potempa et al. 1990). The primary targets of serpins are serine proteases (proteases with a serine residue in their active domain) belonging to the chymotrypsin family. However, a few serpins have been recognised as inhibitors of cysteine proteases such as caspase 1 (Komiyama, Ray et al. 1994), cathepsin L, K, S and V (Schick, Pemberton et al. 1998; Irving, Shushanov et al. 2002), whereas some other serpins, thyroid-binding globulin, corticosteroid-binding globulin (CBG) and angiotensinogen, lack protease inhibitory activity (Potempa, Korzus et al. 1994; Silverman, Bird et al. 2001). In addition, two members of the serpin family, α1-antichymotrypsin and the myeloid and erythroid nuclear termination stage-specific protein (MENT), have been shown to have DNA-binding capacity (Siddiqui, Hughes et al. 1980; Grigoryev, Bednar et al. 1999). Crucial to their protease inhibitory activity, serpins adopt a metastable conformation. This structure is composed of three β-sheets (A, B and C) and at least 7 α-helices (mostly 9 α-helices). The reactive centre loop (RCL), which contains the protease recognition site and is responsible for protease inhibition, is a flexible stretch of about 17 residues, chained between the β-sheets A and C and exposed on the surface of the proteins. The protease inhibition pathway involves an irreversible suicide mechanism (Figure 4), where the cleaved serpin RCL is inserted into β-sheet A and the protease is relocated from the top to the bottom of the serpin. This conformational change leads to a kinetically trapped, covalent serpin-protease complex that severely compromises protease catalytic activity (Stratikos and Gettins 1999; Huntington, Read et al. 2000). The serpin-protease complex is *in vivo* then bound to receptors and swiftly cleared from the system. The ability of the RCL to relocate the protease upon cleavage is critical since if RCL movement is rendered, the protease may escape before it is irreversibly trapped, resulting in an active protease

and a cleaved inactive serpin (Silverman, Bird et al. 2001).
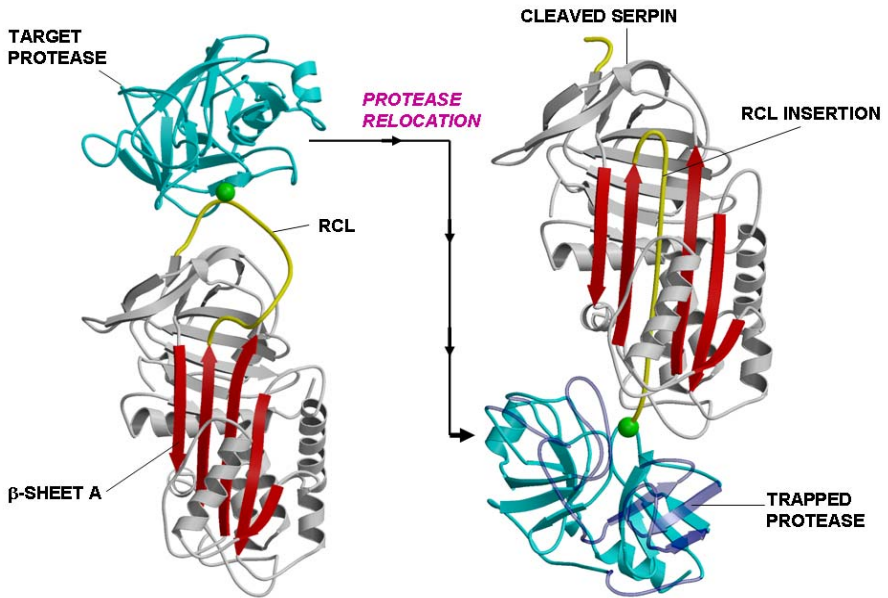
Figure 4. The "suicide mechanism" illustrated by the interaction of the serpin α1-antitrypsin and its' target protease.
Original illustration provided by the courtesy of Dr RW Carrell and adapted by permission from Macmillan Publishers Ltd: Nature (Huntington, Read et al. 2000).

5. Serpins in Alzheimer's disease and dementia with Lewy bodies

To date and prior to the present work, several members of the serpin family have been linked to Alzheimer's disease (Table 1), whereas very few have been found associated with dementia with Lewy bodies (Table 2). So far, the serpin most frequently associated with Alzheimer's disease is α1-antichymotrypsin. However, also α1-antitrypsin and α2-macroglobulin have been associated with the disease. The concentrations of the latter two serpins have in addition been found to be increased in serum from patients with subcortical vascular dementia (Binswanger's disease) in comparison with controls (Wetterling and Tegtmeyer 1994). Further, polymerization of mutant neuroserpin has been shown to underlie the autosomal, dominantly inherited dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB) (Davis, Shrimpton et al. 1999).

Table. 1. Findings linking serpins to Alzheimer's disease, prior to this work

Serpin	Significant links to Alzheimer's disease	Reference
α1-antichymotrypsin (ACT)	Suggested as a biomarker since elevated serum and CSF levels were found versus controls	(Matsubara, Amari et al. 1989) (Matsubara, Hirai et al. 1990)
	Identified in Aβ plaques	(Abraham, Selkoe et al. 1988) (Shoji, Hirai et al. 1991)
	Increased mRNA levels in grey matter versus controls	(Abraham, Shirahama et al. 1990)
	Induces Aβ fibril disaggregation	(Fraser, Nguyen et al. 1993)
	Disease associated ACT gene polymorphisms first reported	(Thome, Baumer et al. 1995) (Kamboh, Sanghera et al. 1995)
	Specific and structural interaction with Aβ1-42	(Janciauskiene, Rubin et al. 1998)
	Enhances AD like pathology in APP mice	(Mucke, Yu et al. 2000)
	Promotes β-sheet Aβ plaque deposition in AD transgenic mice	(Nilsson, Bales et al. 2001)
	Inhibits Aβ degradation *in vitro* and *in vivo*	(Abraham, McGraw et al. 2000)
	Link between CSF levels and cognitive impairment	(DeKosky, Ikonomovic et al. 2003)
	Induces tau phosphorylation in neurons	(Padmanabhan, Levy et al. 2006)
α1-antitrypsin (AAT)	Increased serum levels versus controls	(Giometto, Argentiero et al. 1988)
	Identified in senile plaques and NFTs	(Gollin, Kalaria et al. 1992)
	Disease associated gene polymorphisms first reported	(Kowalska, Danker-Hopfe et al. 1996)
	Greater specific oxidation index of plasma AAT versus controls	(Choi, Malakowsky et al. 2002)
	Elevated plasma levels linked to cognitive function and to iron homeostasis through heme oxygenase-1 suppression versus controls	(Maes, Kravitz et al. 2006)

Table 1. continued

Serpin	Significant links to Alzheimer's disease	Reference
α2-macroglobulin	Identified in cortical senile plaques	(Bauer, Strauss et al. 1991)
	Involved in proteolytic processing of APP	(Ganter, Strauss et al. 1991)
	Suggested as marker for inflammatory cellular processes in neuritic plaques	(Van Gool, De Strooper et al. 1993)
	Shown to attenuate Aβ1-40 fibril formation and neurotoxicity	(Du, Bales et al. 1998)
	SDS-stable complex formation with Aβ1-42 found	(Hughes, Khorkova et al. 1998)
	Genetic disease association found	(Blacker, Wilcox et al. 1998)
	Inhibits Aβ1-40 and Aβ1-42 fibril formation as well as neurotoxicity	(Monji, Yoshida et al. 2000)
	Suggested as biomarker as elevated plasma levels correlated to disease severity	(Hye, Lynham et al. 2006)
Neuroserpin	Associates with Aβ1-42 in a 1:1 binary complex	(Kinghorn, Crowther et al. 2006)
	Accelerates Aβ1-42 aggregation	(Kinghorn, Crowther et al. 2006)
	Reduces Aβ1-42 cytotoxicity	(Kinghorn, Crowther et al. 2006)
Complement C1-inhibitor	The cleaved protein is recognized on dystrophic neuritis in senile plaques	(Walker, Yasuhara et al. 1995)
	Inactivated protein found in astrocytes located near amyloid plaques	(Veerhuis, Janssen et al. 1996)
	Up-regulation of complement activators is not balanced with sufficient up-regulation of complement C1-inhibitor	(Yasojima, Schwab et al. 1999)
	Production is, in contrast to complement activators, refractory to stimulation by plaque associated cytokines in astrocytes and neuronal cells *in vitro*.	(Veerhuis, Janssen et al. 1999)
Antithrombin III	Increased mRNA levels versus controls and protein detected in amyloid deposits, plaque-associated neuritis and in NFTs	(Kalaria, Golde et al. 1993)
Plasminogen activation inhibitor-1	CSF levels increased versus controls	(Sutton, Keohane et al. 1994)

