N-Unsubstituted Glucosamine Residues in Heparan Sulfate and Their Potential Relation to Alzheimer's Disease

BY

CAMILLA WESTLING
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Abstract

Heparan sulfate (HS) is a linear polysaccharide, located on the surface and in the extracellular matrix of most cells, that regulates functions of numerous proteins. HS-protein interaction is mainly mediated by sulfate groups found in N-sulfated (NS) regions of the HS, but may also involve rare HS substituents such as N-unsubstituted glucosamine (GlcNH₂) residues. The location of GlcNH₂ in an HS-epitope recognized by the monoclonal antibody 10E4, that specifically stains the prion lesions in scrapie-infected murine brain, suggests an involvement of GlcNH₂ in prion disease and other amyloid-related disorders. HS in general is strongly associated with amyloidosis, including Alzheimer’s disease (AD). Therefore, the aims of this thesis were to structurally characterize GlcNH₂-containing HS sequences found in native tissues, to further study HS epitopes recognized by 10E4, and to investigate the possible role(s) of GlcNH₂ and other HS structures in binding to amyloid β peptide (Aβ) (core material in AD plaque lesions, also stained by 10E4).

The GlcNH₂ content (0.7-4% of total disaccharide units) varied between HS from different tissues. Most GlcNH₂ units were found in poorly modified N-acetylated (NA-) or NA/NS-domains, located toward the polysaccharide-protein linkage region.

Binding of human cerebral cortex HS to Aβ(1-40) monomers requires N-, 2- and 6-O-sulfation of HS, while binding to Aβ fibrils requires N- and 2-O-sulfation only. GlcNH₂ units do not appreciably contribute to interaction with Aβ. Aβ fibril-binding HS domains also bind to fibroblast growth factor 2 (FGF-2), indicating that Aβ (neurotoxic) and FGF-2 (neuroprotective) may compete for common binding sites in HS. However, Aβ had no effect on FGF-2-induced MAPK signaling in NIH 3T3 fibroblasts.

Continued studies on 10E4-antigenic HS epitope(s) showed that binding of 10E4 to the previously identified antigenic tetrasaccharide, ΔUA-GlcNH₂-GlcA-GlcNAc, requires the nonreducing hexuronic acid (ΔUA) to be 4,5 unsaturated (induced by lyase cleavage), and thus is artificial. Further studies are needed to clarify the potential involvement of GlcNH₂ in 10E4-recognition of the native HS epitope(s).

Keywords: heparan sulfate, N-unsubstituted glucosamine, Alzheimer’s disease, amyloid beta peptide, amyloidosis, 10E4 antibody, fibroblast growth factor 2

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To my family
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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Contents

Introduction ......................................................................................................................... 9

Background ....................................................................................................................... 10
  Glycobiology .................................................................................................................. 10
  Proteoglycans .................................................................................................................. 10
  Heparan sulfate proteoglycans ....................................................................................... 13
    Core proteins .................................................................................................................. 13
  Heparan sulfate biosynthesis ......................................................................................... 14
  Structural diversity of heparan sulfate .......................................................................... 16
  Heparan sulfate-protein interactions .............................................................................. 19
  Biological functions ......................................................................................................... 21
  Turnover of heparan sulfate ........................................................................................... 22
  Heparan sulfate in amyloid disease .................................................................................. 23
    Amyloid diseases ............................................................................................................ 23
  Heparan sulfate in amyloidosis ....................................................................................... 27
  Heparan sulfate in Alzheimer’s disease ........................................................................... 28

Present Investigation ......................................................................................................... 31
  Aims of study .................................................................................................................... 31
    Heparan sulfate in Alzheimer’s disease ........................................................................... 31
    N-Unsubstituted glucosamine residues in heparan sulfate ............................................ 32
  Results ............................................................................................................................. 32
    Common binding sites for -amyloid fibrils and fibroblast growth factor-2 in heparan sulfate from human cerebral cortex (Paper I) ........... 32
    Effect of A protofibrils on heparan sulfate dependent fibroblast growth factor-2 cell signaling? (unpublished data) ........................................ 34
    Location of N-unsubstituted glucosamine residues in heparan sulfate (Paper II) .......... 36
    N-Unsubstituted glucosamine residues in heparan sulfate from human cerebral cortex – Relation to Alzheimer’s disease? (Paper III) ........ 38
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid β peptide</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>aManR</td>
<td>2,5-anhydromannose</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid β precursor protein</td>
</tr>
<tr>
<td>BACE</td>
<td>Beta-site APP cleaving enzyme</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GlcA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>GalNAc</td>
<td>D-galactosamine</td>
</tr>
<tr>
<td>GlcN</td>
<td>D-glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>GlcNH₂</td>
<td>N-unsubstituted glucosamine</td>
</tr>
<tr>
<td>GlcNS</td>
<td>N-sulfate-D-glucosamine</td>
</tr>
<tr>
<td>HexA</td>
<td>Hexuronic acid</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NA</td>
<td>N-acetylated</td>
</tr>
<tr>
<td>NS</td>
<td>N-sulfated</td>
</tr>
<tr>
<td>OST</td>
<td>O-sulfotransferase</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Prion protein, cellular</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
<td>Prion protein, scrapie</td>
</tr>
<tr>
<td>2S, 3S, 6S</td>
<td>2-O, 3-O- and 6-O-sulfate groups</td>
</tr>
</tbody>
</table>
Heparan sulfate (HS) is an anionic linear polysaccharide that belongs to the glycosaminoglycan family. HS is present on the cell surface and in the extracellular matrix of virtually every cell type, from simple invertebrates to humans, and acts as an important regulator of many biological processes. Studies of recent years have for example pointed out fundamental roles of HS in growth factor signaling, morphogenesis and pathophysiological phenomena. HS is mostly found covalently attached to a core protein, *i.e.* in proteoglycan form. HS consists of alternating hexuronic acid and glucosamine residues, that via a complex biosynthetic machinery become sulfated in strictly regulated fashion. Some regions of the HS chain undergo few modifications, while others are extensively modified. The sulfate pattern, as well as the degree of sulfation, varies from one HS sample to another, a difference that can variously be ascribed to cell type, tissue of origin, species, age, or pathophysiological conditions. Due to its sulfate pattern, the HS chain can bind to a protein more or less specifically, and thereby regulate its function. Binding specificity is apparent in the case of unusual HS structures, for example a 3-O-sulfated or N-unsubstituted glucosamine (GlcNH$_2$) residue binding to a protein ligand.

