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Heparan Sulfate in the Amyloidosis and Inflammation of Alzheimer's Disease

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Abstract
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Alzheimer's disease (AD) is a neurodegenerative disorder, with extensive evidence implicating the misfolding, aggregation and deposition of the amyloid-β (Aβ) peptide as central to the pathogenesis. Heparan sulfate (HS) is an interactive glycosaminoglycan, attached to core proteins as HS proteoglycans (HSPGs). HSPGs are present on cell surfaces and in the extracellular matrix where they facilitate multiple signaling functions, but HS is also consistently present in all amyloid deposits, including those of AD. In amyloidosis HS has been studied as an aggregation template, promoting fibril formation and serving a scaffold function in the resulting deposits. The objective of this thesis was to assess how cell surface HS is potentially implicated in Aβ amyloidosis and the associated neuroinflammation of AD.

In AD brain we determined that HS predominantly accumulated in Aβ deposits with dense cores and found glial-expressed HSPGs within these deposits. Aβ elevated HSPG levels in primary glial cultures, implicating activated glia as one source of the Aβ-associated HS. Next, we determined that microglial HSPGs are critical for the upregulation of interleukin-1β and tumor necrosis factor-α following exposure to lipopolysaccharide, an established inflammatory insult. Together these results raise the possibility that Aβ-induced expression of microglial HSPGs may promote neuroinflammation.

Multiple mechanisms of Aβ toxicity have been proposed and different Aβ assemblies exert their toxicity through alternative routes. We found that three different preparations of Aβ aggregates all exhibited HS-dependent cytotoxicity, which in part correlated with Aβ internalization. Furthermore, heparin treatment attenuated Aβ cytotoxicity and uptake. In Aβ-positive AD microvasculature, HS deposited with Apolipoprotein E (ApoE) and its receptor, the low density lipoprotein receptor-related protein 1 (LRP1). In cell culture, HS and LRP1 co-operated in Aβ interactions and the addition of ApoE increased the levels of cell-associated Aβ in a HS- and LRP1-dependent manner. This ApoE-mediated increase in cell-associated Aβ may promote toxicity and vascular degeneration, but equally HS-mediated internalization of Aβ could represent a clearance route across the blood-brain-barrier.

The findings presented here illustrate multiple roles for cell-surface HSPGs in interactions relevant to the pathogenesis of AD.

Keywords: Alzheimer's disease, amyloidosis, amyloid-β, apolipoprotein E, astrocytes, glia, heparanase, heparan sulfate, heparan sulfate proteoglycans, microglia, neuroinflammation

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List of papers

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

I **O'Callaghan P**, Sandwall E, Li JP, Yu H, Ravid R, Guan ZZ, van Kuppevelt TH, Nilsson LN, Ingelsson M, Hyman BT, Kalimo H, Lindahl U, Lannfelt L, Zhang X. (2008) Heparan sulfate accumulation with amyloid-β deposits in Alzheimer's disease and Tg2576 mice is contributed by glial cells. *Brain Pathology*, 18:548-61

II **O'Callaghan P,** Li JP, Lindahl U, Lannfelt L, Zhang X. (2011) Microglial heparan sulfate proteoglycans mediate pro-inflammatory signaling. *Manuscript*

III Sandwall E*, **O'Callaghan P***, Zhang X, Lindahl U, Lannfelt L, Li JP. (2010) Heparan sulfate mediates amyloid-β internalization and cytotoxicity. *Glycobiology*, 20:533-41

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* These authors contributed equally to the study

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Related studies

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The cover illustration depicts heparan sulfate proteoglycans on the plasma membrane. This and all other illustrations in the thesis were drawn by the author. Paul O'Callaghan 2011.

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Abbreviations

AD	Alzheimer's disease
ApoE	Apolipoprotein E
Aβ	Amyloid-β
AβPP	Amyloid-β precursor protein
BBB	Blood-brain-barrier
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CD11b/14/36	Cluster of differentiation 11b/14/36
CHO	Chinese hamster ovary cells
CS	Chondroitin sulfate
DS	Down's syndrome
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GPC	Glypican
GPI	Glycophosphatidylinositol
HEK	Human embryonic kidney cells
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cells
IL-1β	Interleukin-1β
LRP1	Low-density lipoprotein receptor-related protein 1
mAb	Monoclonal antibody
mCD14	Membrane bound CD14
NFT	Neurofibrillary tangles
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PI	Propidium iodide
RAGE	Receptor for advanced glycation end products
RPTPσ	Receptor protein tyrosine phosphatase sigma
sCD14	Soluble CD14
scFv	Side-chain variable fragment
SDC	Syndecan
SDS	Sodium dodecyl sulfate
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α

Introduction

Alzheimer's disease

Background

From the age of 65 the risk of developing Alzheimer's disease (AD) doubles each year[1]. Consequently, as life expectancy increases worldwide, in part due to medical advances in the treatment of other disorders[2], so too does AD. Current estimates suggest that there are 35 million people with dementia worldwide[3], and projection models for 2050 predict that 1 in 85 will suffer from AD[4].

AD is a neurodegenerative disorder where the loss of synapses leads to a progressive decline in cognitive functions. Early symptoms include impaired short term memory and problems with spatial orientation, language and logic. As the disease progresses extensive memory deterioration is associated with a loss of communication and practical skills. These symptoms reflect the location of AD lesions, found throughout the parietal and temporal cortices, typically concentrated in the hippocampus and entorhinal cortex[5]. The patient's experience of AD is difficult to understand or convey. Auguste D was a patient of Alois Alzheimer and is considered the first recognized case of the disease. In an extract from her medical journal from 1901, Alzheimer notes that when asked to write her name she repeated the words *"I have lost myself"*[6]. A sad insight to this loss-of-self is revealed by the artist William Utermohlen (1933-2007), who continued to paint self-portraits following his AD diagnosis. His decline is reflected in the gradual disappearance of almost all facial details from his portraits, accompanied by an inability to sign his paintings, eventually surpassed by a failure to recognize his own name (www.williamutermohlen.org).

Pathology of Alzheimer's disease

In 1907 Alois Alzheimer presented the pathology of the condition that would eventually bear his name [6-8]:

"In the centre of an otherwise almost normal cell there stands out one or several fibrils due to their characteristic thickness…
…Numerous small miliary foci are found in the superior layers. They are determined by the storage of a peculiar material in the cortex…."

These descriptions refer to the intraneuronal accumulation of hyperphosphorylated tau as neurofibrillary tangles (NFT) and the extracellular deposits of amyloid-β (Aβ) (*Figure 1A-C*)[7]. Associated pathologies include cerebral amyloid angiopathy (*Figure 1E, F*), dystrophic neurites (*Figure 1D*) and gliosis. This association of microglia and astrocytes with Aβ deposits has lead to extensive studies regarding the role of neuroinflammation in AD and is discussed later. Multiple other molecules are also found co-deposited with Aβ and NFTs, among these heparan sulfate (HS) and apolipoprotein E (ApoE) are prominent[9-14]. While Aβ and tau have an undisputed association to AD, it has been established that the extent of NFT distribution correlates better with the degree of dementia than Aβ pathology[15]. However, both pathologies are also detected in the brains of cognitively healthy elderly individuals[16,17].

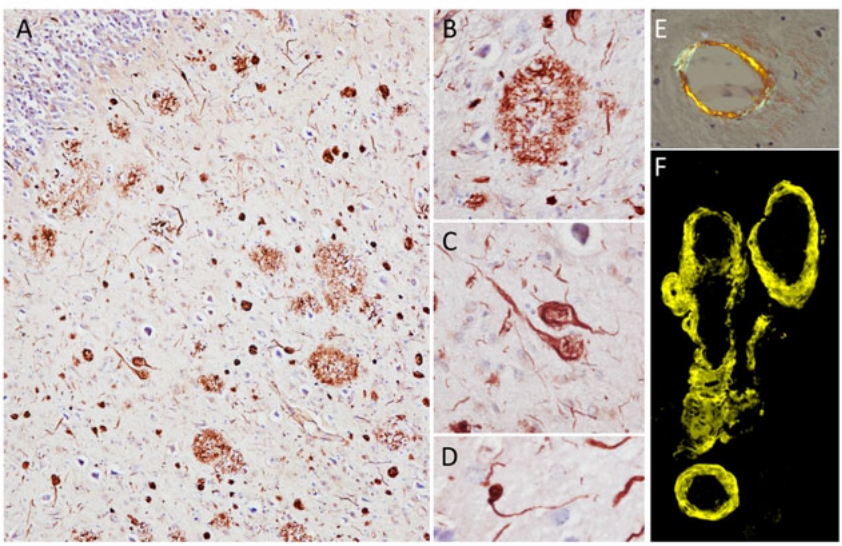

Figure 1. **Alzheimer's disease pathology**. **A.** Heparan sulfate immunostaining (mAb 10E4) of AD hippocampus reveals the signature pathologies of amyloid-β deposits (**B**) and tau tangles (**C**). Dystrophic neurites (**D**) are detected close to these lesions. Amyloid-bound Congo red displays birefringence under polarized light, detecting cerebral amyloid angiopathy in **E**. Extensive vascular pathology is also detected with antibodies against Aβ (**F**). *Original magnification: A, 100X; B-D, 400X; E, 200X; F, 400X.*

Releasing Aβ

The Aβ sequence was determined following the purification of amyloid isolated from the vasculature of AD brain[18]. Aβ is translated as part of the Aβ precursor protein (AβPP), a type I transmembrane protein, reported to promote synaptic plasticity and neurite outgrowth[19,20]. The release of Aβ is dependent on the sequential amyloidogenic processing of AβPP (*Figure 2)*, first by the β-site AβPP cleaving enzyme 1 (BACE1) before position 1 of the Aβ sequence. Next the length of the Aβ peptide (37-40, 42 or 43 amino acids) is determined by intramembranous cleavage at one of the γ-secretase sites[21-24]. These events are thought to occur in intracellular acidic vesicles after the endocytosis of AβPP and BACE1 from the extracellular surface [25].

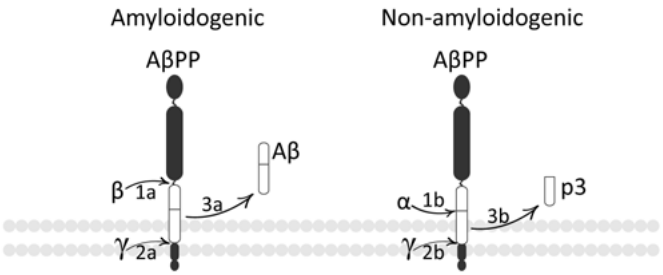

Figure 2. **AβPP proteolysis**. Amyloidogenic processing is initiated by BACE1 cleavage at the β-secretase site of AβPP, exposing the N-terminus of the Aβ sequence (**1a**). The γ-secretase complex releases Aβ by cleaving in the transmembrane domain of AβPP, at the C-terminus of Aβ (**2a-3a**). Non-amyloidogenic processing of AβPP starts with α-secretase cleavage before position 17 of the Aβ sequence (**1b**), γ-secretase then releases the p3 fragment of AβPP (**2b-3b**).

In contrast, Aβ release is precluded if the intra-Aβ α-secretase site is severed first; cleavage at the γ-secretase site will instead release the p3 fragment consisting of the Aβ residues 17-(37-43)[26] (*Figure 2*). BACE1 processing of AβPP is dependent on their co-occurrence in lipid rafts[27]. These are microdomains of the plasma membrane composed of high levels of sphingolipids and cholesterol. Consequently, the proteolytic fate of AβPP can be influenced by the composition of the plasma membrane[28,29].

Genetic determinants of Alzheimer's disease

Apolipoprotein E

In late onset AD the best established genetic risk determinant is an individual's ApoE genotype. ApoE occurs as three major isoforms; ApoE3 is the most commonly expressed isoform, ApoE2 appears to confer a degree of

protection from AD[30], and ApoE4 homozygotes are at greatest risk of developing AD[31-33]. In the brain, ApoE is produced primarily by astrocytes and its functions include the transport of lipids and cholesterol to neurons[34]. To facilitate this role ApoE consists of an N-terminal receptor binding domain linked via a flexible hinge region to a C-terminal lipid binding domain[35] (*Figure 3*). The lipid binding portion of ApoE embeds in lipoparticles while the exposed receptor binding region docks the particles to cell surfaces expressing low-density lipoprotein receptors (LDLR), including the low density lipoprotein receptor-related protein 1 (LRP1) (*Figure 3*).

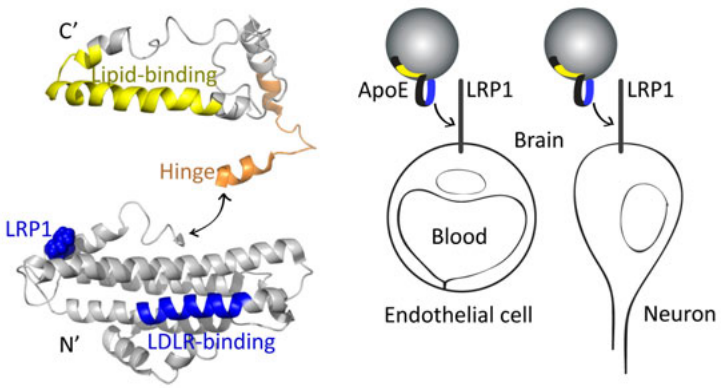

Figure 3. **ApoE structure and function in the brain**. The C-terminal lipid binding domain of ApoE is connected via a hinge region to the N-terminal receptor binding domain. ApoE is processed via thrombin cleavage at the hinge site (double headed arrow). LRP1 is a member of the LDLR family of ApoE receptors and is expressed in the brain on endothelial cells and neurons. ApoE is attached to lipoparticles of cholesterol and lipids (grey sphere) via its C-terminus. The exposed N-terminus of ApoE binds to LRP1 on neurons and transfers its cargo. Endothelial LRP1 is also implicated in the transcytosis of ApoE across the blood-brain-barrier (BBB). *PyMol® model of ApoE3 based on the structure published by Chen et al. 2011*[36].