Table 1. continued

Serpin	Significant links to Alzheimer's disease	Reference
Plasminogen activation inhibitor-1	Identified in plaques	(Rebeck, Harr et al. 1995)
	Identified in ghost tangles	(Hino, Akiyama et al. 2001)
	Up-regulated in AD mouse models and shown to interfer with Aβ clearance of through the tissue plasminogen activator – plasmin system	(Melchor, Pawlak et al. 2003)
Plasminogen activation inhibitor-2	Expression up-regulated versus controls and particularly intense staining identified in plaque associated microglia	(Akiyama, Ikeda et al. 1993)

Table 2. Findings linking serpins to dementia with Lewy bodies, prior to this work

Serpin	Significant links to dementia with Lewy bodies	Reference
α2-macroglobulin	Association of α2-macroglobulin D-allele with DLB in a genetically isolated Dutch population	(Sleegers, Roks et al. 2004)
Plasminogen activation inhibitor-1	Identified in the cerebral cortex of diffuse DLB patients, labelling ubiquitin positive intraneuronal round structures	(Hino, Akiyama et al. 2001)

5.1 *α1-antichymotrypsin*

α1-antichymotrypsin was first described in 1962, without knowledge of its function, and shown to have chymotrypsin inhibitory activity in 1965 (Travis and Salvesen 1983). α1-antichymotrypsin is primarily synthesised by hepatocytes but also by bronchial and breast epithelial cells, and to a lesser extent by monocytes and, in the central nervous system (CNS), by reactive astrocytes (Tokes, Gendler et al. 1981; Stockley 1983; Pasternack, Abraham et al. 1989; Bergman, Kadner et al. 1993; Kordula, Bugno et al. 2000). The protein has a typical serpin structure of three β-sheets surrounded by nine α-helixes (Baumann, Huber et al. 1991; Wei, Rubin et al. 1994). In its native, circulating form, α1-antichymotrypsin is an acute-phase glycoprotein of between 55 and 68 kilo daltons, depending on the heterogeneity in glycosylation of the protein (Tokes, Gendler et al. 1981; Travis and Salvesen 1983).

Upon inflammatory stimuli, the plasma levels of α1-antichymotrypsin increase more than 4-fold within hours (Berninger 1985). Another feature of α1-antichymotrypsin is, together with the myeloid and erythroid nuclear termination stage-specific protein (MENT), the unique property among serpins to bind DNA (Siddiqui, Hughes et al. 1980; Grigoryev, Bednar et al. 1999). *In vitro*, α1-antichymotrypsin inhibits DNA synthesis in permeabilized human carcinoma cells, possibly by either inhibiting DNA polymerase α and/or DNA primase (Tsuda, Masuyama et al. 1986; Takada, Tsuda et al. 1988; Tsuda, Umezawa et al. 1988). Comparisons of the primary sequences indicate that α1-antichymotrypsin expressed in the human hippocampus and in the liver, share more than 90 % homology. However, differential glycosylation studies by sensitivity to N-glycosidase, suggested that different types of glycosylation for α1-antichymotrypsin, derived from the hippocampus and from the liver, might exist with heavier glycosylation of α1-antichymotrypsin synthesised by the liver (Hwang, Steineckert et al. 1999).
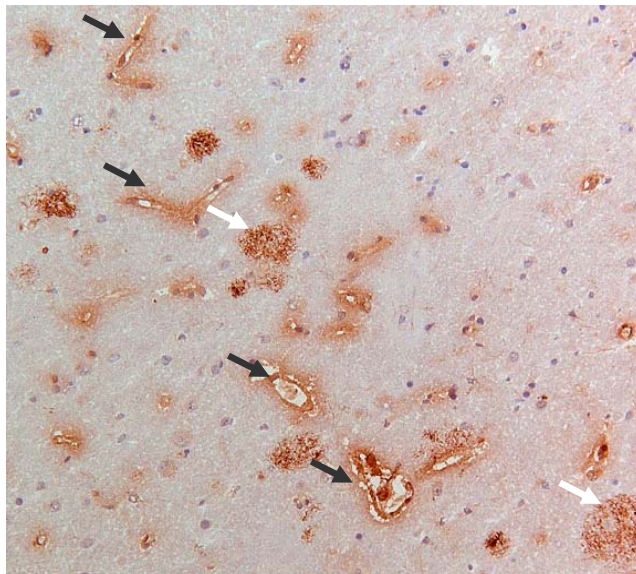

Figure 5. ACT identified with anti-ACT antibodies in amyloid plaques (white arrows) and as lining the cerebral blood vessels (black arrows) in brain tissue from an Alzheimer's disease patient.
Unpublished results, Dr Robert Veerhuis

5.1.1 Link to dementia

Several studies (Table 1) have linked elevated levels of α1-antichymotrypsin in cerebrospinal fluid and plasma to Alzheimer's disease, as the findings that α1-antichymotrypsin is found in the amyloid plaques in the brains of Alzheimer's disease patients (Abraham, Selkoe et al. 1988; Harigaya, Shoji et al. 1995; Licastro, Morini et al. 1995; Licastro, Parnetti et al. 1995; DeKosky, Ikonomovic et al. 2003) (Figure 5). Further, the active state of α1-antichymotrypsin in the amyloid plaques has been debated. In 1991, Rozemuller and co-authors raised antibodies against inactivated α1-antichymotrypsin, with specificity for neoepitopes expressed on α1-antichymotrypsin upon interaction with proteases, and proposed that α1-antichymotrypsin in the amyloid plaques was either proteolytically inactivated or complexed to a protease (Rozemuller, Abbink et al. 1991). Genetically, several α1-antichymotrypsin polymorphisms have been reported as modifying the susceptibility to Alzheimer's disease. The first reports came in 1995 (Table 1) in which one polymorphism was suggested to increase the risk of Alzheimer's disease 2 to 3 fold in Apo E4 carriers (Kamboh, Sanghera et al. 1995). Other studies showed that the frequency of the α1-antichymotrypsin An allele was significantly increased in Alzheimer's disease patients compared to non-demented controls (Thome, Baumer et al. 1995) whereas also the α1-antichymotrypsin TT genotype was linked to increased risk of the disease (Licastro, Pedrini et al. 1999). More recent data suggested that the α1-antichymotrypsin TT genotype affects survival of Alzheimer's disease patients, the age at onset and synaptic plasticity in the Alzheimer's disease brain and the rate of cognitive decline in carriers of the apolipoprotein E4 allele (Licastro, Chiappelli et al. 2004; Licastro, Chiappelli et al. 2005). However, despite the increasing amount of research results linking α1-antichymotrypsin to dementia, there are no reports on its association with dementia with Lewy bodies. Thus, its physiological role in the pathogenesis of Alzheimer's disease, is to date still not clear.

5.2 α1-antitrypsin

Another member of the serpin family is the 394 amino acid glycoprotein and acute-

phase reactant α1-antitrypsin, named in 1962 after having been isolated by Schultze, Heide and Haupt (Schultze, Heide et al. 1962). In the circulation, α1-antitrypsin mainly derives from hepatocytes, but also from circulating monocytes, alveolar macrophages and lung-derived epithelial cells (Koj, Regoeczi et al. 1978; Mornex, Chytil-Weir et al. 1986; Venembre, Boutten et al. 1994; Cichy, Potempa et al. 1997). α1-antitrypsin inhibits several serine proteases, among them chymotrypsin, cathepsin G, plasmin and thrombin. However, the major function of α1-antitrypsin is proposed to be tissue protection against neutrophil elastase (Carrell, Jeppsson et al. 1982). α1-antitrypsin has been implicated in the early development of emphysema and chronic obstructive pulmonary disease as people with α1-antitrypsin deficiency, due to point mutations in the α1-antitrypsin gene (most recently reviewed in (Wood and Stockley 2007)), are more prone to develop these pathologies (Laurell and Eriksson 1963; Eriksson 1965) . In addition, the α1-antitrypsin Z mutation leads to an accumulation of α1-antitrypsin inclusions in the liver, which may cause juvenile hepatitis in about 90 % of homozygous individuals, of which 1-2 % will develop liver cirrhosis and risk developing hepatocellular carcinoma (Sharp, Bridges et al. 1969; Eriksson, Carlson et al. 1986; Sveger 1988).