One of the challenges in the HS research field is to get a deeper understanding of the mechanisms behind the formation of specific motifs in HS. Another is to isolate and characterize specific HS epitopes, and correlate them with cellular function, in health and disease. The latter issue is the main theme of this thesis. More precisely, the thesis work has been focused on two topics. One deals with the location and structural characteristics of the rare GlcNH$_2$ residues in HS. The other topic concerns the possible role(s) of HS in Alzheimer’s disease.
Background

Glycobiology

The term "glycobiology" was coined in 1988 [1] to describe a new frontier in molecular biology, namely the study of structure, biosynthesis, and biological function of complex carbohydrates. Since the era of molecular biology started much earlier, the glycobiology field can be considered rather young. One reason for this is that carbohydrates are difficult to study. Compared to polypeptides or DNA, which are linear and consist of well-defined building blocks, carbohydrates are more complex, often branched and composed of a large number of different, often modified, monosaccharide units. In addition, no biosynthetic "template" has so far been identified for any carbohydrate, which makes it difficult to predict the saccharide structure produced by a certain cell type, or under certain conditions. For this and other reasons molecular biology was for a long time equal to "protein biology". However, with the development of new techniques during the last decades, improving both the detection and identification of carbohydrates, it has become apparent that most proteins, as well as many lipids, actually carry carbohydrate moieties, that influence, or even direct, their functions in many ways. Today glycobiology has become a research field of its own, covering a broad range of saccharides and glycoconjugates, including glycolipids, glycoproteins and proteoglycans. This thesis is focused on one subtype of proteoglycans (PGs), that is the HSPG.

Proteoglycans

A PG is a glycoconjugate that consists of a core protein with one or more covalently attached glycosaminoglycan (GAG) chains (for reviews, see [2-5]). The PGs are produced by virtually all cells, and are found on the cell
surface, in the extracellular matrix (ECM) or intracellularly, usually in secretory granules (Fig. 1). A multitude of functions has been described for PGs (see reviews above, and [6, 7]). In vitro studies have demonstrated important roles of PGs as modulators of normal cell and tissue function (e.g. cell division, adhesion, migration, axon guidance, and matrix assembly), but also as mediators of pathological events, such as viral infection, inflammatory conditions, or cancer. Many of these functions have been confirmed by more recent in vivo studies, using gene knock-out constructs in model organisms such as C. elegans, Drosophila, or mouse [8-10]. This mutation-based strategy has also lead to the discovery of new intriguing functions of PGs, for example as modulators of cell-cell signaling and morphogenesis during development.

![Figure 1 Schematic presentation of some proteoglycans.](image)

How can PGs have such many diverse functions? The protein core, which can be of different types, is of importance, but what makes a certain PG really unique is the attached carbohydrate moieties, the GAG chains. GAGs are linear polysaccharides composed of repeating disaccharide units of an amino sugar and a hexuronic acid (HexA). The GAG chains are sulfated to various extent and can thereby, via the negatively charged sulfate and carboxyl groups, bind to and regulate the function of numerous proteins. Moreover, by attracting counter ions, and thereby causing osmotic imbalance, they provide a hydrated environment in the ECM [11].

The protein-bound GAGs can be divided into three groups: chondroitin/dermatan sulfate, heparin/heparan sulfate, and keratan sulfate.
The fourth GAG member, hyaluronan, occurs only in free form, \textit{i.e.} not as a PG. Chondroitin sulfate (CS) and dermatan sulfate (DS) are both galactosaminoglycans that contain repeating units of [HexA/1,3-GalNAc 1,4]. In CS the HexA is invariably D-glucuronic acid (GlcA), while in DS some of the GlcA residues are epimerized into L-iduronic acid (IdoA). The D-galactosamine (GalNAc) can be O-sulfated at C-4 and C-6, and the HexA at C-2 position. Heparin and heparan sulfate (HS) consist of [HexA/1,4-GlcNα1,4] disaccharide units. The HexA is either GlcA or IdoA, and the D-glucosamine (GlcN) is either N-acetylated, N-sulfated or, in rare cases, N-unsubstituted. Heparin contains higher proportions of N-sulfated GlcN and IdoA units than HS, and is also O-sulfated to a larger extent. The heparin/HS structure will be described in more detail below. Keratan sulfate (KS) is composed of repeating units of [Gal 1,4-GlcNAc 1,3]. Both the galactose (Gal) and the GlcNAc units can be O-sulfated at position C-6. Heparan, consisting of [GlcA 1,3-GlcNAc 1,4], lacks sulfate groups.