Structural studies of ApoE3 demonstrate that lipid-free ApoE assumes a conformation that inhibits its interactions with LDLR[37]. Importantly, ApoE contains HS and Aβ binding sites in both of its structural domains and the possible relevance of this will be discussed later. ApoE is metabolized via thrombin cleavage within the hinge region, and the resulting 22 kDa N-terminal domain has neurotoxic properties[38]. The conformation and interactive potential of ApoE are dependent on the presence of isoform-specific residues. The three major isoforms differ with respect to their isoelectric point due the presence of cysteine (Cys) or arginine (Arg) at positions 112 and 158 as follows: ApoE2, Cys/Cys; ApoE3, Cys/Arg; ApoE4, Arg/Arg. The Cys content in ApoE2 and ApoE3 facilitates the formation of disulfide bridged ApoE dimers[39], while Arg at both positions in

ApoE4 permits an intra-domain interaction. These isoform dependent structural differences also alter interactions between ApoE and Aβ, and this is proposed as one basis for the pathogenic role of ApoE4 in AD[40].

Familial variants of AD

The trisomy of chromosome 21 in Down's syndrome (DS) provides an extra copy of the AβPP gene[41,42]. This increase in gene dose, ultimately leading to an increase in Aβ production, is a direct explanation for the AD pathology and dementia in later life observed in DS[43-46]. In addition, a subset of AD is explained by inherited autosomal dominant mutations in the genes encoding AβPP or the presenilins. Amyloidogenic processing of AβPP is favored in these familial forms of AD (FAD). The Swedish mutation (AβPP KM670/671NL)[47-49] converts AβPP into a substrate that is more favorably cleaved by BACE1. Proteolysis occurs in the Golgi apparatus prior to surface presentation of AβPP, which reduces the potential for non-amyloidogneic α-secretase processing[50]. In contrast, the Flemish AβPP mutation (A692G) produces a substrate that is poorly processed by α-secretase[51]. As part of the γ-secretase complex, pathogenic mutations in the genes encoding presenilin-1 and -2 favor AβPP cleavage that results in the release of Aβ42 - an aggregation-prone form of the peptide[19,52]. One such mutation is the PS1 exon 9 deletion (PS1 Δ9 AD)[53], which leads to a distinctive Aβ pathology, where the parenchyma is full of large round deposits, termed 'cotton wool plaques'. They are rich in Aβ42 and present with significantly less gliosis than that observed for dense-core neuritic deposits[54,55].

There are a number of mutations in the intra-Aβ domain of AβPP, including the Flemish mutation discussed above, and the Arctic (AβPP E693G) mutation at position 22 of Aβ[56,57]. This mutation increases the aggregation propensity of Aβ, promoting the formation of protofibrils, and occurs within a hydrophobic region where glutamic acid is substituted for a more flexible glycine; this presumably accelerates destabilization of the monomer. *In vitro* the Arctic mutation affects the distribution of AβPP, prolonging its time in intracellular vesicles, and increasing the incidence of β-secretase cleavage[58-60].

The Aβ hypothesis

The amyloid cascade hypothesis proposes that overproduction and/or impaired clearance of Aβ drives its accumulation, aggregation and deposition[61,62]. Pathogenic states of Aβ are thought to precede and promote NFT formation[63] and other tissue responses including inflammation[64,65]. The cascade culminates in neuronal dysfunction and loss, which presents as progressive dementia.

This Aβ-based theory requires a tipping-point, an event that unbalances the normal homeostasis, creating conditions that promote Aβ accumulation. In this context, the familial mutations discussed above are in part accountable for early onset AD, before the age of 65[66]. Beyond the increased risk associated with ApoE4, the more common late onset form of AD (~95% of cases) is not readily explained by clearly delineated genetic causes, although it is often associated with strong patterns of familial inheritance[32]. In contrast to the overproduction implicated in early onset AD, a recent comparison of Aβ levels in CSF revealed that, despite equal levels of Aβ production, Aβ clearance was impaired in patients with late onset AD[67]. Therefore, different underlying molecular mechanisms may converge on the common pathogenic pathway that leads to AD.

Neuroinflammation

Aβ deposits are often enclosed by a perimeter of reactive microglia and astrocytes. In CNS injuries these glial scars form barriers around the lesion and isolate it from surrounding healthy tissue[68]. Part of the scars function is to prevent unregulated neurite outgrowth, fulfilled by the release of chondroitin sulfate proteoglycans[68,69]. The need for the formation of these protective scars is in part a reflection of the potent nature of the immune reaction. The activation of a pro-inflammatory response requires tightly regulated risk assessment, as cytokines such as IL-1β and TNF-α have neurotoxic potential[70,71]. Resolution of the inflammatory state is imperative, because chronic immune activation contributes to neurodegeneration. An important factor for the efficiency of the immune response in AD is age, and cellular senescence can offer explanations as to how glial cells negatively alter homeostasis in the aging brain, contributing to neurodegeneration[72]. This idea is supported by observations in a transgenic model of Aβ deposition, where elderly animals revealed a marked decrease in microglial expression of Aβ degrading enzymes and uptake receptors, while IL-1β and TNF-α expression were increased[65].

Microglia, macrophages and astrocytes

During development, monocytes enter various organs, differentiate and assume residence there as tissue-specific macrophages. This facilitates a rapid, acute response to a wide range of insults. In a dynamic resting state their processes are continually surveying the surrounding microenvironment, alert to pathogens and cell death[73]. Inflammatory insults, including Aβ, bacterial endotoxins and cellular debris, induce an active M1 state that is associated with pro-inflammatory cytokine release. In the brain this immune function is fulfilled by microglia. However, in the injured brain the macrophage population is increased by recruitment of blood-borne monocytes across the BBB[74,75]. It is proposed that activated microglia and

recruited macrophages are morphologically comparable, but phenotypically distinct[74]. In this respect, the infiltrating macrophages are thought to contribute to the resolution of the inflammatory response by promoting an M2, anti-inflammatory state, and via their actions as phagoctyes (*Figure 4*). A variety of surface receptors, including CD36, scavenger receptor A[76] and RAGE[77] have been identified as Aβ uptake receptors, and are expressed on macrophages, including microglia. The aggregation state of Aβ seems to determine, or at least confer, preference for a particular uptake route and CD14 has been implicated as a surface receptor for fibrillar Aβ[78,79].

Astrocytes perform numerous support functions in the CNS. Due to their structural role in the neurovascular unit, the presence of astrocytes in glial scars may facilitate vascular repair and increase blood-flow to the injury site[68]. However, astrocytes can produce inflammatory cytokines and are also reported to mediate uptake and clearance of Aβ[80-83].

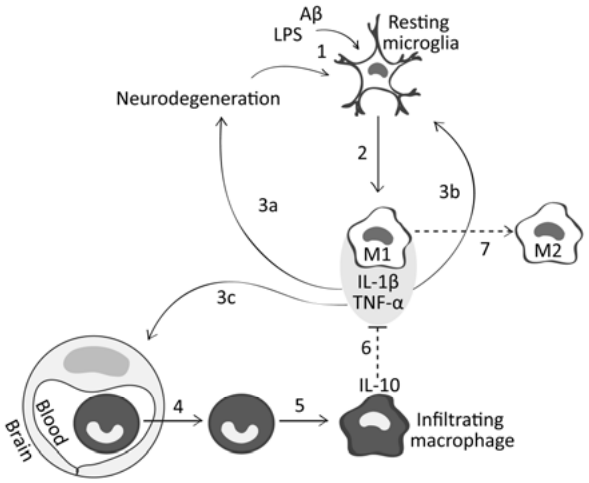

Figure 4. **Microglia and macrophages in neuroinflammation**. **1.** Resting microglia survey their surroundings and can be activated by Aβ, LPS and cellular debris from neurodegeneration. **2.** Microglia adopt an M1 phenotype, characterized by the release of pro-inflammatory cytokines including IL-1β and TNF-α. This acute response is essential for destroying pathogens. **3a**. Prolonged exposure to cytokines can be neurotoxic and promote neurodegeneration. **3b**. Exposure to IL1β and TNF-α can also activate resting microglia and amplify the pro-inflammatory response. **3c**. IL-1β and TNF-α as well as M1 chemokines signal via the endothelium to circulating monocytes. **4**. Monocytes attach to the luminal surface of the BBB and then transmigrate into the brain parenchyma **5**. Recruited monocytes then differentiate to macrophages. **6**. These infiltrating macrophages are thought to facilitate resolution of the inflammatory response by expressing anti-inflammatory cytokines e.g. IL-10, and are efficient phagocytes. **7**. The shift in the cytokine balance can promote M1 microglia to adopt an M2 (anti-inflammatory) phenotype, contributing to the resolution of the inflammatory state. *This model is in part an adaption from Schwartz and Shechter, 2010[74].*

Pro-inflammatory signaling

Pathways that trigger the release of pro-inflammatory cytokines are highly conserved and have been extensively studied. They are also the topic of this year's Noble Prize in Physiology and Medicine, awarded for discoveries regarding the activation of the innate immune system[84-86]. Lipopolysaccharide (LPS) is a gram-negative bacteria endotoxin, which induces cytokine release by binding CD14 and activating Toll-like receptor-4 (TLR4); this engages the NFκB pathway, upregulating IL-1β and TNF-α[73] (*Figure 5*). CD14 is typically glycophosphatidylinositol (GPI) anchored at cell surfaces (mCD14) and facilitates the transfer of LPS to TLR4. Astrocytes lack mCD14 and it has been proposed that TLR4 activation in these cells is mediated through a soluble form of CD14 (sCD14)[87] produced by hepatocytes[88] and macrophages, including microglia[89]. Autocrine signaling loops allow upregulated soluble cytokines to signal back onto the source cell, binding their receptors and amplifying the inflammatory response. In this manner TNF-α can upregulate its own expression as well as the expression of IL-1β and CD14[90] (*Figure 5*).

The direct neuroinflammatory potential of Aβ is observed in its ability to activate the CD14, TLR2 and TLR4 signaling pathways, promoting a pro-inflammatory signal cascade[91,92]. In the context of AD, the inflammatory response has a bad reputation and the neurodegenerative aspects of the process are often emphasized. However, inflammation refers to a wide-spectrum of events, and as mentioned the negative effects must be outweighed by the positives. This equation may be imbalanced if the identity of the inflammatory stimulus influences the intensity and/or duration of the inflammatory response. For example, an intracranial injection of LPS administered to a transgenic model of AD resulted in a microglial-dependent reduction of Aβ load[93]. It is possible that Aβ induces a relatively weak, but prolonged inflammatory response, detrimental to neurons and insufficient to resolve the pathology. In contrast, the rapid and robust response triggered by LPS promotes resolution, possibly through efficient amplification of signaling mechanisms and activation of phagocytic phenotypes. Therefore, therapeutics that modify the inflammatory response could prove valuable supplements to strategies such as anti-Aβ immunotherapy.

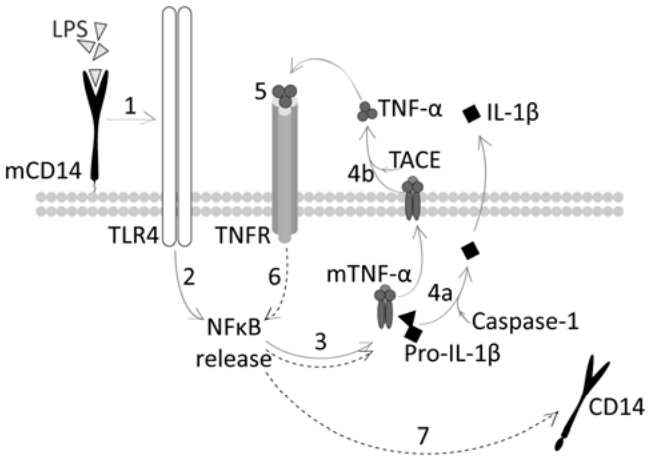

Figure 5. **LPS-induced pro-inflammatory signaling**. **1.** LPS binds membrane bound CD14 (mCD14) and transfers it to Toll-like receptor 4 (TLR4). **2.** Dimers of TLR4 activate the NFκB pathway, upregulating pro-interleukin-1β (Pro-IL-1β) and homotrimers of membrane bound TNF-α (mTNF-α) (**3**). Pro-IL-1β is converted to its active IL-1β form by caspase-1[94] (**4a**) and TNF-α converting enzyme (TACE) releases soluble TNF-α homotrimers[95] (**4b**). **5.** TNF-α binds TNF receptors (TNFR) and can activate the NFκB pathway **(6)**, this autocrine/paracrine signaling upregulates further cytokine production **(3)**. TNF-α signalling is also implicated in the upregulation of CD14 **(7)**.

Aβ aggregation and toxicity

Amyloid

Amyloidoses are protein misfolding disorders and to date some 28 amyloid proteins have been identified in human diseases[96,97]. Early work on transmissible amyloidosis had led many in the field to consider that the pathogenic agent could be a virus[98], and like a virus amyloid proteins are replicators. However, during amyloidosis no genetic data needs to be transferred and no new biological material has to be synthesized. Instead, misfolded amyloid proteins act as templates on which surrounding amyloid proteins associate and conform to the misfolded state[99]. The process is comparable to crystal formation; in which small ordered assemblies act as subunits and platforms from which larger structures can grow. Amyloid proteins form large insoluble fibrils that destroy tissue architecture, causing cell death and ultimately organ failure[100,101].