5.2.1 Link to dementia

The first evidence of a link between α1-antitrypsin, dementia and Alzheimer's disease was published in 1988 when serum levels of α1-antitrypsin were found to be increased in Alzheimer's disease patients in comparison with age-matched, healthy controls (Giometto, Argentiero et al. 1988) (Table 1). These data were confirmed in a later study that also showed increased levels of α1-antitrypsin in subcortical vascular dementia (Binswanger's disease) (Wetterling and Tegtmeyer 1994). A few years after the first report of increased serum levels, α1-antitrypsin was identified in the lesions of Alzheimer's disease (Gollin, Kalaria et al. 1992) (Table 1). Genetically, a link between Alzheimer's disease and α1-antitrypsin was introduced when a study on a small Polish cohort showed an increased frequency of the PI*M3 allele in Alzheimer's disease patients compared to controls from the general population (Kowalska, Danker-Hopfe

et al. 1996). In addition, recent studies have linked altered glycoproteins, such as α1-antitrypsin, to Alzheimer's disease (Puchades, Hansson et al. 2003; Yu, Chertkow et al. 2003). In 2006, Maes and co-workers linked increased plasma levels of α1-antitrypsin to lower MMSE score and also to cerebral iron homeostasis (Maes, Kravitz et al. 2006). Thus, as for α1-antichymotrypsin, the function of α1-antitrypsin in Alzheimer's disease is not known and prior to the present work, no data on links between α1-antitrypsin and dementia with Lewy bodies existed.

5.3 Neuroserpin

Neuroserpin was first characterised as one of two axonally secreted proteins by embryonic chicken neurons and then called axonin-2 (Stoeckli, Lemkin et al. 1989). The new member of the serpin family, later named neuroserpin, turned out to be a 55 kDa protease inhibitory neuronal glycoprotein that is produced and secreted from several neuronal populations in both the peripheral nervous system and the CNS (Stoeckli, Lemkin et al. 1989; Osterwalder, Contartese et al. 1996). Neuroserpin producing CNS cells with large somas, were located in the gray matter, however expression is absent in non-neuronal tissue. Further, neuroserpin was shown to be expressed in most areas of the nervous system during neuronal development in chicken whereas the expression was restricted to relatively few areas in the adult chicken. In the developing nervous system neuroserpin is predominantly expressed during the formation and reorganisation of synapses whereas in the adult CNS, it is expressed in areas of synaptic changes linked to learning and memory. Therefore, neuroserpin was implicated in the reorganisation and formation of synaptic connections in the developing chicken, as well as in synaptic plasticity in the adult (Osterwalder, Contartese et al. 1996). The target proteases of neuroserpin were identified as tissue plasminogen activator (tPA), urokinase and plasmin, but not thrombin (Krueger, Ghisu et al. 1997; Osterwalder, Cinelli et al. 1998). The protein was further proposed to modify proteolysis in processes such as neuronal migration, axogenesis and the formation of mature synaptic connections (Krueger, Ghisu et al. 1997). In addition, neuroserpin expression is post-translationally regulated by thyroid hormone T3, and axonal growth was suggested to be regulated by modulation of neuroserpin levels in

cultured PC12 cells (Parmar, Coates et al. 2002; Navarro-Yubero, Cuadrado et al. 2004). Recently, mutations in the neuroserpin gene have been implicated in epilepsy (Takao, Benson et al. 2000) whereas the wild type neuroserpin has been proposed to have beneficial therapeutic properties in stroke (Vivien and Buisson 2000; Yepes, Sandkvist et al. 2000; Cinelli, Madani et al. 2001)

5.3.1 Link to dementia

Neuroserpin was linked to the autosomal dominantly inherited dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB) by the histological and biochemical identification of neuronal inclusion bodies of neuroserpin polymers, due to point mutations (S49P and S52R) in the neuroserpin gene (Davis, Shrimpton et al. 1999). Further, the formation of inclusion bodies itself, was sufficient to cause neurodegeneration, and the rate and magnitude of neuroserpin aggregation was associated with the onset and severity of the disease (Davis, Shrimpton et al. 2002). The two point mutations, whereof the S49P has been demonstrated to be a poor protease inhibitor (Belorgey, Crowther et al. 2002), were rare in a French cohort of patients with inherited Alzheimer's disease and frontotemporal dementia, as investigated by the French Alzheimer's Disease and Fronto-Temporal Dementia Genetics Study Groups (2000). Still, a link to Alzheimer's disease was established when neuroserpin was reported to bind to and form 1:1 binary complexes with the Aβ1-42, to accelerate the generation of thioflavin-positive, smaller Aβ1-42 species, but at the same time to suppress fibril formation. In addition, *in vitro* and *in vivo*, neuroserpin reduced Aβ1-42 cytotoxicity (Kinghorn, Crowther et al. 2006), suggesting a neuroprotective role in Alzheimer's disease. Prior to this investigation, no data existed on plasma or cerebrospinal fluid levels of neuroserpin, nor did any reports indicating a relationship with dementia with Lewy bodies.

6. Pathogenesis and current hypotheses

6.1 Alzheimer's disease

6.1.1 The amyloid cascade hypothesis

As described in earlier sections, one of the major pathological events in the course of Alzheimer's disease is the formation of extracellular amyloid plaques. On the basis of these observations, in combination with the discovery of the APP mutations, the "amyloid cascade hypothesis" was put forward more than 10 years ago (Hardy and Allsop 1991). This hypothesis, which was strengthened by the occurrence of Alzheimer's disease in trisomy 21 (the APP gene is located on chromosome 21) also states that the rest of the disease processes, such as tau pathology and tangle formation, is a later event in the development of the disease caused by an imbalance between Aβ synthesis and clearance (Hardy and Allsop 1991; Hardy and Higgins 1992). Initially, results pointed to the fibrillar forms of Aβ as the neurotoxic species of Aβ (Lorenzo and Yankner 1994; Howlett, Jennings et al. 1995; Blanchard, Konopka et al. 1997). However, it has later also been shown that soluble Aβ, is cytotoxic to neuronal cells in culture (Lambert, Barlow et al. 1998; Hartley, Walsh et al. 1999; Walsh, Hartley et al. 1999; El-Agnaf, Mahil et al. 2000; Klein, Krafft et al. 2001). The toxicity of monomeric, oligometric and fibrillar forms of Aβ40 and Aβ42 was analysed using Neuro-2A neuroblastoma cells. This study found that oligomers of Aβ42 inhibited neuronal viability 10-fold more than fibrils and approximately 40-fold more than unaggregated protein (Dahlgren, Manelli et al. 2002). The current state of knowledge now points to the Aβ-derived diffusible ligands (ADDLs) as the most potent key player in neurotoxicity.

Even though the number of plaques has been shown to only weakly be correlated to clinical mental impairment, the amyloid cascade hypothesis remains the main hypothesis driving research on Alzheimer's disease. In line with the hypothesis, numerous promising remedy trials on animals have been initiated to enhance the clearance of or prevent the accumulation of Aβ (Schenk, Barbour et al. 1999; Morgan, Diamond et al. 2000; Qu, Xiang et al. 2007). The first anti-Aβ trial, using immunotherapy on humans, was initiated by Elan/Wyeth (Thatte 2001). This study was however halted in phase II, following the reports that 15 out of 300 test subjects (early-moderate stage Alzheimer's disease patients) treated with the AN-1792 antibody developed encephalitis (Senior 2002). However, despite earlier failure, the

approach of immunotherapy is still a pressing issue and several clinical trials are at present in progress (reviewed in (Melnikova 2007)).