The CS/DS and heparin/HS chains are linked to serine residues in the core proteins via a GlcA-Gal-Gal-xylose linkage region. KS is linked either to asparagine residues via GlcNAc ("corneal" KS; type I), or to serine/threonine units via a GalNAc residue (skeletal KS; type II). The number of attached GAG chains (1- >100) varies between different types of PGs, and the number of chains on a specific PG may vary as well, depending on cell type. One core protein can carry more than one type of GAG chain, for example both HS and CS, and the ratio between the two types of GAG chain, as well as the chain lengths, may change with development, aging, or disease. Some PGs are referred to as "part-time" PGs since they do not always carry GAG chains. The mechanism behind the selective substitution of a certain PG with one type of GAG chain, but not the other, is not well understood. However, both the amino acid sequence immediately adjacent to the glycan attachment site in the core protein \cite{12, 13}, and more distal amino acid motifs \cite{14}, seem to be of importance.

In the GAG family HS is the member with the highest structural variability. Owing to its heterogenous distribution of sulfate groups, HS has a pronounced polymorphic nature, and is therefore responsible for most of the numerous GAG-protein interactions described. It should be noted though that also the other GAGs bind to various protein ligands and have important biological functions, some of which overlap with those of HS. However, this matter will not be discussed in the following parts of the thesis, that are confined to the structure and function of HS.
Heparan sulfate proteoglycans

Core proteins

The major HSPGs include the two cell surface-bound subfamilies syndecan and glypican, and the three extracellular HSPGs perlecan, agrin, and collagen XVIII (reviewed in [4, 6, 7, 15]).

The syndecan family has four members (syndecan-1 to syndecan-4) that are expressed in a highly regulated manner in most cells and tissues. Syndecans are all type I membrane-spanning proteins (25-35 kDa), with a characteristic domain structure consisting of a short cytoplasmic region, a hydrophobic transmembrane region, and an extracellular ectodomain (Fig. 1). The HS chains (3-5) are attached to conserved motifs in the ectodomain, which also can, in some tissues, be substituted with CS. A protease cleavage site exists proximal to the ectodomain, which can be cleaved by metalloproteinases (or other “sheddases”), resulting in release of biologically active ectodomains into the ECM [16, 17].

The six glypican forms (glypican-1 to glypican-6; 60-64 kDa) are linked to the cell surface by a glycoporphosphatidylinositol (GPI) anchor (Fig. 1). The protein core consists of an N-terminal signal sequence, a cystein-rich globular ectodomain, an extended region with three HS attachment sites, and a hydrophobic C-terminal involved in the formation of the GPI anchor. The six glypican forms are expressed in a highly tissue-specific manner and their overall expression pattern varies with different developmental stages [18-21].

Perlecan and agrin are two large (400 and 225 kDa, resp.) multidomain proteins present in the basement membrane or in the pericellular space (Fig. 1). They have both three GAG attachment sites used for HS substitution, although perlecan, in some cases, also carries CS. Agrin is mainly found in the brain and kidney, while perlecan shows a broader tissue distribution. The third extracellular PG, collagen XVIII, is the only collagen carrying HS chains, and is a precursor of a 20 kDa polypeptide, known as endostatin. Endostatin is an inhibitor of cell proliferation of endothelial cells, that is thought to be regulated by HS [22]. The number (3 in total) and characteristics of the GAG attachment sites in collagen XVIII were recently reported [23].

In addition to the HSPGs already mentioned, also some minor cell surface HSPGs exist, e.g. CD44/epican, betaglycan, and FGF receptor 2 [24]. CD44/epican and betaglycan can be substituted with both HS and CS, while there has been no report on CS-containing FGF receptor 2 molecules. All of these cell surface HSPGs also exist in non-glycosylated forms, and can therefore be considered “part-time” HSPGs.
Heparan sulfate biosynthesis

HS is produced in the Golgi apparatus by a biosynthetic machinery consisting of multiple glycosyl- and sulfotransferases, an epimerase and probably a set of co-factors and proteins, so far not identified (see reviews by [25, 26]). The enzymes work in highly regulated and concerted fashion, creating a 50-200 monosaccharide long HS chain (10-50 kDa) probably within less than one minute [27]. Heparin, the closest relative to HS, is produced by the same machinery, but only in connective-tissue type mast cells [28], in contrast to the ubiquitously expressed HS. The biosynthesis of HS/heparin starts with the formation of a GlcA-Gal-Gal-Xylose- linkage region, that is attached, via the xylose residue, to a serine unit in the core protein. The following addition of a GlcNAc residue directs the synthesis into the HS/heparin pathway; addition of GalNAc would result in the formation of CS, since the same linkage region is used for production of both GAG types. The HS/heparin chain is polymerized through the action of GlcA and GlcNAc transferases, forming a backbone of [GlcA 1,4-GlcNα1,4] disaccharide units. This backbone is then enzymatically modified (Fig. 2); N-acetyl groups of GlcN residues are replaced by N-sulfate groups, GlcA residues are converted to IdoAs, and O-sulfate groups are added at various positions. The modification procedure of heparin goes almost to completion, generating a polysaccharide that consists largely of IdoA and GlcNS residues, and is extensively O-sulfated (structure not shown). The modification of HS is more moderate and restricted to certain domains of the chain, resulting in a very heterogenous structure (Fig. 2).