Dynamics of Aβ aggregation

As for all amyloid proteins, aggregation of Aβ is a nucleation and concentration dependent reaction. During the lag phase of aggregation, Aβ monomers must first unfold before coalescing to form low molecular weight (MW) oligomers. The unfolding event is dependent on changes in the free

energy state of the peptide[102]. The folding funnel model dictates that in a given environment a proteins native conformation will represent the lowest free energy state[103,104]. Therefore, changes in Aβ's microenvironment e.g. pH, temperature and the local concentration of Aβ and/or other folding chaperones (e.g. ApoE and HS), can alter the peptides conformation. One explanation as to why misfolded monomers proceed to aggregate is provided by the hydrophobic collapse theory[105]: Peptide misfolding can result in conformations that expose hydrophobic functional groups; Aβ has several, including a central hydrophobic cluster at residues 17-22, and at its C-terminus. The exposure of hydrophobic residues is energetically unstable and so several misfolded monomers assemble to enclose them within an aggregate structure. These multimers act as seeds upon which surrounding Aβ molecules rapidly assemble and a variety of resulting high MW structures have been reported. A schematic view of the aggregation process is described in *Figure 6.*

The dose makes the posion

Cell culture studies of Aβ toxicity are dependent on toxicity assay selection, the cell-type tested and the Aβ dose. However, in the context of aggregation-prone Aβ, dose is not necessarily a straightforward concept. As described, Aβ aggregation results in the formation of transient assemblies of increasing molecular weight, but also of altered conformation[106]. It should be noted that many of the reported Aβ aggregates have been derived from *in vitro* studies, although a number of Aβ assemblies have been detected *in vivo*[107-110]. Therefore, the possibility that different assemblies of Aβ exert their toxicity via distinct mechanisms has been explored and several studies have determined that pre-fibrillar multimers of Aβ have greater toxic potential than the end-stage Aβ fibrils[111,112]. This structure-toxicity relationship has been investigated with stabilized Aβ oligomers, where higher order oligomers of Aβ proved more neurotoxic than Aβ dimers, despite being prepared from equivalent molar concentrations of monomeric Aβ[113].

The toxic effects of Aβ are reported to include mitochondrial dysfunction, production of reactive oxygen species, activation of caspases involved in apoptosis and competitive-binding to growth factors[114]. Mechanisms by which specific Aβ structures exert their toxicity include the formation of membrane spanning pores, leading to an imbalance in ion homeostasis[115-118]. The relevance of intracellular Aβ as an initiator of pathogenesis has still to be fully elucidated[119,120], but mechanisms have been proposed by which internalized Aβ and Aβ derived from intracellular processing of AβPP promote cell death[121-123]. More recently, Aβ toxicity has been assessed with functional assays that aim to measure the effects of Aβ on synaptic transmission, and the detrimental effects of AD-derived Aβ on long-term potentiation (LTP) has been demonstrated in tissue slice experiments[124,125].

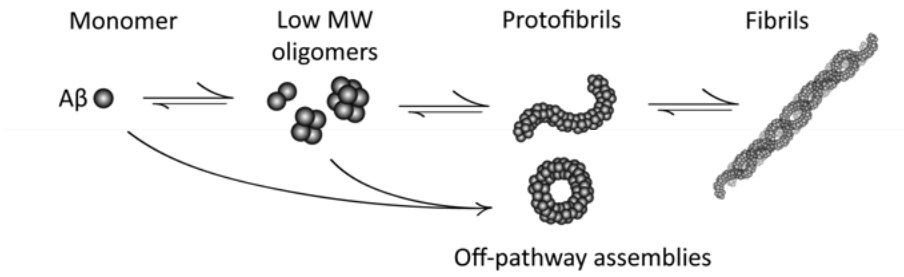

Figure 6. **Aβ aggregation.** Aβ aggregation proceeds as a nucleation dependent reaction. During a lag phase Aβ monomers associate to form low molecular weight oligomers that serve as aggregation seeds. At sufficient concentrations of Aβ the seeds drive a rapid exponential phase of aggregation, producing larger Aβ assemblies (e.g. protofibrils), which proceed to fibrils. Off-pathway Aβ assemblies have also been reported, these do not progress to form amyloid fibrils.

Diagnostics and therapeutics

The precise criteria on which a diagnosis of AD is assigned are currently under review[126,127], and a conclusive diagnosis still requires a post-mortem pathological examination. The clinical assessment involves cognitive testing and a review of the patient's medical history to rule out other potential causes. In addition, biochemical analysis of patient's plasma and cerebrospinal fluid can also reveal alterations in biomarkers that are indicative of AD[128-133]. Advances in neuroimaging techniques using amyloid-binding tracers increase the potential for *in vivo* detection of AD pathology[134-139]. These diagnostics are critical to the success of therapeutic strategies as the ability to intervene prior to the onset of symptoms will be important for a successful outcome.

Today AD can neither be prevented nor cured, but several treatments that temporarily improve cognition are prescribed. For example, the loss of synapses in AD is reflected by a decrease in the neurotransmitter acetylcholine. In an effort to decrease acetylcholine turnover in the synaptic cleft, patients are administered an inhibitor of acetylcholinesterase. In recent years an extensive effort has been invested in the development of anti-Aβ immunotherapeutics. Several treatment strategies are currently in clinical trials with antibodies that have been selected due to their high affinity for specific forms of Aβ[140].

Heparan sulfate proteoglycans and heparanase

Heparan sulfate biosynthesis

The glycosaminoglycan HS is a carbohydrate polymer composed of alternating disaccharide units, specifically glucuronic acid (GlcA)/iduronic acid (IdoA) connected to glucosamine (GlcN) via an α/β1,4 glycosidic bond[141]. HS biosynthesis occurs in the Golgi apparatus, with an initial O-linked tetrasaccharide extending from a serine residue. The serine is part of a core protein and this complex of HS and protein is termed a HS proteoglycan (HSPG)[142]. The HS chain polymerizes by the alternating addition of N-acetylated GlcN (GlcNAc) and GlcA residues, which subsequently undergo a variety of sequential enzymatic modifications, including N-deacetylation and N-sulfation of GlcNAc to N-sulfated GlcN (GlcNS), epimerization of GlcA to IdoA, and O-sulfation reactions[141,143,144] (*Figure 7*). HS chain length ranges from 50-200 disaccharide units[145], and the arrangement of disaccharide domains and sulfation patterns are conserved in specific cell and tissue types[146]. This supports the idea that the consorted efforts of HS biosynthesis enzymes are under strict regulation[147]. HS is critical for development and transgenic models of altered HS-biosynthesis have revealed it to have essential functions in most organ systems and physiological processes[148,149].

Heparan sulfate proteoglycans

HSPGs are present in the extracellular matrix as perlecan, agrin and collagen XVIII[150], and attached to cell surfaces as six GPI-anchored glypicans (GPC1-6) and four transmembrane spanning syndecans (SDC1-4)[151] (*Figure 7*). Several HSPGs, for example SDC1 and SDC3, may also bear chains of the glycosaminoglycan, chondroitin sulfate. Heparin (a highly sulfated analog of HS) can be found attached to the intracellular proteoglycan serglycin, which in mast cells facilitates protease storage[152,153]. Cell surface HSPGs can be endocytosed for lysosomal degradation, but may also be recycled[154]. Importantly, cell-surface HSPGs are also subject to shedding, altering the landscape of the cell surface and creating a soluble pool of HSPG ectodomains. This is observed, for example, during inflammation[155] and wound healing[145].

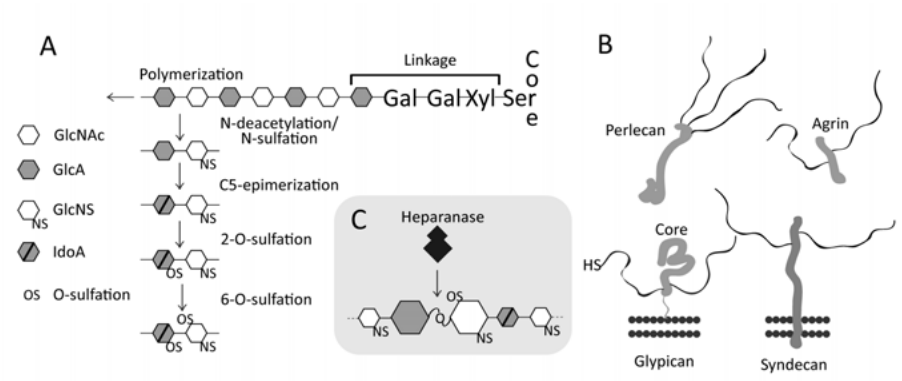

Figure 7. **HS biosynthesis, HSPGs and heparanase**. **A**. HS synthesis commences with the extension of a tetrasaccharide linker (Linkage) to a serine (Ser) in the amino acid sequence of the HSPG core protein (Core). Polymerases assemble the HS chain, which is first comprised of GlcNAc-GlcA disaccharide units. Units are then modified to varying degrees by a sequence of enzymatic reactions, which include N-deacetylation/N-sulfation (GlcNAc to GlcNS), C5-epimerization (GlcA to IdoA) and O-sulfation. **B**. The glypican family of HSPGs is GPI-anchored to cell membranes, while syndecans have a transmembrane spanning domain. HSPGs of the extracellular matrix include perlecan and agrin. **C**. The link between GlcA and 6-O-sulfated GlcNS is reported as a site of heparanase cleavage[156]. *Abbreviations:* GlcNAc, *N*-acetylated glucosamine; GlcA, glucuronic acid; GlcNS, *N*-sulfated glucosamine; IdoA, iduronic acid; Gal, galactose; Xyl, xylose; Ser, serine.

Heparanase

The HS chain can be fragmented by heparanase, an endo-β-glucuronidase that hydrolyses the β-1,4-glycosidic bond linking a GlcA to a GlcNS[157] (*Figure 7C*). The resulting HS fragments are of variable lengths and can subsequently be degraded by exoglycosidases and sulfatases[158]. Only one gene has been identified for heparanase, encoding a 65 kDa pro-enzyme, which undergoes cathepsin-L proteolysis, yielding an active heterodimer consisting of 50 kDa and 8 kDa subunits[159-162]. As indicated by an optimal activity observed at pH 5-6, heparanase is believed to cleave HS within acidic vesicles, specifically endosomes[163]. The 65 kDa form of heparanase is detected extracellularly and *in vitro* experiments have confirmed that internalization of pro-heparanase precedes catalysis to its active form[159]. HSPGs, LRP1 and mannose-6-phosphate receptors can all accommodate heparanase internalization[164-166]. However, while active heparanase is present in the cytoplasm it is also present within the nucleus, where it is believed to function as a transcription factor[167]. Heparanase is implicated in angiogenesis, inflammation and cancer where heparanase expressing tumor cells have enhanced metastatic potential[168]. The proposed physiological functions of heparanase are largely derived from studies in transgenic mice overexpressing heparanase (Hpa-tg) in which phenotypes including

increased vascularization and rapid tissue remodeling are observed[169,170]. Heparanase overexpression in Hpa-tg mice results in expected HS fragmentation, however it also upregulates the extent of chain sulfation and the rate of HSPG turnover[171]. Heparanase is clearly involved in HS metabolism, but the ability of heparanase to remodel cell surface HS may also play a regulatory role in cellular responses to specific stimuli.

Heparan sulfate interactions with proteins

HS interactions with proteins are primarily facilitated by electrostatic exchanges between positively charged amino acid residues and negatively charged sulfate groups of the HS chain[172]. Optimal HS domain structures have been identified for interactions with a number of proteins, including fibroblast growth factor (FGF)[173,174] and antithrombin. In the case of antithrombin, its anticoagulant effects have been traced to interactions with a pentasaccharide sequence with a centrally located 3-O-sulfated glucosamine[175]. Efforts are ongoing to elucidate other potential HS domains that may confer selective binding to specific proteins[147,176].

HS interactions with proteins often mediate the formation of complexes. For example, HSPGs facilitate ternary complex formation between FGF and its receptors, promoting signal transduction[177,178]. Coles *et al.* 2011 recently proposed the existence of a proteoglycan switch, potentially relevant for multiple signaling mechanisms. They found that the tight distribution of HS domains, responsible for binding the neuron growth receptor protein tyrosine phosphatase sigma (RPTPσ), mediated receptor clustering and nerve regeneration[179]. In contrast, the spacing of RPTPσ binding domains on chondroitin sulfate (CS) chains did not promote clustering and therefore inhibited regeneration. This spatial distribution of HS protein binding domains may also be relevant for proteins with two HS/heparin binding sites such as ApoE. A hypothetical protein with two HS/heparin binding sites is presented in *Figure 8*. Without HS/heparin binding the protein assumes a ‘closed’ conformation, this could for example represent a concealed binding site. The ability of HS/heparin to alter the conformation of the protein to an ‘open’ state, where the hypothetical binding site is exposed, may be dependent on the spatial distribution of the protein binding domains positioned on the HS/heparin chain (*Figure 8*). It is also conceivable that two HS chains, present on separate HSPGs, could facilitate dual binding to such a protein. Therefore, the availability of HSPGs on the cell surface and the distribution of protein binding domains along the HS chains are important factors when studying HS interactions with proteins.

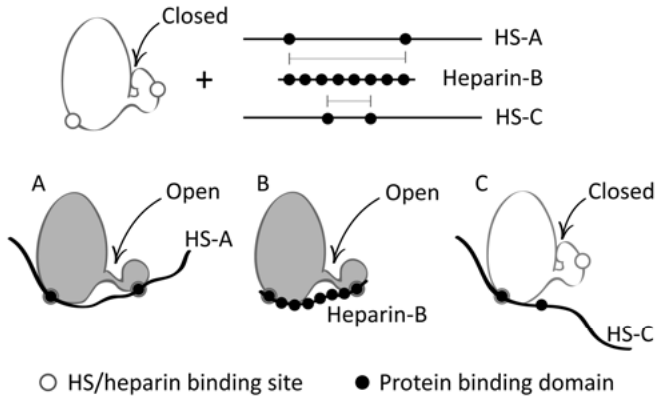

Figure 8. **Spatial distribution of protein-binding HS domains**. Several proteins, including ApoE, have two or more HS/heparin binding sites (open circles, ○). In the hypothetical example presented the unbound protein assumes a closed conformation. This could represent a concealed binding site. **A**. The protein-binding domains (closed circles, ●) of HS-A are optimally spaced to facilitate occupation of both HS/heparin binding sites, inducing an open conformation. **B**. Heparin-B has an excess of protein-binding domains and is of sufficient length to induce the open conformation. **C.** The HS-C chain is equal to HS-A in terms of its total polysaccharide length, but its protein-binding domains are spatially restricted from occupying both sites simultaneously; therefore, the protein remains closed.