6.1.2 The "vascular hypothesis"

Altered cerebral vasculature, reduced cerebral blood flow and increased permeability of the blood-brain-barrier (BBB) has been shown in Alzheimer's disease patients (Su, Arendash et al. 1999; Farkas and Luiten 2001; Engelberg 2004) and the question of vascular damage and its relation to Alzheimer's disease has been debated for a long time. Microvascular pathology is increased in Alzheimer's disease patients, versus normal controls, and parallels the patterns of neuronal loss (Buee, Hof et al. 1994). In fact, cerebral vascular changes have even been proposed to precede the neuronal damage and dementia in Alzheimer's disease patients (Rhodin and Thomas 2001). Therefore, a re-classification of Alzheimer's disease as a vascular disorder was suggested in order to enhance the chances of finding a disease remedy (de la Torre 2002; de la Torre 2002; de la Torre 2002). In line with these reports, decreased parietal regional cerebral blood flow (rCBF) in combination with pathological Alzheimer's disease biomarkers showed increased risk of developing Alzheimer's disease in patients with mild cognitive impairment. Even in itself, reduced parietal rCBF was associated with a more rapid progression to Alzheimer's disease in mild cognitive impairment patients (Hansson, Buchhave et al. 2007). Vascular risk factors often associated with Alzheimer's disease include for example hypertension, cholesterol, atherosclerosis, diabetes mellitus and ApoE genotype. Today, vascular pathology is believed to cause or contribute to Alzheimer's disease in at least 50 % of all demented patients (reviewed in (Breteler 2000)). Results from epidemiologic studies conducted within the Rotterdam Study, suggested that classic cardiovascular risk factors and thrombogenic factors were related to white matter lesions, which in turn were associated with cognitive function in elderly subjects (>65 years) of the general population (Breteler, van Swieten et al. 1994). Further, another study conducted within the Rotterdam Study, evaluated the association between atherosclerosis and dementia, vascular dementia and Alzheimer's disease. This population-based study

found an interaction between the ApoE ε4 allele, and atherosclerosis, which seemed to significantly increase the prevalence of Alzheimer's disease (Hofman, Ott et al. 1997).
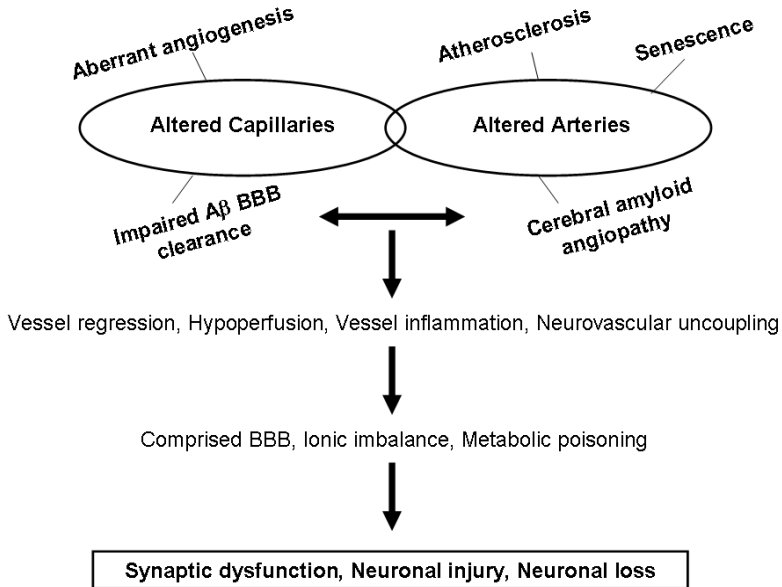
Figure 6. A schematic interpretation of the neurovascular hypothesis including alteration of the cerebral capillaries and arteries (Zlokovic 2005).

In a very recent study, Alzheimer's disease patients devoid of vascular risk factors, compared to healthy controls, exhibited significant endothelial dysfunction when measuring the endothelial flow-mediated dilation. This observation was even enhanced in patients with severe Alzheimer's disease (Dede, Yavuz et al. 2007). On a cellular level, the main character of the amyloid hypothesis, Aβ, directly interacts with endothelial cells and causes activation of apoptotic and necrotic processes, which in the end could lead to vascular endothelial damage (Thomas, Thomas et al. 1996; Suo, Fang et al. 1997). Further, the Aβ species seems to be crucial to endothelium interaction as soluble aggregates of Aβ (ADDLs), in contrast to Aβ monomers and mature fibrils, activate human brain microvascular endothelial cells for adhesion and transmigration of monocyte cells (Gonzalez-Velasquez and Moss 2007). Taken together, a substantial amount of evidence suggests that vascular factors have a prominent role in the aetiology of Alzheimer' disease. According to the neurovascular

hypothesis, pathogenic processes originating from altered cerebral arteries and/or capillaries, can initiate the down-stream pathological events in Alzheimer's disease, including increased levels of Aβ due to faulty Aβ clearance over the BBB (Zlokovic 2005) (Figure 6). However, in what chronological order these events occur is still elusive.

6.1.3 The "inflammatory hypothesis"

The cardinal signs of inflammation, described by Celcus, include dolor (pain), rubor (redness), tumor (swelling) and calor (heat) (McGeer and McGeer 1995). These signs do not show in neuroinflammation as the brain is protected behind the BBB and for instance lacks sensory fibres to detect calor, dolor and rubor (McGeer and McGeer 2001). Alzheimer's disease has a long history of inflammatory associations and brain acute-phase speculations (Vandenabeele and Fiers 1991). However, instead of the four cardinal inflammation signs, acute-phase reactants like α1-antichymotrypsin and α1-antitrypsin, pro-inflammatory cytokines and complement components (Yasojima, Schwab et al. 1999) have been found in elevated amounts in Alzheimer's disease patients (Giometto, Argentiero et al. 1988; Matsubara, Amari et al. 1989; Matsubara, Hirai et al. 1990; Fillit, Ding et al. 1991; Wetterling and Tegtmeyer 1994; Harigaya, Shoji et al. 1995; Kalman, Juhasz et al. 1997; Singh and Guthikonda 1997; DeKosky, Ikonomovic et al. 2003; Heneka and O'Banion 2007). Amyloid plaques are dressed in acute-phase proteins (Abraham, Shirahama et al. 1990; Gollin, Kalaria et al. 1992), complement proteins (Eikelenboom, Hack et al. 1989) and other inflammatory markers like intercellular adhesion molecule-1 (ICAM-1) (Frohman, Frohman et al. 1991), considered a marker of CNS inflammation (Rieckmann, Nunke et al. 1993; Trojano, Avolio et al. 1996). Further, inflammatory processes within the Alzheimer's disease brain includes reactive microglia and astrocytes as well as an up-regulation of complement activating factors. The reactive microglia and astrocytes in turn, produce and release several potential toxic and pro-inflammatory molecules including cytokines, proteases and free radicals, which, when uncontrolled, could damage neuronal cells by "bystander lysis" (Eikelenboom, Zhan et al. 1994). In themselves, inflammatory processes have been, more than Aβ deposition and NFT formation,

shown to account for synapse loss (Lue, Brachova et al. 1996). Whether the inflammatory processes in Alzheimer's disease are causative or a secondary event remains unclear. Epidemiological studies showed that elevated levels of α1-antichymotrypsin, interleukin-6 and to a lesser degree C-reactive protein before clinical onset of dementia was linked to increased risk of dementia and Alzheimer's disease (Engelhart, Geerlings et al. 2004). In addition, several lines of evidence suggest a protective role of treatment with non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of Alzheimer's disease (in t' Veld, Ruitenberg et al. 2001; Etminan, Gill et al. 2003; Szekely, Thorne et al. 2004; Townsend and Pratico 2005). Inflammation in Alzheimer's disease seems to be tightly linked to the aetiology of the disease, however if as cause or result, needs to be firmly established.

6.2 Dementia with Lewy bodies

Dementia with Lewy bodies is a relatively new dementia diagnosis with strong clinical and pathological overlaps with Alzheimer's disease and Parkinson's disease dementia, both of which still have an unclear aetiology and pathogenesis. As extensive elaboration of the potential mechanisms underlying dementia with Lewy bodies, is beyond the scope of the present investigation, and as major hypotheses supported by epidemiological research as well as *in vitro* and *in vivo* data are lacking, the pathogenesis of dementia with Lewy bodies will here be only briefly discussed in relation to Alzheimer's disease.