The biosynthetic enzymes have been purified and cloned (reviewed in [26]). At least five separate enzymes are involved in the formation of the linkage region and the addition of the first GlcNAc unit [29]. The chain elongation is catalyzed by a HS copolymerase with dual glucuronyl/N-acetylglucosaminyltransferase activity. In vivo, the enzyme activity is believed to depend on complex formation between its presumed subunits EXT1 and EXT2 [30], although in vitro HS polymerization can also be carried out by EXT1 alone, as shown in a recent report [31]. The first sulfation step is catalyzed by another enzyme with dual activity, the N-acetylglucosamine N-deacetylase/N-sulfotransferase (NDST). Four vertebrate NDST isoforms have been identified, each showing characteristic tissue-specific and developmentally regulated expression pattern (reviewed in [32]). The C5-epimerization and the following 2-O-sulfation step are carried out by single enzymes, each found in only one form, while the 6-O- and 3-O-sulfation reactions are catalyzed by multiple isoforms. Three 6-O-sulfotransferase (6-OST) and six 3-O-sulfotransferase (3-OST) members have been identified, and both enzyme families show isoform-related differences regarding both tissue distribution and, to various extent, their substrate specificity [26, 33-37].
**Figure 2** Scheme of heparan sulfate chain modifications. Adapted from Esko and Lindahl, see ref. [25]. The symbols used are defined by the structures shown below the scheme. PAPS, adenosine 3'-phosphate 5'-phosphosulfate (sulfate donor). For residual abbreviations, see the text.
Structural diversity of heparan sulfate

There are at least two levels of structural variability in HS; the variation in content and relative proportions of different types of modified disaccharide units, and the variation in size and distribution of the three domain types in HS; N-acetylated (NA)-, N-sulfated (NS)-, and the interspersing NA/NS-domains, consisting of alternating N-acetylated and N-sulfated disaccharide units (Fig. 2; for reviews see [25, 26, 38].

To date ~23 different disaccharides, distinguished by their GlcA/IdoA content and sulfation pattern, have been identified in heparin, HS, or as intermediates in the biosynthesis. In theory, an even larger number would be possible, but due to restrictions in the biosynthesis, reflecting the high substrate specificity of the biosynthetic enzymes, these disaccharides are never expressed in native HS. The most common HS disaccharides, GlcA-GlcNAc and IdoA+/−2S-GlcNS+/−6S (Fig. 2), are found in all HS samples, but in various proportions. Less frequent are disaccharide species that contain rare HS structures such as 2-O-sulfated GlcA, 3-O-sulfated GlcN, or N-unsubstituted GlcN residues. Examples of 3-O-sulfated disaccharide units are the GlcA-GlcNS3S+/−6S (Fig. 2) found in the antithrombin-binding pentasaccharide [39], the IdoA2S-GlcNS3S structure present in marine clam heparin and renal HS [40, 41], and the more recently identified tetrasulfated disaccharide IdoA2S-GlcNS3S6S expressed in bovine intestinal HS [33]. 3-O-Sulfation has also been detected in disaccharide species containing a GlcNH2 residue [42]. The GlcNH2 unit, of major interest in this thesis work, was earlier generally believed to be an experimental artifact, reflecting loss of N-sulfate groups due to improper handling of HS samples. However, during the early 90s experimental evidence appeared that clearly demonstrated the presence of these units in native tissues [43-45]. The mechanism of GlcNH2-formation is still unclear though. The free amino groups could be the result of incomplete N-sulfotransferase activity of the NDST enzyme (see “Present Investigation”).

The various disaccharide units described are located in specific regions of the HS chain [46-48]. The NA-domains consist mainly of GlcA-GlcNAc repeats, while the NA/NS-domains include GlcA-GlcNAc and more than half of the 6-O-sulfated disaccharide species found in HS (Fig. 2). NA/NS-domains also contain a large proportion of IdoA residues. 2-O-Sulfated disaccharides are mainly found in the highly modified NS-domains, including the trisulfated IdoA2S-GlcNS6S disaccharide that is restricted to this domain type. Disaccharides containing the rare 3-O-substitution can be located to both NA/NS- and NS-domains [49], while N-unsubstituted GlcNH2 residues are mainly found in NA- or NA/NS-domains (see "Present Investigation"). The proportions of the three domain types vary between different HS samples [48, 50], as well as the domain lengths (NA- and NS-domains are typically 2-10 disaccharide units in length) [51, 52].
of NS-domains have been shown to influence the degree of 2-O-sulfation (more efficient 2-O-sulfation with increased domain length), but not 6-O-sulfation [52].

In what contexts does the HS structure vary? Several studies have shown that the variation does not correlate with core proteins, rather the cell type of origin [53-55]. Immunostaining with monoclonal antibodies, recognizing specific HS epitopes, has demonstrated differential expression of HS structures in certain cell types or tissue compartments, as well as differences between various tissues, species, or developmental stages [56-61]. For example, certain HS epitopes containing GlcNH₂ residues are found only in restricted areas of the rat kidney, while other renal HS epitopes are widely expressed [43]. Structural information has also been obtained from chemical analyses, based on controlled deaminative or enzymatic cleavage of the HS chains (see reviews above). By these means age-related differences in HS structure have been demonstrated, both at the stage of embryonic development [62-64], and postnatally [65, 66]. Furthermore, changes of HS structure have been correlated with various pathological conditions, such as cancer [67, 68], amyloidosis [69] and diabetes [70].