HS interactions with amyloid

Aβ deposits, like all amyloid lesions also contain glycosaminoglycans (GAGs), predominantly heparan sulfate (HS)[12]. In amyloidosis, HS has been implicated as a scaffold molecule on which amyloid fibrils can aggregate and deposit[180-182]. The HHQK motif at Aβ residues 14-17 has been identified as a HS/heparin binding site that promotes fibrillization, but also mediates microglial interactions with Aβ[183]. We have established a similar HS/heparin binding site for the amyloid protein transthyretin, it too consists of basic residues and promotes fibrillization[180]. HS binding to Aβ is demonstrated to protect against protease degradation[184] and could therefore contribute to increased levels of Aβ in the brain. Beyond a scaffold role, heparin has been assigned a catalytic function in its interaction with fibronectin, a prominent component of the extracellular matrix in the CNS. Heparin induces conformational changes in fibronectin that expose otherwise cryptic binding sites for vascular endothelial growth factor (VEGF). The change in fibronectin conformation results in heparin release, but without a return to the cryptic (or closed) state[185]. It would be interesting to deduce if this catalytic potential of HS/heparin may also have relevance for HS/heparin-induced conformational changes in amyloid proteins, where a transient interaction between HS and e.g. Aβ may result in a permanent conformational change towards a more aggregation-prone state.

Additionally, N-sulfated HS domains with critical 2-O-sulfated iduronic acid residues have been implicated in HS interactions with Aβ fibrils, while HS binding to Aβ monomers requires additional 6-O-sulfated glucosamines[186,187]. Therefore, HS domains that interact with Aβ monomers, perhaps promoting Aβ aggregation and/or Aβ uptake[188,189], may be distinct from those that bind and potentially stabilize existing Aβ fibrils.

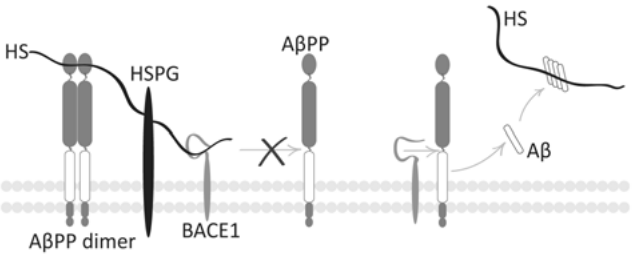

Figure 9. **HSPG regulation of Aβ release.** HS inhibits the β-secretase activity of BACE1 by inducing a closed conformation in the active site. Once the HS restriction is relieved (right) amyloidogenic processing of AβPP frees Aβ monomers and these may interact with surrounding HS chains that can act as templates for aggregation. HSPGs have also been found to promote dimerization of AβPP (far left). This complex is reported to mediate signaling events and as a dimer AβPP is less susceptible to amyloidogenic processing.

HS regulation of Aβ release

As BACE1 is the rate limiting enzyme in amyloidogenic proteolysis of AβPP, the discovery that HS is a natural inhibitor of its activity is of therapeutic relevance[190-192] (*Figure 9*). HS has also been reported to increase the activity of pro-BACE1, suggesting it can regulate both precursor and mature forms of the enzyme[193]. The physiological function of BACE1 is seen during early development where its expression is upregulated to mediate axon myelination[194]. In later life, functions of BACE1, beyond AβPP processing, are less well understood and heparin-derived BACE1 inhibitors have been designed as potential AD therapeutics[195,196].

AβPP has established HS/heparin binding domains and GPC1 has been shown to inhibit AβPP-mediated neurite outgrowth[20]. GPC1 has also been proposed to directly interact with Aβ, promoting aggregation that results in neuronal death[197]. A recent report on heparin-bridged dimers of AβPP, proposes that HSPG-mediated dimerization of AβPP facilitates signal transduction and receptor binding events[198] (*Figure 9*). Dimerization of AβPP is also associated with reduced Aβ production[199], suggesting that HS/heparin-based therapeutics could reduce Aβ levels in AD brain via multiple mechanisms.

Present investigations

Aim of this thesis

The overall objective of this thesis was to investigate potential roles for cell surface HS in the pathogenesis of AD. The specific aims for each paper were as follows:

I Map the distribution and elucidate a potential cellular source for HS in the Aβ deposits of Alzheimer's disease.

II Elucidate the requirement for cell surface HS in microglial inflammatory signaling and assess heparanase as a potential regulator of this process.

III Investigate a role for cell surface HS in the internalization and cytotoxicity of Aβ.

IV Study the effect of ApoE on Aβ interactions with cell surface HS and LRP1 as an explanation for Aβ accumulation in the vasculature.

A glial source for HS accumulation with Aβ deposits

The initial aim of **paper I** was to investigate the distribution of HS with the Aβ deposit pathology of AD. Dense-core Aβ deposits have a fibrillar amyloid core that is detectable with amyloid staining dyes such as Congo red[200] and are commonly associated with dystrophic neurites and gliosis. Diffuse deposits have less well defined morphology and fail to meet the definition of true amyloid as they are not readily stained with Congo red[97]. Some reports suggest that diffuse deposits mature into dense-core deposits[24,201], while others propose that they are distinct pathologies, forming independently of each other[202,203]. The latter concept is arguably supported by the fact that specific Aβ pathology is associated with certain familial AD variants. For example, carriers of a deletion mutation in exon 9 of the *presenilin-1* gene (PS1 Δ9 AD) present with an unusual neuropathology of large diffuse deposits of Aβ, described as 'cotton wool plaques'[204]. The mutation results in increased γ-secretase cleavage after Aβ position 42 in AβPP, and the deposits are primarily composed of Aβ42/43, with little Aβ40[205]. Therefore, specific Aβ peptide compositions may determine a deposit's morphological fate. Recently, this idea was experimentally supported in bitransgenic mice, in which low level expression of the aggregation-prone Arctic Aβ peptide with wild-type Aβ, resulted in an increase in diffuse, but not Congo red-positive deposits[203]. Importantly, the presence of glial cells surrounding Aβ deposits has also been suggested to influence Aβ deposit morphology[202] and glial HSPGs are implicated in interactions with Aβ[206].

Here we assessed HS accumulation with different Aβ deposit types and sought to identify its cellular source. HS has been attributed a critical role in the formation of other amyloid deposits[207] and we considered that HS-related differences between Aβ-deposit types could serve to explain their distinct morphologies.

Results and discussion

Using an immunohistochemical and image analysis approach we concluded that in AD brain, HS preferentially accumulated in deposits containing an Aβ40-positive dense core. In contrast, HS levels were significantly decreased in diffuse deposits and typically absent from the Aβ42-rich 'cotton wool plaques' detected in PS1 Δ9 AD (**paper I**, Figure 1 and 2). We determined that the co-occurrence of HS with Aβ deposits was recapitulated in the Tg2576 mouse model of Aβ deposition; although, the pattern of HS distribution within these deposits, as well as the deposit morphology were distinct from that observed in AD (**paper I**, Figure 1-3). We suggested that diffuse deposits, particularly 'cotton wool plaques', may form in the absence of HS, perhaps due to the higher aggregation propensity of Aβ42 relative to

Aβ40. This concept is supported by Lord *et al.* 2011 where the diffuse deposits, increased in bitransgenics expressing Arctic and wild-type Aβ, also proved to be HS-negative[203].

In the dense-core Aβ deposits of AD brain, we found astrocytes in close proximity to the central core of the deposits that were positive for the HSPGs glypican-1 (GPC1) and syndecan-3 (SDC3) (**paper I**, Figure 4). Immunostaining in Tg2576 brain revealed that the majority of Aβ deposits in this model were also positive for GPC1 and SDC3 (**paper I**, Figure 3) and deposit-infiltrating microglia and astrocytes were identified as a probable source for these HSPGs (**paper I**, Figure 5). In contrast, the 'cotton wool plaques' of PS1 Δ9 AD, which lack pronounced gliosis, also proved negative for intra-deposit GPC1 or SDC3 (**paper I**, Figure 4). This led us to consider that the accumulation of HS with dense-core Aβ deposits may in part be derived from HSPGs expressed by Aβ-associated glial cells. To assess this potential effect of Aβ on glial HSPG expression, we exposed primary microglia and astrocytes, isolated from C57BL mice, to Aβ fibrils and found elevated levels of GPC1 and SDC3 (**paper I**, Figure 6). We concluded that glial cells surrounding Aβ deposits express elevated levels of HSPGs. HS interactions with Aβ have been reported to be mutually protective[208], suggesting that the Aβ/HS interaction could persist after glial cell death, and present as the HS accumulated with dense-core Aβ deposits.

Microglial HS proteoglycans facilitate the inflammatory response

The contribution of the inflammatory response to the resolution and progression of AD is an extensive area of study. HS has been implicated in various aspects of inflammation[209]. For example, endothelial HSPGs play important functional roles in recruitment of leukocytes to sites of tissue injury. The chemokine CCL2 bound to HSPGs is transcytosed into the vascular lumen where it is presented to circulating immune cells. HS-bound CCL2 interacts with its receptor CCR2, expressed on blood-borne rolling leukocytes. This tethering event decelerates the leukocyte. Next, L-selectin on the leukocyte membrane binds to endothelial HSPGs[210] facilitating transmigration into the injured tissue. HS chains are also important in mediating the biological activity of chemokines[211], and HS can cross-link oligomers of APRIL (a member of the tumor necrosis factor superfamily) to promote B cell activation[212]. In addition, the intensity of the inflammatory response to endotoxins is downregulated by shedding of the HSPG syndecan-1, as TNF-α and IL-6 bound to its HS chains are thus removed from cell surfaces[213]. A greater understanding of the mechanisms underlying

the inflammatory reaction in the brain may assist in elucidating the extent to which this tissue response plays a role in AD.

Results and discussion

In **paper II** we tested the hypothesis that HS and/or heparanase is integral to the pro-inflammatory machinery that facilitates cytokine upregulation. In the brain, microglia are the number one cytokine producers, and in AD chronic exposure to pro-inflammatory cytokines such as IL-1β and TNFα is thought to contribute to neurodegeneration[64,214,215]. Astrocytes are also implicated in the inflammatory response, but are typically activated downstream of microglia[216]. Primary microglia and astrocytes were isolated from the brains of neonatal mice overexpressing heparanase (Hpa-tg) and non-transgenic controls (Ctrl). HS chain analysis revealed that heparanase overexpression had resulted in truncation of HS chains in both these cell types (**paper II**, Figure 1 and 3). Microglia or astrocytes were then exposed to the bacterial endotoxin lipopolysaccharide (LPS), an extensively studied stimulus of cytokine upregulation (*Figure 5*).

The levels of LPS-induced TNF-α and IL-1β were reduced in Hpa-tg microglia compared to Ctrl. In addition, Hpa-tg microglia failed to upregulate the LPS-receptor CD14 (**paper II**, Figure 2). This finding attributes microglial HSPGs an integral role in this pro-inflammatory signaling pathway. In contrast, no difference was found between TNF-α levels in Hpa-tg and Ctrl astrocytes, despite heparanase overexpression and HS fragmentation (**paper II**, Figure 3). This cell-type specific effect is possibly due to differences in the types and levels of receptors expressed on microglia and astrocytes e.g. it has been reported that astrocytes lack CD14[87]. HSPGs on each cell-type may consequently serve alternative co-receptor functions. Finally, heparin treatment of LPS-stimulated Ctrl microglia inhibited IL-1β upregulation, and inhibited TNF-α release from the microglial cell line EOC20. However, heparin failed to suppress TNF-α release from Ctrl microglia (**paper II,** Figure 4). This may reflect differences in HS-dependence for the upregulation of specific cytokines. IL-1β and TNF-α can promote their own upregulation via paracrine/autocrine signaling loops[217] and CD14 expression is induced by TNF-α binding to TNF receptors[90]. Therefore, it is possible that LPS-induced TNF-α amplifies the IL-1β response; consequently, heparin interactions with TNF-α may impair an autocrine signaling loop and thus primarily suppress the IL-1β response (**paper II**, Figure 4). In conclusion, we propose that the attenuated inflammatory response in Hpa-tg microglia is due to impaired interactions of TNF-α with its receptors. We suggest that this event is dependent on microglial HSPGs and may be regulated by heparanase. The proposed HSPG-dependent inflammatory signaling pathway is outlined in **paper II**, Figure 5.

Alternative roles for HSPGs in inflammatory signaling

The inflammatory mechanism studied in **paper II** has multiple components that interact in a variety of signaling loops. Given this complexity there are a number of interactions, potentially HS-dependent, that could have been disrupted in Hpa-tg microglia. For example, HSPGs may act as co-receptors for exchanges between CD14 with TLR4, and/or for the formation of TLR4 dimers. Furthermore, delipidized LPS retains affinity for CD14, but is not pro-inflammatory. Therefore, the conformation of CD14 enfolds a polysaccharide binding site[218,219] distinct from the region of CD14 that binds the lipid A component of LPS[219]. It is possible that HS fragments, derived from Hpa-tg microglia, could compete with LPS for this polysaccharide binding site, thus attenuating the inflammatory response. Consequently, while one HS-regulated mechanism of microglial inflammation is outlined in **paper II**, there are other interesting possibilities yet to be investigated.

HS and inflammation-associated amyloid

A similar Hpa-tg mouse was used to demonstrate that HS is critical for amyloid deposition in an experimental model of serum amyloid protein A (AA) amyloidosis[207]. Amyloidosis is initiated in this model by simultaneous administration of a seed of AA fibrils and an inflammatory stimulus (silver nitrate). Inflammation upregulates acute phase proteins including AA, primarily produced by the liver, and in the presence of AA-seeds the protein fibrillizes and deposits as amyloid in systemic organs. Given the attenuated inflammatory response in the Hpa-tg model studied in **paper II**, it could be relevant to investigate if the upregulation of acute phase proteins is also attenuated by heparanase-mediated fragmentation of HS. Although it was shown in Li *et al.* 2006 that organs failing to overexpress heparanase remained susceptible to amyloid deposition[207]. Nonetheless, acute phase upregulation of AβPP and Aβ has been reported in response to traumatic brain injury[220-224] and considering the ability of HS to regulate β-secretase activity, and therefore Aβ release[190], the potential connection between inflammatory responses and HS-dependent amyloid protein upregulation merits further investigation.