Genetic factors as a cause of dementia with Lewy bodies have been postulated in the disease aetiology (see earlier sections) and in addition, Alzheimer's disease pathology may play a role in the disease pathogenesis. Similar to the aggregation of Aβ in Alzheimer's disease, the aggregation of α-synuclein seems to be a central event in the development of dementia with Lewy bodies. In addition, recent studies showed that levels of Aβ1-40 plaques might be involved in or even enhance Lewy body formation (Lippa, Lippa et al. 2005; Pletnikova, West et al. 2005). Further, α-synuclein lesions are rare in aged control brains, but are more frequent in brains with neuritic plaques,

suggesting similar pathogenic mechanisms underlying the accumulation of Aβ and α-synuclein (Mikolaenko, Pletnikova et al. 2005).

6.2.1 Inflammatory aspects

Whereas inflammation is recognized as part of the mechanisms in the pathophysiology of Alzheimer's disease, few studies have focused on inflammatory aspects of dementia with Lewy bodies. Anti-inflammatory treatment, as mentioned earlier, seems to delay or prevent the onset of dementia on Alzheimer's disease (McGeer, Schulzer et al. 1996). Compared to Alzheimer's disease however, a post-mortem study showed less inflammation in the form of human leukocyte antigen-DR (HLA-DR) reactive microglia in brains from dementia with Lewy bodies patients. It was therefore proposed that anti-inflammatory treatment strategies seem unlikely to be effective in pure dementia with Lewy bodies (with little Alzheimer's disease pathology) (Shepherd, Thiel et al. 2000). By contrast, more recent data (reviewed in (Mrak and Griffin 2007)) suggest inflammatory involvement in the dementia with Lewy bodies pathogenesis as activated microglia were indeed found adjacent to α-synuclein containing neurons and glia cells in several α-syncleinopathies. The authors therefore suggested that neuroinflammatory processes might be involved in mechanisms driving the disease progression in both dementia with Lewy bodies and Alzheimer's disease (Mrak and Griffin 2007).

6.2.2 Vascular aspects

The involvement of vascular pathology in the pathogenesis of dementia with Lewy bodies is like the inflammatory mechanisms, not very well investigated. One study indicated that patients fulfilling the clinical criteria of dementia with Lewy bodies, also exhibited clinical features of possible vascular origin (Londos, Passant et al. 2000). The same authors also showed significantly decreased arterial blood pressure during the course of dementia and a more pronounced orthostatic drop in dementia with Lewy bodies compared to Alzheimer's disease patients (Londos, Passant et al. 2000). A post-mortem study including brains of patients with Parkinson's disease and patients diagnosed with dementia with Lewy bodies, aimed to investigate the prevalence of

vascular lesions. In this study, no acute ischemic strokes or haemorrhages were present in the investigated dementia with Lewy bodies brains. Moreover, the frequency of cerebrovascular lesions of various intensity was lower in dementia with Lewy bodies than in Parkinson's disease and control brains. The author therefore suggested a protective effect, of unclear reasons. against stroke in the dementia with Lewy bodies patients included in the study (Jellinger 2003). However, pathology and imaging studies suggested that white matter lesions and lacunes may be present in up to 30 % of autopsy confirmed dementia with Lewy bodies cases (McKeith, Dickson et al. 2005). A more recent study presented data derived from post-mortem investigations of brains from Alzheimer's disease and dementia with Lewy bodies patients (Isojima, Togo et al. 2006). In this comparative study, it was shown that microinfarcts were more commonly found in Alzheimer's disease while more head-trauma-derived, grossly identified haemorrhages were found in dementia with Lewy bodies patients. These haemorrhages were possibly due to more frequent falls in dementia with Lewy bodies than in Alzheimer's disease. Within the group of dementia with Lewy bodies patients, the study found no difference in vascular complications when comparing cases with mild and severe neurofibrillary pathology. Also, dementia with Lewy bodies cases who were lacking vascular complications, presented with memory disturbances as the initial symptom, whereas parkinsonism was more frequent in patients with vascular complications. Therefore vascular complications might affect the intial symptoms of the clinical manifestations (Isojima, Togo et al. 2006). Compared to Alzheimer's disease, there is no clear line of evidence pointing to the involvement of vascular pathology in the pathogenesis of dementia with Lewy bodies.

7. The present investigation

7.1 Specific Aims

A combination of several pathogenesis hypotheses and a multidisciplinary scientific approach will most likely be the key to understanding the causes of Alzheimer's disease and other dementias without known aetiology. The present investigation therefore includes studies addressing the main pathogenesis hypotheses used to explain Alzheimer's disease. As the pathologies of Alzheimer's disease and dementia with

Lewy bodies share many features, we were interested in documenting data that preferably would point to a clear tool to help distinguishing between the two dementia types, provide more knowledge about their pathogenesis and link markers to cognitive function. Our specific aims were therefore:

1. To elucidate the molecular interaction between the Aβ1-42 peptide and the plasma derived wild-type α1-antichymotrypsin in regard of kinetics (PAPER I).
2. To investigate the global gene expression of primary human astrocytes upon exposure to soluble and fibrillar forms of Aβ1-42 alone as well as in combination with α1-antichymotrypsin (PAPER II).
3. To measure plasma and cerebrospinal fluid levels of three serpins, α1-antichymotrypsin, α1-antitrypsin and neuroserpin, in addition to the well-documented standard Alzheimer's disease biomarkers Aβ1-42, total-tau and phosphorylated tau (threonine-181) in a cohort including groups of patients diagnosed with Alzheimer's disease and dementia with Lewy bodies and non-demented controls (The Malmö Alzheimer Study) (PAPER III).
4. To measure plasma and cerebrospinal fluid levels of angiotensin converting enzyme and three soluble cell adhesion molecules in the same cohort as mentioned above. The chosen adhesion molecules were: intercellular adhesion molecule-1, vascular cell adhesion molecules and platelet endothelial adhesion molecule-1 in the same cohort as mentioned above (PAPER IV).
5. To investigate the ability of primary human astrocytes (fetal and adult astrocytes derived from Alzheimer's disease patients and non-demented controls) to bind and internalise aggregates of the Aβ1-42 peptide (PAPER V).

8. Major findings

8.1 Aβ1-42 and ACT form an SDS-unstable complex (PAPER I)

Before the present work, it was proposed that α1-antichymotrypsin sequence-specifically binds to and destabilizes preformed fibrils of Aβ1-40 (Fraser, Nguyen et al. 1993). In addition, Aβ1-42 has been shown to form SDS-stable complexes with

recombinant α1-antichymotrypsin, leading to transformation of the latter from a protease inhibitor to protease substrate (Janciauskiene, Rubin et al. 1998). In order to investigate the Aβ binding properties of the native α1-antichymotrypsin, we have in the present investigation (PAPER I) characterised the interaction between plasma derived wild-type α1-antichymotrypsin and Aβ1-42. In contrast to earlier findings, we have now identified the formation of an SDS-unstable complex with a dissociation constant of 8.2 μM, as determined by using rates of loss of α1-antichymotrypsin inhibitor activity and fluorescence spectrophotometry. This interaction appears to change the properties of both Aβ1-42 and α1-antichymotrypsin. In accordance with previous studies (Ma, Yee et al. 1994; Janciauskiene, Rubin et al. 1998), α1-antichymotrypsin protease inhibitory activity and formation of the characteristic serpin-protease SDS-stable complex was rendered or diminished. Further, upon exposure to α1-antichymotrypsin, we found evidence of a shift in the SDS-stable Aβ1-42 oligomer distribution favouring dimers instead of trimers. This finding could be of major physiological importance as trimers of naturally secreted Aβ inhibits hippocampal long-term potentiation, leading to impairment of recall of complex learned behaviours in rats (Townsend, Shankar et al. 2006). In the presence of stoichiometric excess of the protease chymotrypsin over α1-antichymotrypsin in the α1-antichymotrypsin-Aβ1-42 complex, Aβ1-42 is protease degraded. However, at substoichiometric ratios of chymotrypsin over α1-antichymotrypsin, α1-antichymotrypsin appears to partially protect Aβ1-42 from chymotrypsin digestion.