How are diverse HS structures formed? Two important factors are the lack of template in the HS biosynthesis, and the incompleteness of the enzymatic modification reactions. Instead of a template, the availability and activity of the biosynthetic enzymes seem to determine what HS structure to be produced. Since there are multiple isoforms of the NDST, 3-O-, and 6-O-sulfotransferase enzymes, and they show different tissue distributions, the set-up of enzymes probably varies from one cell type to another. To date it is not clear how the various isoforms might direct the synthesis of a specific HS structure, but there is experimental evidence that some of them act on different precursor sequences and generate different products [42, 71-73]. Interestingly, a shorter splice-variant of the human 6-O-sulfotransferase 2 (6-OST-2) enzyme was recently found to differ from the original enzyme with respect to both tissue expression and substrate preferences [74]. Can tissue-dependent differences in HS fine structure be generated by regulated expression of cDNA splice variants encoding a certain biosynthetic isofrom?

Structural variation in HS may also be created by "post-biosynthetic modification", i.e. by enzymes acting on ready-made HS chains after completed biosynthesis. A 6-O-endosulfatase, Sulfl, has for example been identified, that remodels the 6-O-sulfation state of cell surface HS [75-77]. Moreover, it was recently reported that two isoforms of the biosynthetic NDST enzyme, NDST-1 and NDST-2, can act on already modified HS sequences [78].

Factors such as the availability of sulfate donor, PAPS, or the presence of modulators, either stimulating or inhibiting the biosynthetic enzymes, could also effect the HS structure produced [38]. However, so far no such cofactors or inhibitors have been identified.
Table 1: Examples of heparan sulfate/heparin-binding proteins. The table is adapted from ref. [26, 79].

<table>
<thead>
<tr>
<th>Proteins/esterases</th>
<th>Physiological/pathological role</th>
</tr>
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<tbody>
<tr>
<td>antithrombin III</td>
<td>coagulation cascade serpin</td>
</tr>
<tr>
<td>SLP1</td>
<td>inhibits elastase and cathepsin G</td>
</tr>
<tr>
<td>VCP</td>
<td>inhibits complement activation</td>
</tr>
<tr>
<td>Growth factors/morphogens</td>
<td></td>
</tr>
<tr>
<td>FGFs</td>
<td>cell proliferation, differentiation, morphogenesis, and angiogenesis</td>
</tr>
<tr>
<td>VEGF</td>
<td>angiogenesis and vasculogenesis</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>cell proliferation, motility, morphogenesis, and protection from apoptosis</td>
</tr>
<tr>
<td>Wnts</td>
<td>developmental morphogens</td>
</tr>
<tr>
<td>hedgehog</td>
<td>developmental morphogen</td>
</tr>
<tr>
<td>Chemokines</td>
<td></td>
</tr>
<tr>
<td>platelet factor 4</td>
<td>inflammation and wound healing</td>
</tr>
<tr>
<td>interleukin 8</td>
<td>pro-inflammatory mediator</td>
</tr>
<tr>
<td>Lipid-binding proteins</td>
<td></td>
</tr>
<tr>
<td>lipoprotein lipase</td>
<td>key enzyme in catabolism of plasma lipoprotein triglycerides</td>
</tr>
<tr>
<td>apolipoprotein E</td>
<td>cholesterol transporter, AD risk factor</td>
</tr>
<tr>
<td>Adhesion proteins</td>
<td></td>
</tr>
<tr>
<td>selectins</td>
<td>adhesion, inflammation, and metastasis</td>
</tr>
<tr>
<td>fibronectin</td>
<td>adhesion and traction</td>
</tr>
<tr>
<td>thrombospondin</td>
<td>platelet aggregation, cell migration, and proliferation</td>
</tr>
<tr>
<td>HB-GAM</td>
<td>enhances synaptogenesis, modulator of synaptic plasticity</td>
</tr>
<tr>
<td>Viral proteins</td>
<td></td>
</tr>
<tr>
<td>HIV-1 gp120</td>
<td>viral entry into host cell</td>
</tr>
<tr>
<td>Tat protein</td>
<td>transactivating factor, primes cells for HIV infection</td>
</tr>
<tr>
<td>HSV gB and gC</td>
<td>viral entry</td>
</tr>
<tr>
<td>HSV gD</td>
<td>viral entry and fusion</td>
</tr>
<tr>
<td>Amyloid proteins$^b$</td>
<td></td>
</tr>
<tr>
<td>APP</td>
<td>neuroprotective and neurotrophic precursor of the AD-associated amyloid beta peptide</td>
</tr>
<tr>
<td>cellular prion protein</td>
<td>normal non-toxic conformer of the prion protein; cellular function unknown, associated with metal transport</td>
</tr>
</tbody>
</table>

$^a$SLP1, secretory leukocyte protease inhibitor 1; VCP, vaccinia virus complement control protein; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; HGF/SF, hepatocyte growth factor/scatter factor; Wnts, Wingless family of growth factors; HB-GAM, heparin-binding growth associated molecule; HIV-1 gp120, human immunodeficiency virus glycoprotein 120; Tat, twin arginine translocation; HSV gB, herpes simplex virus glycoprotein B; APP, amyloid beta precursor protein. $^b$More examples of amyloid proteins are given in Table 2.
Heparan sulfate-protein interactions