HS-dependent macrophage recruitment mediates Aβ clearance

In parallel with **paper II** we have also investigated the importance of HS in the recruitment of macrophages into the inflamed CNS. The Hpa-tg model was subjected to an intracerebral injection of Aβ42 fibrils and we found that fibril deposits persisted in Hpa-tg brain at time-points when complete clearance was achieved in Ctrl brains. (Zhang *et al.* 2011. *Submitted manuscript*)[225]. Immunohistochemical analysis revealed poor recruitment of glial cells, and significantly lower levels of CD45+ cells at the injection site. This cell marker is often used to identify macrophages that have been

recruited across the blood-brain-barrier (BBB) to the site of injury. We constructed an *in vitro* model of the BBB from primary endothelial cells derived from cerebral vasculature and established that impaired recruitment of macrophages across the Hpa-tg BBB was responsible for the reduced presence of CD45+ cells at the Aβ injection site. We concluded that endothelial HS is essential for efficient recruitment of immune cells across the BBB and propose that heparanase can regulate this process.

Conclusions (paper I and II)

It has yet to be determined what processes or factors determine the morphology of Aβ deposits. As discussed, dense-core deposits are associated with gliosis, but also contain dystrophic neurites. The order of events is difficult to determine i.e. does Aβ deposition drive dystrophy, which activates a glial response or, is neurodegeneration the result of a glial response to pre-deposited forms of Aβ. The latter concept is supported in a transgenic model of Aβ and tau pathology, where microglial-mediated elimination of neurons preceded Aβ deposition[226]. Microglia targeted and removed neurons at time-points when the prominent pathology in this model consisted of intraneuronal Aβ, suggesting that the microglial response can be triggered by early events in Aβ-mediated pathogenesis. What relevance these differences in Aβ deposit morphology may have for AD has yet to be elucidated. As discussed, the extent of Aβ pathology is not a good correlate to the degree of cognitive decline in AD, but perhaps the ratio between different Aβ deposit types could better reflect disease status[227]. In this context, it has been suggested that while Aβ deposits are present in cognitively healthy individuals they are typically of a diffuse type, while neuritic dense-core deposits are more prominent in late onset AD brain[228]. Therefore, the prevalence of specific deposit-types may reflect a pattern of disease onset, and as demonstrated in **paper I,** high levels of HS-positive Aβ deposits are likely to reflect elevated glial activation, and possibly an associated risk for neuroinflammation. In models of acute brain injury GPC1[229,230], SDC1 and SDC3[231] are upregulated in glial cells at injury sites. In this setting syndecans are believed to promote neurite outgrowth, while GPC1 inhibits axon regeneration. In the glial cultures prepared in **paper I,** fibrillar Aβ-alone induced elevated levels of GPC1 and SDC3, and while we did not confirm that the elevated HSPG levels were a result of increased protein expression, a subsequent report using human pericytes demonstrated that Aβ fibrils upregulated GPC1 mRNA and protein levels[232]. Accordingly, it is worth considering that Aβ deposits may inadvertently play a neuroprotective role, promoting expression of glial scar components. However, in **paper II** we established that microglial HSPGs are a critical component of the acute pro-inflammatory signaling response, involved in the upregulation of TNF-α and IL-1β. These cytokines are implicated in the

neurodegeneration in AD[233] and based on the ability of Aβ to elevate microglial HSPG levels, it is conceivable that Aβ promotes and/or aggravates a pro-inflammatory phenotype *in vivo* (*Figure 10*).

The proposed mechanism of microglial HSPG-mediated pro-inflammatory signaling, in combination with the function of endothelial HSPGs in macrophage recruitment, described in Zhang *et al.* 2011 (*Submitted manuscript)*[225], are relevant to neuroinflammation in a variety of conditions. These studies identify heparanase and HSPGs as targets for modulating the inflammatory response in the CNS.

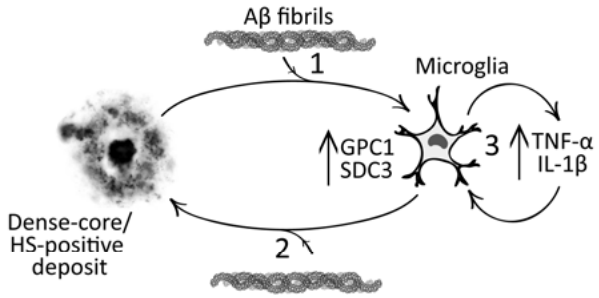

Figure 10. **Possible roles for HSPGs in Aβ deposition and neuroinflammation based on paper I and II**. Aβ fibrils and early stages of Aβ deposition may stimulate HSPG (e.g. GPC1 and SDC3) expression in glial cells, including microglia (**1**). The glial response to these forms of Aβ may promote the formation of dense-core Aβ deposit types by supplying co-deposit factors including HS (**2**). The Aβ-mediated upregulation of microglial HSPGs may increase microglial sensitivity to pro-inflammatory stimuli (including Aβ) and promote release of TNF-α and IL-1β (**3**).

HS-dependent Aβ cytotoxicity

Multiple mechanisms of Aβ toxicity have been proposed and a number of them have implicated HS. For example, Aβ interactions with microglial HS activates a neurotoxic phenotype[206], while low molecular weight heparin protects neuronal cells from Aβ toxicity[234]. In **paper III** we used three distinct cell systems to investigate the possibility that cell surface HS facilitates Aβ cytotoxicity.

Results and discussion

We confirmed that Aβ toxicity was attenuated in HS-deficient Chinese hamster ovary (CHO) cells, heparanase-overexpressing human embryonic kidney (hpa-HEK) cells and heparin-treated human umbilical vein endothelial cells (HUVEC), compared to their control counterparts. In parallel, we determined that Aβ internalization was also a HS-dependent event and propose that Aβ interactions with cell surface HS decrease cell viability **(paper III)**.

Preparation of toxic Aβ40 aggregates

In **paper III** we explored the toxicity and internalization of three different aggregation states of Aβ40. Using native PAGE we determined that each preparation protocol gave rise to a distinct and reproducible aggregate profile (**paper III**, Supplementary Figure 1). Non-aggregated Aβ40 (na-Aβ40) contained predominantly monomeric Aβ with a small population of low molecular weight (MW) aggregates, aggregated Aβ40 (a-Aβ40) contained less monomer and a population of mid MW aggregates, and aggregated-by-agitation Aβ40 (aa-Aβ40) contained less monomer than a-Aβ40 and a population of high MW aggregates. Subsequent to the publication of **paper III** the presence of Aβ40 protofibrils was confirmed in both the a-Aβ40 and aa-Aβ40 preparations[235], as defined by the mAb158 sandwich ELISA[140]. As we assume that Aβ in each preparation continued to aggregate during the cell incubation period, we did not aim to define a specific toxic intermediate, but importantly all three preparations proved cytotoxic in a HS-dependent manner (**paper III**).

HS-mediated mechanisms of Aβ toxicity

The attenuation of na-, a- and aa-Aβ40 toxicity in HS-deficient CHO cells clearly implicated cell surface HS as a mediator of toxicity (**paper III**, Figure 1 and 2). As HS functions as a co-receptor in multiple settings it is also possible that Aβ interactions with cell surface HS represents a common initiation point for several mechanisms of Aβ toxicity. The attenuated Aβ toxicity in hpa-HEK cells is open to interpretation (**paper III**, Figure 3). For example, heparanase degradation of cell surface HS may prevent toxic

interactions with Aβ. Alternatively, heparanase overexpression often leads to upregulation of HS sulfation[171] and it is possible that such HS fragments released into hpa-HEK cell medium have higher affinity for Aβ, sequestering it and inhibiting cytotoxic interactions with cell surface HS. Released HS-fragments may also bind Aβ and promote accelerated/altered aggregation of Aβ, potentially reducing or even bypassing aggregation intermediates that have greater toxic potential. However, these explanations are not mutually exclusive. HS interactions with Aβ fibrils are reported to occur via HS domains that are involved in binding to neurotrophic fibroblast growth factor (FGF)[187]. It is possible then that in the brain Aβ competes with FGF for HS-binding sites, essentially starving cells (*Figure 11*).

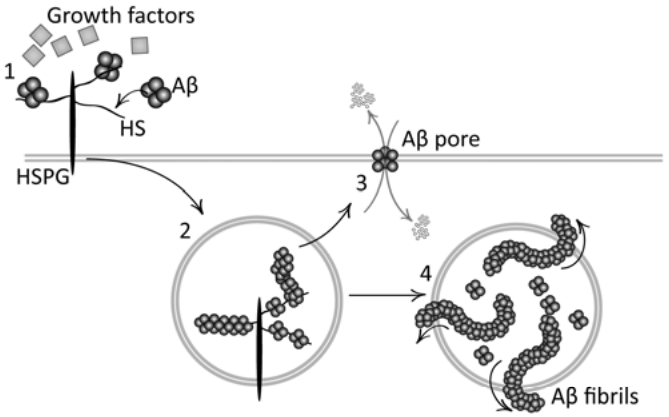

Figure 11. **Mechanisms of Aβ toxicity**. Aβ may compete with other ligands for HS binding sites on the cell surface; this effectively cuts off the cells access to growth factors (**1**). HS-mediated internalization of Aβ may facilitate a number of toxic events (**2**). Internalization of Aβ may facilitate the embedding of membrane spanning Aβ pores, implicated in the disruption of ion homeostasis (**3**). Aggregation of internalized Aβ is reported to result in the formation of fibrils that compromise the integrity of membranes (**4**).

Aβ toxicity may also be influenced by Aβ solubility, as insoluble Aβ fibrils often prove to be less toxic than soluble Aβ assemblies[111,113]. However, a recent report presents a mechanism of toxicity whereby internalized Aβ forms membrane-piercing fibrils, resulting in cell death and subsequent release of amyloid-like deposits into the extracellular space[121]. This finding demonstrates how the dynamic act of Aβ aggregation can itself be destructive, while extracellular exposure to the end-stage fibril may be relatively innocuous. The relevance of this mechanism is supported by the recent detection of intra-synaptic fibrils in transgenic models of Aβ deposition[123]. In **paper III** we used immunocytochemistry to assess Aβ uptake and found that na-Aβ40 and a-Aβ40 were internalized in a HS-

dependent manner (**paper III**, Figure 1 and 2). We proposed that HS-mediated internalization of Aβ represents part of the toxic mechanism and considering the findings of Friedrich *et al.* 2010[121] it is possible that continued aggregation of internalized Aβ could contribute to cell death (*Figure 11*). In contrast, aa-Aβ40 was not readily detected inside the cells. This lack of immunosignal may reflect a concealment of epitopes in this highly aggregated preparation of Aβ40. Alternatively, this Aβ preparation may exert its toxicity via an extracellular mechanism, which nonetheless involves HS.

Heparin protection against Aβ

In **paper III** heparin treatment of Aβ-exposed HUVEC cells attenuated toxicity and reduced Aβ internalization. The protective effect required a minimal heparin chain length of 18-mer or greater (**paper III**, Figure 4). Enoxaparin, a low MW derivative of heparin, attenuates Aβ deposition in a mouse model of Aβ amyloidosis[236]. However, in the Aβ transgenic setting and in the treatment of Aβ-producing cells, the protective effects of heparin may be multiple. Endogenous HS controls Aβ production by regulating BACE1 proteolysis of AβPP. In addition, the administration of heparin-derivatives inhibits BACE1 activity, thus reducing Aβ levels[190,192]. This has prompted the design of heparin analogs that efficiently inhibit BACE1 activity, are permeable to the BBB and lack anti-coagulant activity[196]. Accordingly, the protective effect of heparin does not necessarily require a direct interaction with Aβ.

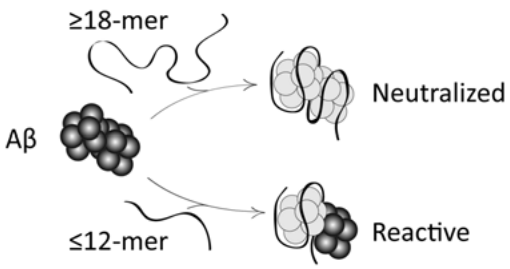

Figure 12. **Theoretical protective effect of heparin**. Heparin derived 18-mers may be the minimum chain length that can access and neutralize the reactive surfaces (e.g. residues that promote aggregation or cell-surface interactions) of toxic Aβ aggregates. In contrast, the heparin derived 12-mers may be too short for this purpose, allowing reactive surfaces to remain exposed and toxic.

In the context of **paper III**, the administration of exogenous Aβ circumvents the possibility that heparin protects against Aβ toxicity by inhibiting Aβ production. A direct interaction between heparin and Aβ, reducing Aβ uptake by cell surface HS, is one possible explanation for the protective

effect. However, why a minimal heparin chain length is required remains to be fully elucidated. HS and heparin chains are often discussed as linear polyanions, but they are also flexible molecules capable of assuming non-linear conformations[237]. If heparin protects HUVEC by interfering with the formation of toxic Aβ intermediates it is conceivable that a minimal chain length is required to 'neutralize' Aβ assemblies (*Figure 12*). Heparin-mediated neutralization may then inhibit interactions between Aβ aggregates that are precursors to toxic species and/or reduce the ability of toxic assemblies to interact with the cell surface (*Figure 12*). Alternatively, a minimum chain length may be required to facilitate an aggregation pathway that bypasses more toxic intermediates, discussed above in the context of the hpa-HEK results.