8.2. Combinations of ACT and Aβ1-42 alter the global gene expression and the uptake of aggregated Aβ1-42 in primary human astrocytes

Astrocytes are implicated in neuroprotection as mediators of the anti-oxidant defence and neurotransmitter metabolism as well as production and secretion of neurotrophic substances (reviewed in (Takuma, Baba et al. 2004)). Under physiological conditions their proposed functions include for example scar tissue formation after neuronal damage, generation and maintenance of the BBB, regulation of synapse formation. In addition, they have been inferred as immune effector cells within the brain as they

express cytokines and chemokines upon activation (Dong and Benveniste 2001; Ullian, Sapperstein et al. 2001; Ballabh, Braun et al. 2004; Takuma, Baba et al. 2004). Recently, astrocytes have been attributed involvement in the inflammatory processes associated with Alzheimer's disease pathogenesis and clearance of the Aβ peptide (Meda, Baron et al. 2001; Wyss-Coray, Loike et al. 2003; Heneka and O'Banion 2007).

8.2.1 Global gene expression (PAPER II)

Using Microarray gene technology, we showed that α1-antichymotrypsin, in combination with both soluble and fibrillar forms of Aβ1-42, alters the global gene expression of primary human astrocytes more extensively than α1-antichymotrypsin and both forms of Aβ1-42 alone, in comparison to non-treated cells (PAPER II). Using a cut-off of 1.2 fold change, we identified the highest number of genes (n=274) with altered expression upon stimulation with soluble Aβ1-42 in combination with α1-antichymotrypsin. Of these genes, n=193 were down-regulated, predominantly genes involved in transcription and signal transduction. Also, genes involved in neurogenesis, Numb and the collapsing response mediator protein 2 (CRMP2), and the protein degradation system, were found to be down-regulated. Decrease in proteasome activity has been linked to Alzheimer's disease. However, loss of activity was suggested to be induced by posttranslational modifications rather than a decrease in proteasome expression (Keller, Hanni et al. 2000). The question of proteasome acitivity in Alzheimer's disease stays elusive as in our study, the gene expression of the proteasome component 26S alpha, was down-regulated in all conditions compared to un-treated controls, as verified with RT-PCR.

In contrast to soluble Aβ1-42 with α1-antichymotrypsin, exposure to fibrillar Aβ1-42 in combination with α1-antichymotrypsin, yielded a higher number of up-regulated than down-regulated genes (n=6 versus n=58). These genes included inflammatory response genes like chemokine ligands 1, 6 and 24, chemokine receptor 1, monocyte chemoattractant protein I (MCP-1) and interleukin 1 receptor antagonist. An inflammatory response initiated by the fibrillar Aβ1-42-α1-antichymotrypsin

combination, was supported by elevated levels of MCP-1 in the conditioned cell supernatants. This was the only condition that remarkably differed from medium controls. MCP-1 release after stimulations with soluble and fibrillar Aβ1-42 and α1-antichymotrypsin alone, and the combination of α1-antichymotrypsin with soluble Aβ1-42, were similar.

Interestingly, fibrillar and soluble Aβ1-42 alone affected a rather small number of genes. We found an increased expression of 16 versus 9 genes and a decreased expression of 5 genes versus 10 genes. Among the down-regulated genes, upon fibrillar Aβ1-42 treatment, was the terminal complement component C5. When cleaved in the activated complement cascade, the complement component C5 generates the products C5a and C5b, of which the first is a potent anaphylatoxin and the latter part of the cytolytic membrane attack complex (Jose, Forrest et al. 1983; Kirschfink 2001). The gene of which expression was the most down-regulated, more than 4-fold upon soluble Aβ1-42 treatment, was the serum amyloid P component gene. This protein is found in amyloid deposits in Alzheimer's disease and plays an interesting role by protecting Aβ fibrils from proteolysis and promoting Aβ deposition (Tennent, Lovat et al. 1995; Botto, Hawkins et al. 1997). The only gene which expression was enhanced during treatment with both Aβ forms, was the gene encoding Golgin-67, a novel Golgi protein. Golgin-67 is inferred to be involved in organization of the Golgi apparatus and in docking/thethering of vesicles (Jakymiw, Raharjo et al. 2000).

To summarise, we found that the global gene expression was the most altered when treating astrocytes with the α1-antichymotrypsin – soluble Aβ1-42 combination. However, a substantial part of the total number of genes (soluble Aβ1-42: 15.8%, fibrillar Aβ1-42: 33.3 %, α1-antichymotrypsin: 22.2 %, soluble Aβ1-42 - α1-antichymotrypsin: 22.6 % and fibrillar Aβ1-42-α1-antichymotrypsin: 26.6 %) with rendered expression, still had no documented function. Until the functions of these, in total n=93 genes, have been elucidated it is not possible to in fully understand the effects of the treatments used.

8.2.2 Binding and uptake of aggregated Aβ1-42 by human primary astrocytes (PAPER V)

The role of astrocytes in Aβ clearance has been debated as immunohistochemical and immunolabelling studies have found Aβ inside astrocytes associated with senile plaques (Kurt, Davies et al. 1999; Thal, Schultz et al. 2000). Prior to the present investigation, it was shown that mouse astrocytes migrate in response to MCP-1, present in Aβ plaques, and that they bind and degrade Aβ *in vivo* and *in situ* (Wyss-Coray, Loike et al. 2003). We investigated whether primary human fetal astrocytes and adult astrocytes, derived from n=3 Alzheimer's disease cases and n=4 non-Alzheimer's disease cases could do the same and whether α1-antichymotrypsin would have an affect on these processes.

Using flow cytometry and fluorescence labelled Aβ1-42 aggregates, we found that primary human adult non-Alzheimer's and Alzheimer's astrocytes, as well as fetal astrocytes, dose and time-dependently become Aβ1-42 positive upon exposure to over-night formed Aβ1-42 aggregates. Further, we documented a trend in Aβ1-42 binding efficiency as fetal astrocytes and adult Alzheimer's disease astrocytes were the most prone to become Aβ1-42 positive. At 10 μM Aβ1-42 over night treatment, the majority of the Aβ1-42 aggregates were bound to the surface of the cells. However, we also found indications of cellular uptake, by using confocal laser scanning microscopy. By adding α1-antichymotrypsin to the Aβ1-42 mixture, which was incubated over night for aggregate formation, Aβ1-42 aggregate formation was modified. At 10:1 and 100:1 molar ratios of Aβ1-42 to α1-antichymotrypsin, less dense Aβ1-42 aggregates with a more heterogeneous oligomer profile were formed, as determined with electron microscopy and western blotting. In addition, upon treatment with 10 μM 10:1 Aβ1-42-α1-antichymotrypsin over night, the percentage of Aβ1-42 positive cells, increased in all cell populations, especially in the adult astrocytes, compared to Aβ1-42 treatment alone. In contrast, less fetal astrocytes became Aβ1-42 positive when treated over night with 10 μM 100:1 and 1000:1 Aβ1-42- α1-antichymotrypsin, versus 10 μM Aβ1-42 alone. At a lower Aβ1-42 dose, 2.5 μM over night, also the amount of adult astrocytes becoming Aβ1-42 positive decreased compared to the amount of

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positive astrocytes upon treatment with 2.5 μM Aβ1-42 alone. Using the enzyme linked immuno-sorbent assay (ELISA) technique we measured the release of MCP-1 into the cell supernatants, as a marker of pro-inflammatory activation of the cells. At the standard over night treatment of 10 μM Aβ1-42, the MCP-1 levels, in the cell supernatants, were similar in treated and non-treated cells, indicating that the Aβ1-42 binding and uptake at this concentration, takes place without an apparent inflammatory response.