All HS protein-interactions that have been discovered so far involve binding of basic amino residues in the protein to various sulfated motifs in the HS chain [6, 79-81]. A major part of these interactions were first identified through the use of heparin. Heparin can, due to its high degree of modification, be considered as an elongated NS-domain, and is therefore a convenient tool to study the involvement of sulfate groups in protein binding. It is also, in contrast to HS, easy to obtain in large amounts at a low cost. Another GAG that is frequently used in binding studies is the *Escherichia coli* K5 polysaccharide. K5 has the same structure as the unmodified HS building block, consisting of \([\text{GlcA}\ 1,4-\text{GlcNAc}\ 1,4]\) disaccharide units, and can be N- and O-sulfated by chemical or enzymatic means. By the use of these HS analogues, as well as purified HS NS-domains, in for example "in solution" binding assays, affinity chromatography, or enzyme-linked immunosorbent assays (ELISAs), hundreds of HS-binding proteins have been identified to date. A few of those proteins are shown in Table 1.

Structural analysis of protein-bound heparin/HS ligands, either by disaccharide composition analysis or by more modern sequencing techniques (reviewed in [82]), suggests that HS-protein interactions may involve unspecific electrostatic binding between saccharide and protein. Examples of such interactions are the binding of HS to lipoprotein lipase [83], laminin [84], and thrombospondin-1 [85]. However, the binding can also be highly specific.

Specific protein-binding sites in HS are usually 3-9 disaccharide units long, and are generally of two types: HS motifs that have sulfate groups distributed in a certain pattern, and HS motifs that contain rare modifications (reviewed in [25, 26, 38, 86]). A well known example of the latter type, and perhaps the most specific heparin/HS motif identified so far, is the antithrombin binding sequence found in HS produced by endothelial cells [87], and in heparin produced by connective tissue mast cells [39]. The sequence is a pentamer containing a critical 3-O-sulfate group (Fig. 3a), added either by the 3-OST 1 enzyme, or by the newly discovered isoform 3-OST 5 [35]. By binding to antithrombin, the HS sequence enhances the inactivation of Factor Xa, thrombin, and other factors of the coagulation cascade [88, 89]. Another, more recent, example of a specific HS epitope containing rare modifications, is the HS sequence mediating binding of the herpes simplex virus type 1 glycoprotein D, resulting in enhanced viral infection [90] (Fig. 3b). In addition to a 3-O-sulfate group, this HS sequence contains a GlcNH₂ unit, which serves as a substrate for both 3-OST 3A and 3-OST 5 [35]. A third example of a specific HS epitope is the sequence recognized by fibroblast growth factor 2 (FGF-2). In contrast to the two other epitopes mentioned, binding to the growth factor is mediated by a
certain pattern of sulfate groups, and not unique HS components (Fig. 3c). The minimal FGF-2-binding sequence is a N-sulfated pentasaccharide containing one 2-O-sulfated IdoA unit. However, for the biological function of FGF-2 a longer HS domain is required, that binds to the FGF-2 receptor as well. Thus the biological active HS sequence consists of the pentamer adjacent to a segment of 2-O- and 6-O-sulfated monosaccharide units [91].

Figure 3 Three specific protein-binding heparan sulfate motifs. The minimal binding sequence is for both antithrombin (sequence A) and FGF-2 (sequence C) a pentasaccharide, whereas the minimal length of the sequence binding to the herpes simplex virus glycoprotein D (HSV-1 gD; sequence B) has yet not been determined. X = H or SO$_3^-$.

Specificity in HS-protein interaction can also be achieved through a certain domain distribution pattern. Some examples are binding of interleukin-8 [92], interferon-γ [93], platelet factor 4 [94] and endostatin [95]. In all these cases the proteins bind to an HS motif that consists of two NS-domains interspersed by a less modified, GlcNAc-rich region. This type of HS motif, sometimes called SAS-domain, is believed to span over the protein, or even two subunits of a dimer, by binding of the NS-domains to patches of basic amino acids (mainly arginines or lysins).

What other factors than sulfation could be important for binding of HS to a protein? Non-ionic interactions, such as hydrogen bonding and van der Waals forces, are involved to various extent [96]. The absence of sulfate groups in certain positions could be crucial, if there are acidic amino acid residues within or in the close vicinity of the HS-binding region of the
protein, that otherwise would be repelled by the negatively charged sulfate groups. Furthermore, the secondary structure of the HS chain has been proposed to be of importance (reviewed in [97]). HS forms a helical coil, the overall shape of which is largely determined by the rotations about the glycosidic bonds. However, it also has internal mobility, which is explained by the unusual flexibility of the pyranose ring in the IdoA unit [98]. IdoA can adopt different conformations and thereby influence the spatial orientation of attached sulfate, carboxyl, and hydroxyl groups, as well as of neighboring sugar residues. This in turn influences the protein binding properties of the HS chain.

Biological functions

Protein binding to HS can serve many different purposes [4, 6, 7, 99]. HS-protein binding can be utilized for storage of proteins on the cell surface or in the ECM, protection of proteins against proteolytic degradation, modulation of protein conformation, or enhancement of protein-protein interaction, for example as a co-receptor for binding of growth factors or cytokines to cell-surface receptors. HS can also regulate protein transport through basement membranes, mediate internalization of proteins (reviewed in [100]) and has been suggested to direct diffusion of proteins, thereby promoting molecular encounters [101].