ApoE increases cell-associated Aβ via cell-surface HS and LRP1

The result of Aβ interactions with cell surfaces will presumably vary depending on the cell-type in question. Immune cells are capable of internalizing and degrading Aβ[238], while receptors on endothelial cells can transcytose Aβ across the blood brain barrier (BBB)[239]. Given that Aβ is constitutively expressed by all individuals it is not surprising that the brain is equipped to metabolize and clear it. Therefore, investigating potential mechanisms of Aβ deposition and clearance can increase our understanding of events that contribute to the accumulation of Aβ and the pathogenesis of AD. The E4 isoform of Apolipoprotein E (ApoE) is a major risk factor for AD, implicated in increased Aβ deposition and reduced Aβ clearance[240]. LRP1 is an ApoE receptor, but also facilitates neuronal uptake and endothelial transcytosis of Aβ[239,241]. In addition, recent evidence from Kanekiyo *et al.* 2011 asserts that HSPGs co-operate with LRP1 during neuronal uptake of Aβ[189]. Importantly, LRP1 and HSPGs also work together in interactions with ApoE[242-244].

Results and discussion

To ascertain the context in which interactions between Aβ, ApoE, HSPGs and LRP1 are of potential importance in AD brain we performed a qualitative immunohistochemical study. We established that HS, ApoE and LRP1 co-occurred in numerous Aβ40-positive microvascular structures throughout AD hippocampus (**paper IV**, Figure 1). We speculated that Aβ alone or in complex with ApoE may be transported from the brain across the BBB via endothelial HSPGs and LRP1, and that an imbalance in this process may explain why Aβ deposits in capillaries. To test the affect of ApoE on

cell-associated Aβ and the relevance of cell surface HSPGs and LRP1 in this process we first established that Aβ40 and a sample of human ApoE could form native complexes in solution (**paper IV**, Figure 3). Next, Aβ40 and or ApoE were incubated with Wt, HS-deficient or LRP1-deficient CHO cells. Cell associated levels of Aβ40 were analyzed by an Aβ-sandwich ELISA (**paper IV**, Figure 3). We found that the association of Aβ40 with cells was dependent on LRP1 and to a greater extent on cell surface HS, supporting the findings of Kanekiyo *et al.* 2011[189]. In addition, we discovered that the ability of ApoE to increase cell associated Aβ levels was also dependent on LRP1, but again demonstrated greatest dependence on cell surface HS (**paper IV**, Figure 3). To assess the impact of HS- or LRP1-deficiency on ApoE interactions with the cells, cell lysates and medium were analyzed for ApoE levels following a 3 h incubation. We determined that HS-deficiency reduced the presence of a 22 kDa ApoE band, which represents the N-terminal domain and has been reported to have neurotoxic properties[38,245,246](**paper IV**, Figure 2). This altered ApoE processing in the absence of HS is in keeping with previous observations attributing HS roles in lipoprotein metabolism[242,247]. The results presented in **paper IV** support a co-operative role for cell surface HS and LRP1 in interactions with Aβ and complexes of Aβ and ApoE. We have yet to elucidate if these interactions reflect steps in a normal Aβ clearance route, or if disturbances in this pathway, due to Aβ aggregation and/or ApoE4, contribute to Aβ accumulation in the microvasculature.

ApoE interactions with HS and Aβ

ApoE contains two HS/heparin binding sites, one in each of its functional domains (*Figure 13*). A two-step binding event has been proposed for HS interactions with ApoE and structural studies suggest that conformations induced by HS and/or Aβ regulate ApoE interactions with its receptors and lipids[36,248-250].

Hydrophobic residues in the ApoE C-terminal domain are believed to be involved in binding to Aβ, but N-terminal Aβ binding sequences have also been identified[251-253]. Analysis of the binding domains indicate that HS and Aβ binding sites do not overlap, so ApoE could in theory interact with both simultaneously (*Figure 13*). However, ApoE bound to HS or Aβ may adopt conformations that hinder access to other binding sites[36]. It is also possible that Aβ-bound ApoE may assume conformations comparable to its lipid-bound state. ApoE-binding receptors such as LRP1 and HSPGs may then 'recognize' Aβ-bound ApoE as lipidated and promote uptake, resulting in Aβ internalization.

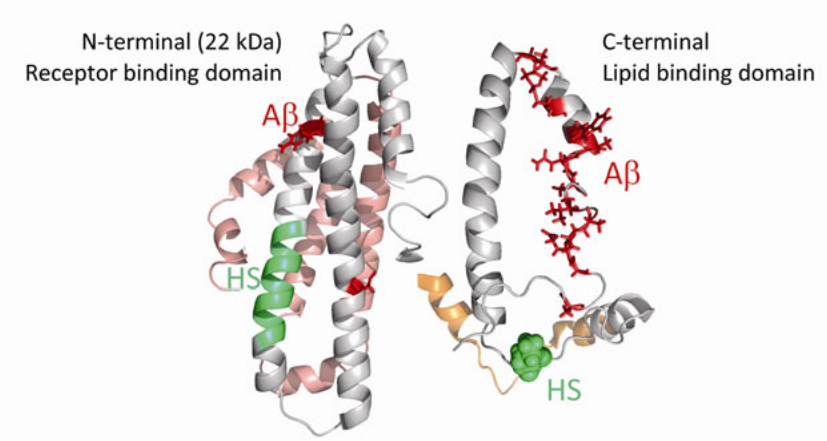

Figure 13. **The interactive potential of ApoE**. HS/Heparin and Aβ binding sites have been reported in the N- and C-terminal domains of ApoE. The HS/heparin binding sites at 136-147 (green helix) overlaps with the receptor binding domain. The presence of Cys at 112 and/or 158 (N-terminal red sticks) increases ApoE affinity for Aβ, while removal of 2-81 abolishes Aβ binding (light-red helix). The C-terminal HS/heparin binding domain is dependent on Lys at position 233 (green spheres) and the hydrophobic residues of the lipid-binding domain are involved in Aβ binding (red sticks). *PyMol® model of ApoE3 based on the NMR structure published by Chen et al. 2011*[36].

Aβ uptake as a means of amyloid transport

In **paper III** we discussed Aβ internalization as part of a toxic mechanism and as mentioned, a recent report confirms that HSPGs are a major pathway for neuronal uptake of Aβ[189]. In a separate context the internalization of Aβ by phagocytes is considered an important process for eliminating Aβ from the brain[254]. However, the inability of amyloid-laden macrophages to cross the BBB has been suggested to contribute to Aβ deposition in the vasculature[255]. To test the capacity of phagocytes to internalize Aβ, we air-dried Congo red-positive Aβ42 fibrils to high-binding glass slides (*Figure 14A*). Next we isolated primary peritoneal macrophages from C57BL mice and incubated them on top of the immobilized fibrils. After 3 days the cells were fixed and the intracellular Aβ was analyzed by immunocytochemistry, Congo red-staining and confocal microscopy (*Figure 14B-H*). We found macrophages to be highly efficient fibril-phagocytes, essentially engulfing all of the immobilized Aβ (*Figure 14D*). The internalized fibrils (Congo red-positive) presented as a relatively large perinuclear collar, but morphologically the cells appeared healthy (*Figure 14F*). The ability of monocytes to internalize amyloid fibrils has been implicated in the transmission of AA amyloidosis[256] and the results presented in *Figure 14* suggest that macrophages are capable of containing substantial Aβ-fibril loads. This undoubtedly contributes to Aβ clearance, but macrophage

migration to the vasculature and subsequent death could also result in the re-deposition of amyloid. Therefore, while in **paper IV** we discuss how unregulated interactions of endothelial HSPGs and LRP1 with Aβ:ApoE complexes may promote the deposition of Aβ in the vasculature, there are several clearance routes that can contribute to its presence there.

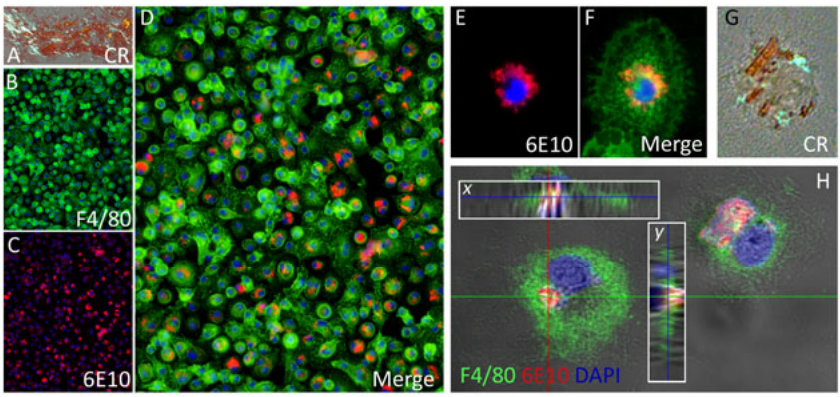

Figure 14. **Macrophage uptake of fibrillar Aβ.** Fibrils of Aβ42 were air-dried onto glass slides and Congo red-stained. Under polarized light red/green birefringence confirmed the presence of adhered amyloid-like Aβ fibrils (**A**). Primary peritoneal macrophages were isolated from C57BL mice and cultured on the Aβ fibrils for 3 days (**B-H**). Macrophage-associated Aβ was detected by co-immunostaining with the macrophage marker F4/80 (**B, D**) and the anti-Aβ antibody 6E10 (**C, D**). Halos of perinuclear Aβ (**E**) were detected within the macrophages (**F**). Congo red staining determined the intracellular Aβ to be fibrillar, seen as red/green birefringence under polarized light (**G**). Z-scans through Aβ-positive macrophages, prepared by confocal laser scanning microscopy, confirmed the Aβ to be internalized (**H**; *x* and *y* represent cross sections of the z-stacks). *Unpublished data.*

Conclusions (paper III and IV)

Cell surface HS mediated the cytotoxicity of three Aβ preparations with 'small', 'medium' and 'large' populations of Aβ aggregates (**paper III**, Supplementary Figure 1), this suggests that Aβ binding to cell surface HS could represent an initial event in multiple mechanisms of Aβ toxicity. We determined that Aβ toxicity in part correlated with HS-dependent internalization of Aβ. However, following the observation that ApoE, HS and LRP1 could be localized to Aβ deposits in the microvasculature of AD brain (**paper IV**, Figure 1), we considered the possibility that under healthy conditions this HS-mediated uptake of Aβ may represent a route of Aβ clearance. In cell culture we determined that ApoE increased cell-associated Aβ in a HS- and LRP1-dependent manner. However, the consequence of this event is difficult to predict and is presumably cell-type specific. It may represent a transcytosis mechanism, mediate Aβ degradation, facilitate Aβ

toxicity and/or contribute to Aβ accumulation (*Figure 15*). The potential for toxicity is supported by the findings in **paper III**, and a previous report demonstrates that the addition of HS or heparin attenuates Aβ toxicity in cultured cerebrovascular cells[257]. However, the presence of ApoE has been shown to protect astrocytes and pericytes, components of the neurovascular junction, from Aβ toxicity[258]. Despite the increased risk of AD associated with ApoE4 the precise molecular mechanism(s) underlying the connection have yet to be elucidated. Distinguishing potential physiological interactions of ApoE and Aβ, from pathogenic events will be necessary before ApoE based therapeutics can be developed for the treatment of AD.

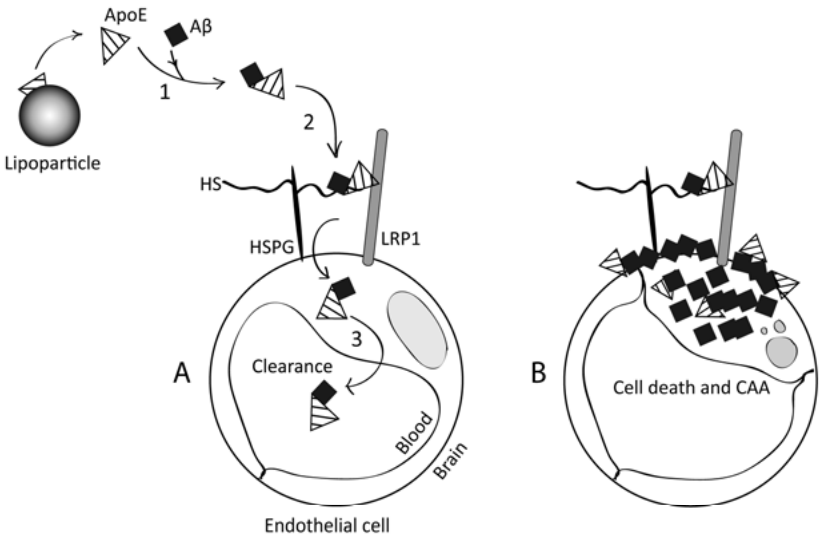

Figure 15. **Possible consequences of ApoE, HS and LRP1 interactions with Aβ at the blood-brain-barrier based on the findings in paper III and IV. A.** ApoE must separate from lipoparticles to interact with Aβ (**1**). ApoE and Aβ in complex can then interact with cell surface HS and LRP1 (**2**). This mediates internalization of the complex and may represent a transcytosis mechanism that facilitates Aβ clearance across the endothelial layer of the BBB (**3**). **B.** Alternatively, internalization of Aβ via this route may prove cytotoxic, equally Aβ accumulation on the cell surface may contribute to cell death and cerebral amyloid angiopathy (CAA). A version of this model, lacking HSPGs, has previously been proposed by Bu G. 2011[31].

Method considerations

According to the Oxford English Dictionary an experiment is an action or operation undertaken in order to discover something unknown. In the following section a number of the actions and operations used in this thesis will be discussed with respect to their influence on results.

The aggregation propensity of Aβ requires careful consideration, especially when results are a product of antibody-based detection systems. Transgenic models, in addition to the intended phenotype, may exhibit associated modifications that can have relevance to the interpretation of results, and therefore require careful characterization. Isolating specific cell-types is often necessary to simplify mechanistic studies, especially in situations where cross-talk between cell-types is known to influence a response. However, conclusions from such studies must take into account the complexity and diversity of the *in vivo* setting. Functional assays are designed based on known or assumed principles; consequently, if the target-of-study interferes with assay-dependent processes in an unpredicted manner results may be misrepresentative. Finally, the ability of Aβ to bind to an array of surfaces requires consideration when selecting materials for cell culture studies.