8.3 Levels of serpins and markers of Alzheimer's disease and endothelium activation in non-demented controls, Alzheimer's disease and dementia with Lewy bodies patients (PAPER III, IV)

We measured the plasma and cerebrospinal fluid levels of three serpins, neuroserpin, α1-antitrypsin and α1-antichymotrypsin, whereof the latter two are acute-phase proteins, in a cohort (The Malmö Alzheimer Study) of well characterised clinically diagnosed Alzheimer's disease and dementia with Lewy bodies patients, relative to non-demented controls (PAPER III) (Table 3). All of these proteins have been linked to dementia. Whereas normal plasma levels of α1-antichymotrypsin (340-620 mg/L) (Lorier, Hawes et al. 1985) and α1-antitrypsin (1.3 g/L) (Jeppsson, Laurell et al. 1978) are known, the concentrations of neuroserpin have, to our knowledge, prior to this investigation never been determined before. We also measured the concentrations of the three standard Alzheimer's disease biomarkers, phosphorylated (threonine 181)-tau, total tau and Aβ1-42, in the same patients and controls. These are widely accepted to reflect the pathological processes in Alzheimer's disease, including neuronal degeneration (total-tau), formation of neurofibrillary tangles (phosphorylated tau) and the depositions of Aβ1-42 in the plaques. (Blennow and Hampel 2003; Hampel, Mitchell et al. 2004; Andreasen and Blennow 2005) (Table 3).

In addition, by measuring the plasma and cerebrospinal fluid levels of three soluble adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) in combination with angiotensin converting enzyme (ACE), we aimed to

elucidate the endothelium in the same patient cohort (The Malmö Alzheimer Study) (PAPER IV) (Table 3). As a large amount of attention is devoted to vascular pathology and inflammation in the pathogenesis of Alzheimer's disease, we hoped that the outcome of this study would lend us better comprehension of the interplay endothelium-inflammation in the investigated patient cohort.

Table 3. Marker profile in dementia patients versus non-demented controls (%)

Markers	Alzheimer's disease		Dementia with Lewy bodies	
	Elevated (%)	Decreased (%)	Elevated (%)	Decreased (%)
Plasma ACT	19.5[#]	-	12.6[NS]	-
CSF ACT	44.0[#]	-	36.0[#]	-
Plasma AAT	11.8[NS]	-	16.9[NS]	-
CSF AAT	42.1[#]	-	34.2"	-
CSF Neuroserpin	25.5[#]	-	8.8[NS]	-
CSF T-Tau	75.6[#]	-	7.5[NS]	-
CSF P-Tau	28.1[§]	-	19.3"	-
CSF Aβ1-42	-	47.4[#]	-	38.6[#]
Plasma ICAM-1	38.7[#]	-	102.5[#]	-
CSF ICAM-1	-	14.0[NS]	43.7[#]	-
Plasma VCAM-1	7.8[NS]	-	9.2[NS]	-
CSF VCAM-1	-	29.4[#]	-	38.6[#]
Plasma PECAM-1	23.0[#]	-	29.6[#]	-
Plasma ACE	0.6[NS]	-	-	2.4[NS]
CSF ACE	-	6.4[NS]	-	17.0[NS]

% values are obtained by comparing median values of markers levels from the two dementia groups with median values of the control group. NS=not significant. #, § and " indicate a significant difference at the $p< 0.001$, $p<0.01$ and $p<0.05$ level.

8.3.1 Serpins and Alzheimer's disease markers in relation to cognitive function, disease pathological processes and diagnostic accuracy (PAPER III)

By using correlation analysis, we aimed to investigate whether any of the markers correlated to cognitive function (MMSE score) and if levels of the measured serpins possibly could be linked to some of the pathological processes through correlation with the known Alzheimer's disease biomarkers (Table 4). In addition, we aimed to clarify whether serpins could add to the diagnostic accuracy, in combination with the known Alzheimer's disease biomarkers, in distinguishing between the two investigated dementia types.

Table 4. Serpin correlation with standard Alzheimer's disease markers

Markers	Alzheimer's disease			Dementia with Lewy bodies		
	Total-Tau	P-Tau	Aβ1-42	Total-Tau	P-Tau	Aβ1-42
Plasma ACT	-	-	-0.129"	-	-	-
CSF ACT	-	-	-	-0.358"	-	-
CSF Neuroserpin	0.486#	0.406#	-	0.517#	-	-

-) not significant. # and " indicate a significant difference at the p< 0.001 and p<0.05 level

Interestingly, increased levels of plasma α1-antichymotrypsin, cerebrospinal fluid total-tau and phosphorylated tau correlated with lower MMSE scores in Alzheimer's disease patients, whereas increased levels of α1-antitrypsin and total tau were linked to lower cognitive function in dementia with Lewy bodies patients. We could also link some of the serpins to the pathological processes, represented by decreased Aβ1-42 and elevated total and phosphorylated tau (table 4). Elevated neuroserpin levels were strongly associated with elevated levels of tau in both dementia groups, indicating a role as marker of neuronal damage, similar to levels of the tau protein. In dementia with Lewy bodies patients, increased levels of cerebrospinal fluid α1-antichymotrypsin were linked to lower levels of cerebrospinal fluid total-tau, suggesting a neuroprotective role of α1-antichymotrypsin in dementia with Lewy bodies. Elevated plasma, but not cerebrospinal fluid α1-antichymotrypsin, was in the

Alzheimer's disease patients correlated to decreased Aβ1-42, of which the latter is a proposed marker of amyloid deposition in the brain.

In regard of disease specificity, the three highest odds ratios (OR) obtained for Alzheimer's disease were related to decreased cerebrospinal fluid levels of Aβ1-42 (OR 102.9, p<0.001), increased levels of cerebrospinal fluid total-tau (OR 10.89, p<0.001), and neuroserpin (OR 10.75, p<0.001), closely followed by elevated levels of cerebrospinal fluid α1-antichymotrypsin (OR 10.59, p<0.001). For dementia with Lewy bodies, the highest odds ratios were related to decreased cerebrospinal fluid levels of Aβ1-42 (OR 12.0 p<0.001), elevated cerebrospinal fluid levels of α1-antichymotrypsin (OR 10.0, p<0.001) and elevated plasma levels of α1-antitrypsin (OR 4.6, p<0.01). However, though we believed that we documented disease specific differences, we could not enhance the discriminative ability compared to the well-documented standard Alzheimer's disease markers.

8.3.2 Surrogate markers of endothelilal activation, relation to cognitive function and BBB integrity (PAPER IV)

Several of the measured soluble adhesion molecules were elevated in Alzheimer's disease and dementia with Lewy bodies patients (Table 3), however none of the endothelial markes were associated with cognitive function (MMSE score). Both cerebrospinal fluid levels of sICAM-1 and sVCAM-1 exhibited a positive correlation with the cerebrospinal fluid/serum albumin ratio, which indicated that at signs of comprised BBB (elevated albumin ratios), levels of these to markers increase. Only one marker was found to be decreased in the dementia patient groups compared to controls, cerebrospinal fluid sVCAM-1 (Table 3). This is an interesting result, as the generation of sVCAM-1 has partly been attributed to proteolytic cleavage by serine proteases (Levesque, Takamatsu et al. 2001). As levels of the serpins α1-antichymotrypsin and α1-antitrypsin are elevated in both dementia groups (Table 3), we speculate that lower sVCAM-1 levels might partly be due to protease inhibition. The physiological significance of this results is however not clear.

9. Major conclusions

In vitro studies

- Plasma derived wild-type α1-antichymotrypsin forms SDS-unstable complexes with Aβ1-42, upon which the chymotrypsin inhibitory activity of α1-antichymotrypsin is diminished. α1-antichymotrypsin also renders Aβ1-42 oligomer formation, favouring dimers instead of trimers, which *in vivo* have been shown to inhibit hippocampal long-term potentiation in rats.

- α1-antichymotrypsin potentiates the altering effect on gene expression of soluble Aβ1-42 in primary fetal human astrocytes

- At certain ratios of Aβ1-42 to α1-antichymotrypsin, α1-antichymotrypsin influences the percentage of primary human astrocytes binding and potentially internalising Aβ1-42 *in vitro*.

- At substoichiometric ratios of chymotrypsin over α1-antichymotrypsin, α1-antichymotrypsin appears to partially protect Aβ1-42 from chymotrypsin digestion.

Whether α1-antichymotrypsin is a pathologic or non-pathologic player in the pathogenesis of Alzheimer's disease might be dependent on the local ratios of Aβ1-42-α1-antichymotrypsin in the brain.