One of the most well studied functions of HS is the mediation of growth factor signaling, especially the signaling pathways involving members of the fibroblast growth factor (FGF) family. Cell-surface bound HS involves in ternary complex formation with FGF and its receptor, and FGF receptor oligomerization, which results in potentiation of receptor phosphorylation and thereby signal transduction (reviewed in [102-105]). FGF-induced signaling evokes various cellular responses, depending on which FGF member that has been activated (at least 23 vertebrate members exist). FGFs have important roles in embryonic development by regulating cellular processes such as proliferation, differentiation, and migration. These processes are to bye extent determined by the HS fine structure [62, 73]. In the adult organism, FGFs function as homeostatic factors and are involved in angiogenesis, tissue repair, neuronal regeneration and neurite outgrowth. FGFs can also contribute to disease such as cancer (see reviews above and [106]).

Other growth factors that are dependent on HS for their activity are for example the members of the hepatocyte growth factor/scatter factor, and the vascular endothelial growth factor families. The role of HS in receptor binding and signaling of these growth factors is not as well understood, but seems to differ from that in FGF-induced receptor signaling [107-109]. Like the FGFs, these growth factors are regulators of various cellular processes.
Vascular endothelial growth factors have for example, together with the FGFs and HSPGs, important roles in angiogenesis [109-111].

A few additional functional aspects, all linked to disease, will be mentioned here. HS thus has been implicated in the development of atherosclerosis, by binding protein ligands involved in the lipoprotein metabolism, such as low density lipoproteins [112]. HS has a complex role in inflammation, by modulating leukocyte-endothelial interaction and extravasation, chemokine function, and tissue repair processes [113]. Binding of HS to viral surface proteins mediates internalization of viruses into the host cell [100, 114-116]. Moreover, different roles of HS in cancer [117], diabetes [118], and amyloidosis [119] have been described. The involvement of HS in amyloidosis, one of the main themes in this thesis, will be discussed in more detail below.

Turnover of heparan sulfate
HS is not only a molecule with a complex heterogeneous structure, it is also highly dynamic. After biosynthesis and transport of a HSPG to its site of action, it is, at some time point, processed by degrading enzymes [120]. HSPGs at the cell surface have a half-time of 3-8 h, and are either endocytosed for intracellular degradation, or, less commonly, shedded into the extracellular space. In the latter case, membrane-bound HSPGs are released by proteolytic cleavage of the core protein, while GPI-anchored glypicans are cleaved by either proteases or phospholipase C. Shedding of HSPGs can serve different purposes. It has shown to enhance cell migration by disrupting matrix anchorage [121], it maintains the proteolytic balance of acute dermal wound fluids [122], and can modulate growth factor activity [123]. Endocytosis, on the other hand, is the major route of metabolic turnover. Membrane-bound and GPI-anchored HSPGs are processed in different catabolic pathways [124]. The degradation of membrane-bound HSPGs is initiated by a step-wise partial processing of the HS chains by intracellular endoglycosidases (heparanases), followed by complete digestion in the lysosomes by the action of proteases and HS-degrading enzymes, including various sulfatases and exo-glycosidases. In contrast, GPI-anchored HSPGs are more rapidly degraded since they are directly transported to the lysosomes, and not subjected to intermediate processing by heparanases. Moreover, studies by L-Å Fransson and co-workers have shown that endocytosed GPI-anchored HSPGs also can follow another route, and be recycled. Recycling has been demonstrated for glypican-1 expressed by fibroblasts, as well as by transformed cells [21, 125]. The recycling process involves modification of the core protein, and combined partial cleavage of the HS chains by intracellular heparanases (cleave at certain glycosidic bonds) and, interestingly, by deamination at GlcNH₂ residues by
endogenous nitrite [126, 127]. The resulting HS stubs, still attached to the core protein, are then used for de novo synthesis of HS. Recycling of glypican-1 is believed to be important for transport of proteins and other ligands, such as polyamines, in and out of the cell. By the de novo synthesis of HS chains, but not the whole HSPG, the cell can quickly adapt to environmental changes by altering the HS structure, and thereby its ligand-binding properties [128].

Heparanase-mediated degradation of HSPGs can also take place in the ECM. The extracellular heparanase activity has important roles in for example extravasation of immune cells during inflammation [129], tumor metastasis, and angiogenesis [130].

Heparan sulfate in amyloid disease

Amyloid diseases

Amyloid disease denotes a heterogenous group of disorders that are all characterized by formation of insoluble fibrillar aggregates of misfolded protein. The aggregates interfere with various organ functions, which, in at least some of the amyloidoses, may be explained by the cytotoxic properties of the aggregates, although the mechanism(s) behind the cytotoxicity are poorly understood. Today more than 20 distinct forms of amyloid have been identified, each comprising a certain type of protein, and associated with a unique clinical syndrome (reviewed in [131-136]). Some examples of amyloid diseases are shown in Table 2, including neurodegenerative disorders such as Alzheimer’s, Huntington’s, Parkinson’s, and prion encephalopathies, and various forms of systemic amyloidoses, affecting multiple organs and tissues of the body. In all amyloids, the aggregated protein forms fibrils that are ordered in a characteristic β-sheet conformation. In most amyloids, the fibrils are made up of a polypeptide that is the degradation product of a larger precursor protein (Table 2). The various precursor proteins show no obvious sequence similarity, but contain, either in their wild-type or mutated form, certain amino acid motifs or substitutions that predispose to amyloid fibril formation. In vitro, many of the polypeptides spontaneously form fibrils, without the help from co-factors or chaperones, while the fibrils found in amyloid deposits in tissues in vivo, are always associated with other molecules that may enhance the fibrillization process and/or stabilize the already formed fibrils. Two components that are consistently present are the blood protein designated
serum amyloid P component (SAP) and HSPGs [137, 138]. The role of HSPGs in amyloid disease is discussed in more detail in the two following sections. However, first Alzheimer’s disease will be described, since this disorder is of major interest in the thesis work.