Image analysis and Aβ deposit morphology

As the findings in **paper I** and **paper IV** are in part derived from image analysis of immunostained tissue sections, a number of practical considerations relating to the detection, quantification and architecture of Aβ pathology will be discussed.

Single deposit image analysis

Figure 16A-E outlines the general aspects of the image analysis strategy used in **paper I**. In the example presented, sections were stained with a rabbit pAb against the C-terminus of Aβ40 (Aβ40, *Figure 16A*) and a mouse mAb against an N-terminal Aβ neoepitope (82E1, *Figure 16B*). Species-specific fluorescent secondary antibodies with distinct emission wavelengths were used to detect Aβ(1-40) and 82E1 (*Figure 16A and B*). The individual signals were then merged to detect areas of overlap (*Figure 16C*). The merged images were imported into the image analysis software ImageJ and used to construct an analysis map, created by selecting and labeling individual Aβ deposits. This map was then overlaid on the Aβ(1-40) or 82E1

channel (*Figure 16D and E*) and statistics such as the area of specific channel signals relative to the total deposit area can be recorded. This approach was used to identify HS-positive deposits and determine the area of HS staining/Aβ deposit in AD and Tg2576 brain (**paper I**, Figure 2).

Depth dependent morphology

The preparation of tissue sections and the optics of bright-field and fluorescence microscopy have resulted in Aβ deposits being typically described as plaques, a word synonymous with flat structures that lack depth. Confocal laser scanning microscopy (CLSM) allows for the three-dimensional structure of Aβ deposits to be appreciated. In CLSM fluorescent signals emitted from the labeled specimen are collected via a pinhole that is calibrated to deflect signals from optical planes that are not in focus. A motor controls sequential downward shifting of the lens and a series of in-focus images are collected along the depth axis (z-plane). Aligning these optical sections in imaging software such as Imaris® Bitplane allows for orthogonal viewing and three-dimensional modeling. Analyzing optical sections through Aβ deposits can reveal complications with regard to classifying a given deposits morphology as dense-core or diffuse. To illustrate this issue a 25 μm thick section of AD hippocampus was immunostained with an Aβ42 antibody. Optical sections were imaged at 1 μm depth intervals resulting in 25 frames (0-24) (*Figure 16F-H*). To optically represent two 13 μm thick sections, frames 0-12 were reassembled and found to present with dense-core deposit morphology, while reassembly of 12-24 resulted in diffuse deposit morphology (*Figure 16F and G*). In the 25 frame composite image the dense-core morphology prevails (*Figure 16H*). Accordingly, the morphology of an Aβ deposit in a tissue section is depth dependent, a fact which requires consideration when analyzing images of Aβ pathology.

Epitope accessibility and deposit architecture

In contrast to the diffuse outer halo, the core of the Aβ deposit presented in *Figure 16I is* intensely stained. However, analysis of the 18 optical sections (1 μm intervals) that include images of the core, suggests that the high intensity signals (red) are derived from its outer surface (*Figure 16I 7-24*). This distribution of immunosignal is open to several interpretations. Aβ within the central component of the core may be densely packed, thus reducing antibody diffusion and consequently binding. Alternatively, Aβ fibrils within the core may be less susceptible to antigen retrieval techniques and antibodies may be sterically hindered from accessing their epitopes. Aβ truncation and modifications resulting in e.g. pyroglutamate-^{200}, or the recently detected, nitrated-forms of Aβ^{259} may also remove or mask epitopes. Tissue fixation may irreversibly cross-link Aβ in complex with other deposit components e.g. HS or ApoE, obscuring the antibody-binding site.

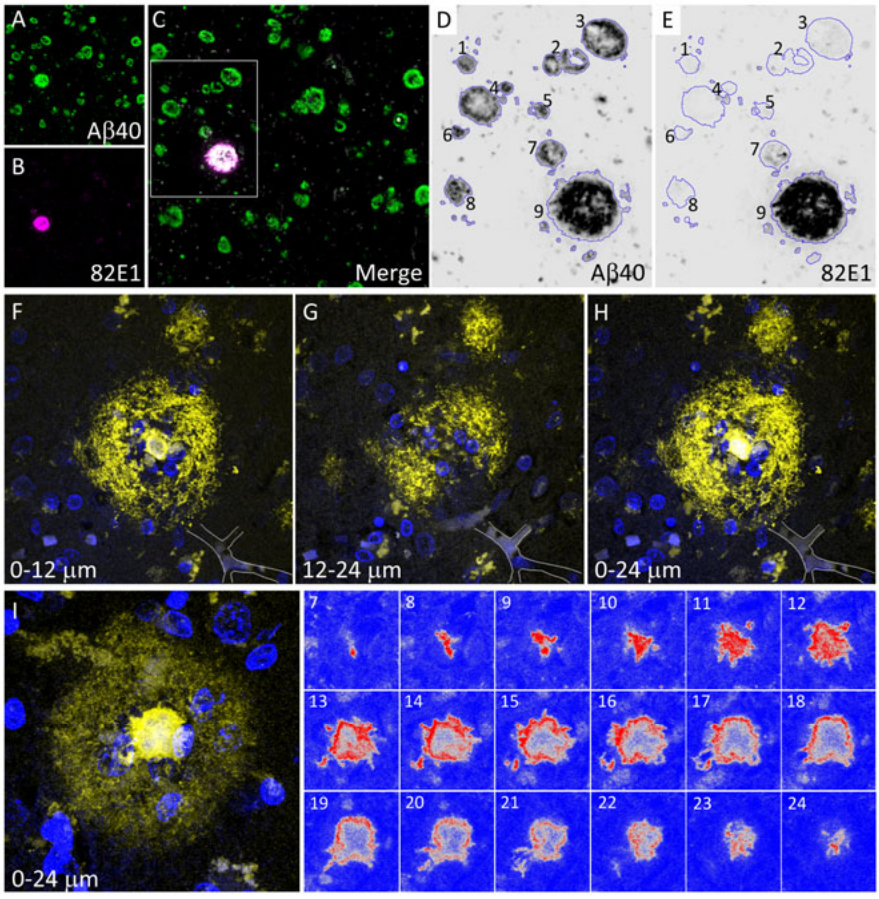

Figure 16. **Image analysis and depth-dependent morphology**. **A-E**. Outline of image analysis protocol applied in **paper I**. Sections were double immunostained and merged images were prepared (**A-C**). Using ImageJ software the merged images formed the basis of an analysis map in which individual Aβ deposits were outlined and labeled. This map was then overlaid on the individual Aβ(1-40) (**D**) or 82E1 (**E**) channels, after which ImageJ was used to calculate the area of each immunosignal/deposit. **F-H**. 25 µm thick sections of AD hippocampus were immunostained with an anti-Aβ42 antibody. Z-scans were captured at 1 µm depth intervals by confocal laser scanning microscopy, resulting in 25 frames (0-24). Assembling z-scans 0-12 results in a dense-core deposit morphology (**F**), while z-scans 12-24 present with diffuse deposit morphology (**G**). The dense-core morphology of the deposit prevails when all 25 z-scans are combined (**H**). The capillary in the bottom right corner is outlined to identify a common location in each image. (**I**) A dense core deposit rendered from 25 z-scans. The intensity and distribution of the immunostaining around the core is represented by 18 frames (from 7-24 µm depth), where the high intensity pixels are red and low intensity pixels are blue. *Original magnification: A-C, 100X; F-I, 630X. In F-I immunosignal fluorescence is presented in yellow for best contrast.*

It could of course also be the case that the centre of the core is in fact devoid of Aβ. In fact, in **paper I** we used CLSM to analyze the fluorescence from Congo red-stained Aβ deposits in Tg2576 mice and concluded that the centre of these deposits was often weakly/not stained with Congo red (**paper I**, Figure 3), suggesting a lack of fibrillar material in this location.

Recent studies suggest that fibrillar Aβ deposits form rapidly[260], possibly within synapses[123]. Another proposal is that parenchymal Aβ deposits can be derived from microvascular structures, where Aβ accumulation on basal surfaces of capillaries causes collapse and occlusion. This isolated segment then serves as a platform around which further deposition can occur[261]. It is in fact possible to view the frames (7-24) of the Aβ core in *Figure 16I* and regard them as cross-sectional views through an occluded capillary.

Epitope-dependent detection of HS

In **paper I** HS was detected using the side-chain variable fragment (scFv) HS4E4. HS4E4 is one of several scFvs derived from a phage display library; those described to date have HS-epitopes that are predominantly dependent on degrees of sulfation[262-264]. HS4E4 is reported to detect N-sulfated forms of HS, and may be inhibited by the presence of 2-O- and 6-O-sulfate groups[265]. Accordingly, it would have been more accurate to describe the distribution of HS with dense-core and diffuse Aβ deposits in terms of the presence/absence of the HS4E4-eptiope of HS. Subsequent to the publication of **paper I** came a report by Bruinsma *et al.* 2009 in which five scFvs, including HS4E4, were used to study the distribution of HS with Aβ pathology[264]. They determined that all of the scFvs tested detected HS with fibrillar Aβ deposits, as defined by thioflavin S staining. However, they also found that scFvs that recognized more sulfated forms of HS immunostained non-fibrillar deposits that were not detected by HS4E4. They concluded that a greater variety of HS epitopes are associated with fibrillar than diffuse deposits. Relevantly, their image analysis revealed that no single scFv co-detected all of the Aβ-immunostained pathology. This suggests that at least a subset of non-fibrillar Aβ deposits could in fact be devoid of all five HS epitopes.

Heparanase expression

The Hpa-tg mice used in **paper II** express a chimeric form of heparanase in which a signal peptide sequence, identified in chicken pro-heparanase, precedes the human heparanase sequence. Human heparanase is typically localized to intracellular acidic vesicles; the chicken signal sequence promotes secretion and re-localization of heparanase to the cell surface[266]. To assess the distribution of heparanase in Hpa-tg microglia and astrocytes we performed immunocytochemistry with pAb 733, which recognizes the 50 kDa subunit of heparanase. Hpa-tg astrocytes presented with extensive intracellular granular staining, which was absent from Ctrl astrocytes (**paper**

II, Figure 3). In contrast, 733 immunocytochemistry did not clearly distinguish Hpa-tg from Ctrl microglia and no granular structures were detected *(data not shown)*. Western immunoblotting with 733 confirmed heparanase overexpression in Hpa-tg microglia (**paper II**, Figure 1), but heparanase was readily detected in Ctrls, suggesting that these neonate-derived microglia have higher endogenous levels of the enzyme than neonatal astrocytes.

Transgenic and induced expression of heparanase has been reported to modify HS metabolism beyond chain fragmentation[171,267]. In a previous Hpa-tg model (encoded with human heparanase only) it was demonstrated that heparanase overexpression resulted in upregulated HS sulfation and increased HSPG turnover[171]. In tumors and under hypoxic conditions, heparanase expression is also increased and again upregulates HS sulfation[171,268]. These factors warrant consideration in the interpretation of the findings in **paper II**. For example, it would be relevant to determine if the LPS-induced inflammatory state alters microglial levels of heparanase and the length and/or structure of HS. Disaccharides of HS have been proposed to inhibit macrophage secretion of TNF-α[269]; therefore, extracellular, potentially high-sulfated, HS fragments in Hpa-tg microglial medium may also function to inhibit cytokine production, beyond the mechanisms previously discussed.

Aβ and toxicity assays

The membrane of a viable cell is impermeable to propidium iodide (PI), a DNA staining dye. Equally, in a healthy cell phosphatidylserine is anchored to the inner leaflet of the plasma membrane and is not accessible to binding proteins such as Annexin V. Exposure of phosphatidylserine on the outer cell surface is considered an early apoptotic event, which permits Annexin V binding. As the cell dies the permeability of the plasma membrane increases, permitting entry of PI and staining of the nucleus. These events can be quantified by flow cytometry and were used in **paper III** to assess Aβ40 cytotoxicity. In this assay cell death is essentially expressed as a function of membrane permeability and it is worth considering how Aβ could affect such analysis. It has been proposed that Aβ aggregates can form membrane spanning pores (*Figure 11*), disrupting ion homeostasis and increasing membrane permeability[270]. Such structures may permit the influx and intracellular binding of PI (668.4 Da) and Annexin V (36 kDa). Consequently, while the Annexin V/PI readout will correctly indicate that cells are in a state of distress it is not necessarily occurring in accordance to the logic of the assay, so interpretations regarding specific modes of cell death should be made cautiously.

Aβ may also influence interpretations of the MTT assay used in **paper III**. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow tetrazolium salt, which is impermeable to cell membranes. MTT is

endocytosed, reduced and exocytosed to the cell surface as a water insoluble purple formazan. This reaction is dependent on electron donors and the assay is essentially designed to reflect metabolic activity. However, Aβ has been found to decrease MTT reduction and increase formazan exocytosis[271], and the assay readout does not distinguish these events. While Aβ-mediated effects on reduction and exocytosis are unlikely to be positive, the apparent viability of the cultures may in fact be the product of several contributing processes. Due to such limitations it is advisable not to rely on one single method for assaying toxicity.

Cell-associated Aβ assay

In **paper III** internalized Aβ40 was detected in adherent cells by immunocytochemistry and quantified by image analysis. Advantages of this approach include analysis of Aβ distribution at the level of single cells. However, populations of native Aβ aggregates may be underestimated by antibody-dependent methods due to concealment of epitopes in the aggregate structure[272]. Antigen retrieval techniques can be applied to fixed cell cultures, but due to practical limitations these tend to be mild in comparison to the denaturing protocols applied before analysis by e.g. SDS-PAGE. Furthermore, detecting single epitopes of Aβ (i.e with mAbs 6E10 or 82E1 in **paper III**) does not inform on how intact the peptide is. A common alternative is to analyze cell lysates by a denaturing method.