Clinical studies

- Neuroserpin, which earlier was linked to the familial dementia FENIB, showed a specific association with Alzheimer's disease by elevated cerebrospinal fluid levels in Alzheimer's disease patients versus both dementia with Lewy bodies patients and non-demented controls.

- Release of neuroserpin into the cerebrospinal fluid appears linked to neurological processes reflected by increased levels of total tau and phosphorylated tau, as these were highly correlated in Alzheimer's disease and dementia with Lewy bodies.

- Elevated levels of cerebrospinal fluid α1-antitrypsin and plasma α1-antichymotrypsin correlated with lower cognitive function (MMSE scores) in dementia with Lewy bodies and Alzheimer's disease patients respectively. Thus, different members of the serpin family might be linked to disease specific mechanisms affecting cognitive function in different dementia types.

- Elevated levels of α1-antichymotrypsin and α1-antitrypsin in dementia patients might partially be responsible for the decreased cerebrospinal fluid levels of the soluble VCAM-1 in the investigated patients with Alzheimer's disease and dementia with Lewy bodies. The physiological significance is however unclear.

- Despite of earlier findings of a link between lower activity of the angiotensin converting enzyme and higher susceptibility to Alzheimer's disease (Kehoe, Katzov et al. 2003), no difference in actual protein concentrations was related to dementia diagnosis.

- Increased levels of plasma and cerebrospinal fluid sICAM-1 and plasma sPECAM-1 in dementia patients, especially in dementia with Lewy bodies, supports the notion of inflammatory and vascular mechanisms in dementia pathogenesis of Alzheimer's disease and dementia with Lewy bodies.

Serpins appear to reflect on distinct disease processes, depending on dementia type, which may include the regulation of cell adhesion molecules. Whether altered levels of the investigated serpins have a causative role in the dementia pathogenesis, remains to be elucidated.

10. Concluding remarks

This work included studies on the three serpins α1-antitrypsin, neuroserpin and α1-antichymotrypsin, whith special attention devoted to the latter, in the setting of neurodegenerative dementia. Serpins in general, are proteins which many crucial physiological processes depend on. Many of them are found in the circulation,

produced mainly by the liver and leukocytes, whereas others are tissue specific and only produced locally (Travis and Salvesen 1983). Neuroserpin appears to be one of these tissue specific serpins as the production of this protein so far only has been attributed to cells within the nervous system (Osterwalder, Contartese et al. 1996; Hastings, Coleman et al. 1997). This was in our investigation supported as we could not detect neuroserpin in plasma. α1-antichymotrypsin is found in the circulation, derived from the liver. This serpin is however also locally produced by reactive astrocytes under the influence of pro-inflammatory cytokines within the brain (Das and Potter 1995; Kordula, Rydel et al. 1998; Kordula, Bugno et al. 2000; Abraham 2001). The glycosylation of α1-antichymotrypsin found in the circulation and in the brain, slightly differs (Hwang, Steineckert et al. 1999) and even though its role as a protease inhibitor seems to be its main function in the circulation, the function of the locally produced protein might be another. We speculate that different serpins might be involved in the neurodegenerative processes in different types of dementia, which is in our investigation was supported by the finding that elevated levels of α1-antichymotrypsin and α1-antitrypsin correlates with cognitive function in Alzheimer's disease and dementia with Lewy bodies respectively. Longitudinal patient and healthy control studies might answer the question whether altered levels of serpins are primary or secondary events in the processes leading to cognitive dysfunction. More basic research is warranted on brain cellular responses to elevated or decreased physiological levels of the serpins involved in dementia. First by understanding the physiological and non-pathological functions of serpins in the brain, we might get a clue to as what mechanisms underly the pathogenesis neurodegeneration and dementia.

11. Populärvetenskaplig sammanfattning

11.1 Bakgrund

Demens är en obotlig sjukdom som drabbar 7 % av befolkningen över 65 år och hela 30 % av dem som är äldre än 80 år. Orsaken till utvecklandet av demens är okänd, men teorier rörande inflammatoriska processer, blodkärlsrelaterade skador och framför

allt ansamling av amyloidproteinet som delar i sjukdomsförloppet, är välkända. De två vanligaste neurodenerativa demenstyperna är Alzheimers sjukdom och demens med Lewykroppar, vilka till viss del har en liknande symptombild. Dessutom hittar man ofta en liknande patologi i hjärnvävnaden från avlidna patienter med dessa sjukdomar. De två huvudsakliga fynden i hjärnvävnad från Alzheimerspatienter är proteinansamlingar utanför hjärncellerna (plack), bestående av aggregerat amyloidprotein och fibrillära nystan av tau protein inuti nervcellerna. I demens med Lewykroppar hittar man också, av varierande grad, dessa två karakteristiska fynd, men man hittar även Lewykroppar. Dessa består i sin tur till största delen av ett annat protein, alfa-synuclein. I denna avhandling har tre proteiner, alfa1-antikymotrypsin, alfa1-antitrypsin och neuroserpin ur proteinfamiljen serpiner undersökts för deras roll i de två beskrivna demenssjukdomarna. Dessa tre proteiner har tidigare blivit associerade med demens då man bland annat hittat alfa1-antikymotrypsin och alfa1-antitrypsin i placken hos Alzheimerspatienter, medan genetiska mutationer av neuroserpin har visats sig vara orsaken till en annan demenssjukdom (FENIB).

11.2 Resultat

Vi har med våra studier visat att alfa1-antikymotrypsin påverkar aggregeringen av amyloidproteinet, vilket anses påverka utvecklingen av plack i hjärnvävnaden och att alfa1-antikymotrypsin samtidigt, under vissa omständigheter, skyddar amyloidproteinet från nedbrytning av enzymer. Dessutom har vi kommit fram till att en kombination av alfa1-antikymotrypsin och den lösliga formen av amyloidproteinet påverkar uttrycket av olika gener hos en viss hjärncellstyp (astrocyter) i större omfattning än proteinerna var för sig. I pågående kliniska studier försöker man att eliminera amyloidproteinet, vilket man anser vara giftigt för bland annat nervceller, från hjärnvävnaden hos Alzheimerspatienter. I våra studier visade det sig att om man tillsätter alfa1-antikymotrypsin till amyloidproteinet, innan man får detta att bilda aggregat, så påverkar man bindingen och möjligtvis också upptaget av dessa amyloidaggregat hos cellerna som nämnts ovan (astrocyter).

I två studier, i vilka vi inkluderade patienter med Alzheimers sjukdom och demens med Lewykroppar, men även äldre icke-dementa kontrollpersoner, mätte vi halterna av flera inflammatoriska proteiner associerade med funktionen hos blodkärlsväggarna, halterna av de tre serpinerna nämnda ovan och dessutom nivåerna av tre kända markörer för Alzheimers sjukdom. För första gången visade vi resultat på nivåerna av neuroserpin i ryggmärgsvätska, vilket inte har blivit uppmätt i människor tidigare. Dessa nivåer var dessutom förhöjda hos Alzheimerspatienter jämfört med kontroller och patienter med demens med Lewykroppar. Vi fann även att förhöjda nivåer av alfa1-antikymotrypsin och alfa1-antitrypsin korrelerade med lägre mental funktion hos demenspatienterna i respektive diagnosgrupp. De två demensgrupperna uppvisade dessutom förhöjda nivåer av markörerna för blodkärlsfunktion, vilket vi tror beror på inflammtoriska processer och en delvis störd kärlfunktion.

11.3 Slutsatser

Våra resultat stöder teorierna om inflammatoriska och blodkärlsrelaterade mekanismer i utvecklandet av demens. Våra studier visar dessutom att serpiner med största sannolikhet är involverade i de processer som leder till den mentala försämringen hos demenspatienter. Hos dessa patienter rör det sig då möjligen om lokala skillnader i serpinkoncentrationer och den därpå följande (eller föregående) interaktionen mellan serpiner, amyloidprotein och hjärnceller. Hur serpiner förhåller sig till alfa-synucleinet i Lewykroppar är inte känt. Framtida studier bör utröna de exakta mekanismerna för hur serpiner deltar i de neurodegerativa processerna vid utvecklandet av demens.

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