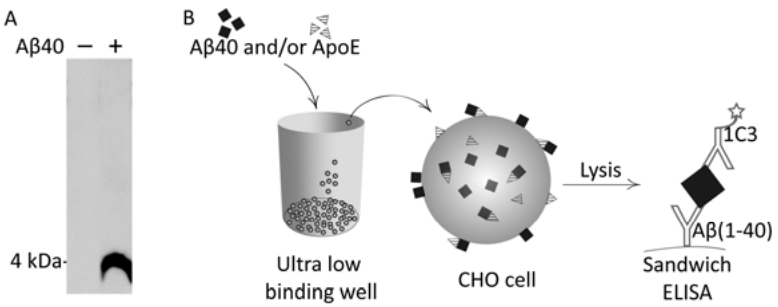

Figure 17. **CHO cell assay**. **A**. Western immunoblotting (mAb 6E10) reveals levels of non-specifically bound Aβ recovered in lysis buffer from a standard tissue culture well, in a cell-free setting. **B.** In **paper IV** CHO cell suspensions were treated with Aβ and/or ApoE, cells were recovered by centrifugation, lysed and analyzed by Aβ(1-40):1C3 sandwich ELISA.

In **paper IV** we planned to treat adherent CHO cells with Aβ40 in the presence or absence of ApoE. After treatment, the washed cell layer was to be lysed directly from the wells and cell-associated levels of Aβ were to be analyzed in a sandwich ELISA. In control experiments we first assessed the

degree of Aβ binding to standard tissue culture plates. Cell-free wells were blocked with 10% FBS in cell culture medium (24 h, 37^{o}C). This was aspirated and a solution of Aβ40, prepared in serum free culture medium, was added. After 3 h incubation (37^{o}C) the solution was removed, the cell-free wells were washed and then incubated with lysis buffer. Western immunoblot analysis with mAb 6E10 detected substantial levels of Aβ in this cell-free lysate, confirming that Aβ readily bound to the FBS-blocked tissue culture plates (*Figure 17A*). Consequently, cell lysates prepared directly from these wells would contain 'cell-bound' and 'well-bound' Aβ. These specific and non-specific fractions could be separated by first detaching cells with trypsin treatment. This would permit isolation of the cell-specific fraction by centrifugation, but trypsin would likely remove Aβ attached to the cell surface, which was of interest to us.

Conveniently, CHO cells are readily cultured in suspension, so we proceeded with the experiments in ultra-low binding tissue culture plates. Non-specific Aβ binding was reduced in these plates, but more importantly as CHO cells settled, but did not attach to these wells, they could be easily collected without the need for enzymatic detachment. Lysates were prepared and levels of cell-associated Aβ40 were quantified by sandwich ELISA. This ELISA detects near full-length forms of Aβ40, as pAb Aβ(1-40) captures at the C-terminus and mAb 1C3 detects the N-terminal residues 3-8 (*Figure 17B*).

Concluding remarks

Alzheimer's disease is a devastating condition, erasing a lifetime of memories and experiences. Memories are dependent on synaptic connections between neurons; consequently, neuronal and synaptic loss leads to their irreversible deletion. Therefore, detecting pre-symptomatic states of AD will be essential to ensure that preventative treatments, under development, can be pre-emptively administered.

The discovery of familial AD mutations greatly contributed to the formation of the amyloid cascade hypothesis, which in turn provided the framework for an array of transgenic models of Aβ deposition. In this setting the importance of Aβ in the pathogenesis is inherent to the models design and therefore strategies that aim to modulate Aβ production, aggregation or deposition can be logically tested. However, while relatively clear-cut genetic explanations do underlie familial variants of AD, the most common late-onset form of AD presents as a far more complex condition. The discovery in the early 90's that an ApoE4 genotype increased an individual's risk for AD was perhaps the first concrete target directly associated with late onset AD, but nearly 20 years later the specific role of ApoE in AD has yet to be fully elucidated.

The AD field has come a long way from the observations presented by Alzheimer in 1907 and is now at one of the most promising and hopeful states it has ever been[228]. Several immunotherapy strategies are in the final stages of clinical trials and diagnostic techniques, both biomarker- and neuroimaging-based, are advancing rapidly. Nonetheless, it is important to continue to investigate the as yet elusive biology that underlies the pathogenesis of late onset AD, as such an understanding may yield new therapeutic targets and/or facilitate the improvement of current experimental approaches.

During the course of this thesis a number of mechanistic roles for HS in AD pathogenesis were explored and while the potential diagnostic or therapeutic value of these findings were not evaluated, the following sections aim to place the studies in this broader context.

Diagnostic imaging of amyloid-associated HS

Given the recent *in vivo* imaging studies of amyloid-associated HS, it is worth considering the findings of **paper I** in the context of amyloid diagnostics. Smits *et al.* 2011 report on NS4F5, a newly identified scFv, that

binds a highly sulfated HS motif $(GlcNS6S\text{-}IdoA2S)_3$. MicroSPECT imaging of radiolabeled NS4F5 detects amyloid deposits *in vivo* in a mouse model of AA amyloidosis, and *ex vivo* autoradiography demonstrates that NS4F5-binding correlates with Congo red-positive amyloid deposits[262]. The resolution of neuroimaging techniques is rapidly improving and ligands that identify pre-fibrillar Aβ pathology are under development. Amyloid imaging ligands that detect non-Aβ components of deposits may prove useful when assessing patients that are simultaneously undergoing e.g. anti-Aβ immunotherapy. Based on the selective affinity of the scFv HS4E4 for HS in dense-core Aβ deposits and its association with gliosis (**paper I**) it may prove useful in the differential diagnostic imaging of Aβ deposit types. In a therapeutic context it would also be interesting to explore the possibility of using these scFvs to selectively inhibit specific HS-domain interactions with Aβ.

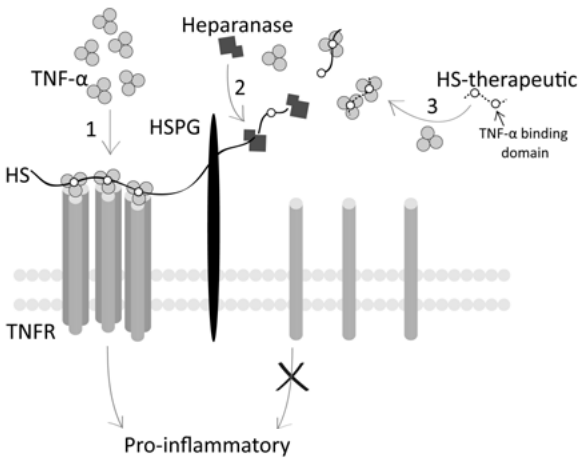

Figure 18. **Heparanase and HS regulation of inflammation**. In **paper II** we concluded that pro-inflammatory signaling of TNF-α was dependent on microglial HSPGs (**1**). Heparanase may attenuate the inflammatory response by remodeling the HS chains required for the signaling event (**2**). Designing HS-analogs that interfere with TNF-α interactions with microglial HSPGs may function as anti-inflammatory therapeutics (**3**). Circles on the HS chains and the HS-therapeutic represent potential TNF-α binding domains.

Heparanase and HS-analogs as regulators of inflammation and Aβ cytotoxicity

Heparanase is typically discussed in terms of HS degradation, but its ability to remodel the HS landscape on cell surfaces may serve to regulate the interaction of certain ligands, with specific cell-types under conditions such as inflammation (*Figure 18*). Strategies that inhibit or enhance heparanase

may therefore function as modulators of such processes. In **paper II** we proposed that heparin sequesters LPS-induced TNF-α, impairing its interactions with HSPGs and TNF receptors, and thus reducing the levels of IL-1β induced by TNF-α. Detailed analysis of microglial HS chains may reveal specific features involved in optimal interactions with TNF-α e.g. domain sulfation patterns and minimal chain lengths. These could facilitate the design of anti-inflammatory HS-analogs (*Figure 18*). Equally, in **paper III** heparin administration attenuated Aβ toxicity and the design of HS oligosaccharides that compete with cell-surface HS for Aβ binding may prove beneficial in attenuating neurodegeneration. Inhibiting Aβ interactions with ApoE may potentially impair an Aβ clearance mechanism, but it is also possible that Aβ deposition in the microvasculature could be reduced by a HS mimetic. Such agents may also function as chaperone-like molecules and Aβ bound to a HS-oligosaccharide may be less likely to be internalized, making it a more susceptible target for current anti-Aβ immunotherapeutic strategies.

The interactive nature of HS undoubtedly makes it a complicated drug candidate. However, beyond their well established role as anticoagulants, HS/heparin-derived therapeutics are under development in a variety of settings and heparanase inhibitors designed to hinder tumor metastasis are being explored. The HS-mediated mechanisms described in this thesis may prove to be viable disease-modifying targets, but in the context of this work, they primarily contribute to our basic knowledge of molecular interactions relevant to the pathogenesis of AD.

Sammanfattning på svenska

Alzheimers sjukdom är en neurodegenerativ sjukdom där felveckning, aggregering och plackinlagring av peptiden amyloid-β (Aβ) spelar en central roll i patogenesen. Heparansulfat (HS) är en kolhydrat som förekommer dels på cellytan och dels extracellulärt i form av HS proteoglykaner (HSPG). HSPG medverkar vid flera signaleringsfunktioner, men HS är även närvarande i alla amyloidinlagringar, inklusive de som påträffas vid Alzheimers sjukdom. Vid amyloidos främjar HS fibrillbildning samt är en strukturell komponent av amyloidinlagringar. Syftet med avhandlingen var att studera HS roll vid Alzheimers sjukdom; dels interaktionen med Aβ och dels inblandningen i den inflammatoriska processen.

I hjärnor från patienter med Alzheimers sjukdom upptäckte vi HS ackumulerat i specifika typer av Aβ-plack. I samma placktyp hittades även glialceller som uttryckte HSPG. Vidare, ökade Aβ HSPG-nivåerna i primära gliaceller, vilket antyder att Aβ-associerat HS kommer från aktiverade gliaceller. Mikroglialt HSPG visade sig dessutom vara viktigt för uppregleringen av cytokiner. Sammantaget visar dessa resultat på möjligheten att Aβ-inducerat HSPG-uttryck i mikroglia kan främja inflammation i centrala nervsystemet

Flera mekanismer för hur Aβ orsakar toxicitet har föreslagits och olika typer av Aβ-aggregat utövar sin toxicitet genom alternativa mekanismer. Vi upptäckte att tre olika typer av Aβ-preparationer samtliga uppvisade HS-beroende cytotoxicitet, vilken delvis korrelerade med Aβ-upptag. Dessutom ledde heparinbehandling till reducerad Aβ-cytotoxicitet och reducerat Aβ-upptag. I Aβ inlagringar i kapillärer ansamlades även HS, apolipoprotein E (ApoE) och dess receptor, LRP1 (low-density lipoprotein receptor-related protein 1). ApoE ökade nivåerna av cellassocierat Aβ i cellkultur på ett HS och LRP1-beroende sätt. Det är möjligt att denna ApoE-medierade effekt kan främja Aβ-toxicitet och vaskulär dysfunktion. Alternativt, kan det också vara en HS-medierad clearance-väg av Aβ över blod-hjärnbarriären.

Resultaten som presenteras i avhandlingen illustrerar HSPG-medierade interaktioner som är relevanta för patogenesen vid Alzheimers sjukdom.

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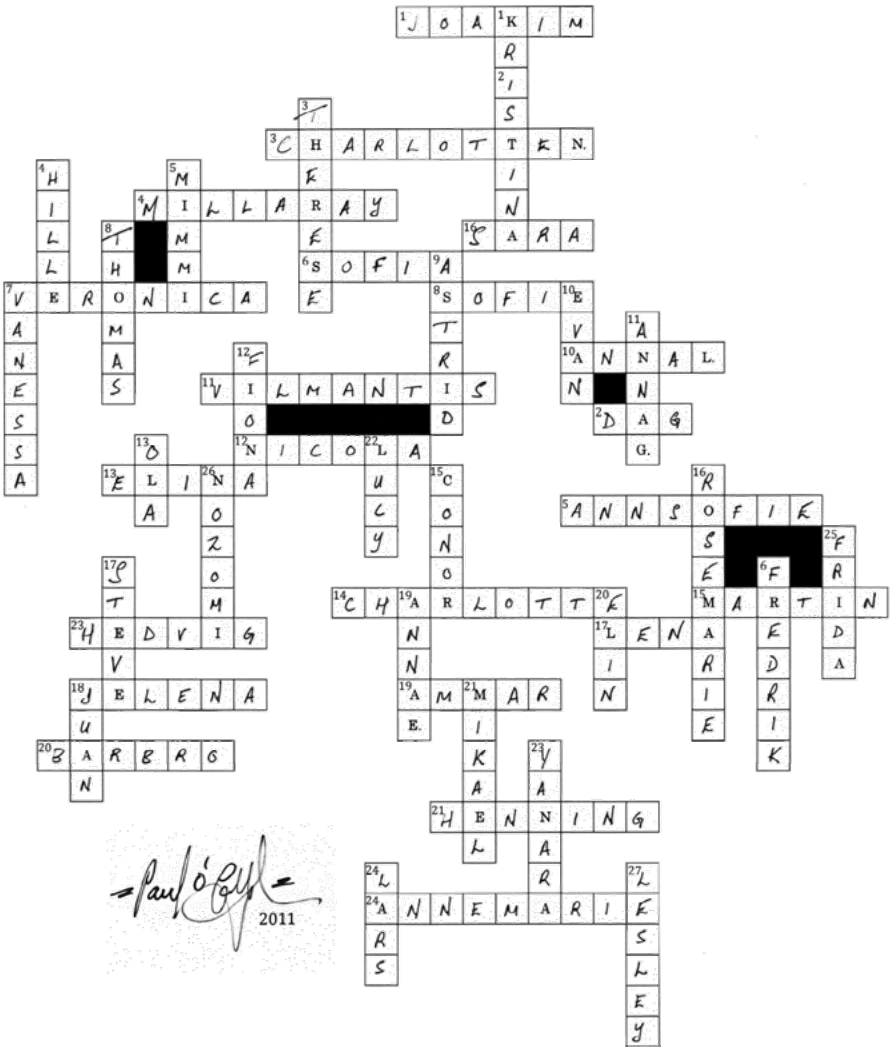
Thanks/Tack



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