Table 2: Proteins associated with amyloid deposits. The table is adapted from K.Ohasi, Pathol. Int. 2001 [139].

<table>
<thead>
<tr>
<th>Amyloid Protein</th>
<th>Precursor Protein</th>
<th>Clinical Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Serum amyloid A protein (SAA)</td>
<td>Inflammation-associated amyloidosis (secondary amyloidosis)</td>
</tr>
<tr>
<td>AL</td>
<td>light chains</td>
<td>Primary amyloidosis</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin (TTR) with various mutations or normal TTR</td>
<td>Familial amyloid polyneuropathy</td>
</tr>
<tr>
<td>AApoA-1</td>
<td>Apolipoprotein-1 (Arg26)</td>
<td>Familial amyloid polyneuropathy, Iowa</td>
</tr>
<tr>
<td>AGel</td>
<td>Gelsolin (Asn 187)</td>
<td>Familial amyloidosis, Finnish</td>
</tr>
<tr>
<td>A 2M</td>
<td>2-microglobulin</td>
<td>Hemodialysis associated amyloidosis</td>
</tr>
<tr>
<td>ACal</td>
<td>Calcitonin</td>
<td>Medullary carcinoma of thyroid</td>
</tr>
<tr>
<td>AANF</td>
<td>Atrial natriuretic factor</td>
<td>Isolated atrial amyloid</td>
</tr>
<tr>
<td>A1APP</td>
<td>Islet amyloid polypeptide (IAPP amylin)</td>
<td>Diabetes type II, insulinoma</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystatin C (Gln 68)</td>
<td>Hereditary cerebral hemorrhage with amyloidosis, Icelandic</td>
</tr>
<tr>
<td>A</td>
<td>A precursor protein (various mutations)</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein precursor, cellular form (several mutations)</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>Huntingtin</td>
<td>Huntingtonin (mutated; polyglutamine expansion), see ref. [140, 141]</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>α-Synuclein</td>
<td>α-Synuclein, either mutated or wild-type, see ref. [135]</td>
<td>Parkinson’s disease, dementia with Lewy bodies</td>
</tr>
<tr>
<td>Tau protein</td>
<td>Tau protein, see ref. [142]</td>
<td>Alzheimer’s and tauopathies, e.g. Pick’s disease</td>
</tr>
</tbody>
</table>

*Intact precursor protein. "Either intact precursor protein or fragment. "Additional diseases exist, see review by Ohasi.

Alzheimer’s disease (AD) is the most common cause of senile dementia, today affecting more than 20 million people worldwide (for reviews, see [135, 143-146]). Typical symptoms of the disease are progressive decline in memory function, behavioral problems, and cognitive impairment. The time of onset of the disease varies. The most common sporadic form (≥90% of the cases) usually shows up in individuals past their mid 60s, while the rare familial forms can start as early as late 20s. Pathologically, AD has two major hallmarks: extracellular senile plaques, mainly consisting of aggregated amyloid peptide (Aβ), and intracellular neurofibrillar tangles (NTFs), primarily containing hyperphosphorylated tau protein. Other
characteristics are dystrophic neurites, neuronal atrophy, and cerebral amyloid angiopathy. The cause of disease has been a matter of debate ever since the identification of the Aβ and tau proteins during the 1980s. Currently, the most strongly supported idea is the amyloid hypothesis, which claims that accumulation of Aβ is the primary influence driving AD pathogenesis, while tau is more associated with the consequences, such as neurodegeneration [144].

Aβ is a 40-43 amino acid residue peptide (4 kD; Aβ (1-40) and Aβ (1-42) the most common species) that is formed by proteolytic cleavage of the amyloid precursor protein (APP) (see reviews above). APP is a transmembrane protein that is expressed by different cell types of various tissues, and is found both at the cell surface, and in intracellular compartments such as the endoplasmatic reticulum and the Golgi apparatus [147]. APP occurs as several isoforms, of which APP695 is most commonly expressed by neurons. APP is known for its neuroprotective and neurotrophic functions [148-150], whereas no normal physiological role for the Aβ peptide has yet been described. Thus, the Aβ peptide might be merely a by-product resulting from the release of a biologically active soluble form of APP. The APP-processing enzymes have all been cloned and characterized. Cleavage of APP by the β-secretase (beta-site APP cleaving enzyme; BACE), followed by the action of γ-secretase (presenilin in complex with the co-factors nicastrin, APH-1 and PEN-2; see [151]) results in the formation of the soluble form of APP and the Aβ peptide (Fig. 4), the latter most often being secreted from the cell. The action of α-secretase (ADAM; a disintegrin and metalloproteinase) rather than γ-secretase, precludes formation of the amyloidogenic Aβ. Formation and secretion of Aβ is a normal cellular event, i.e. the peptide can be detected in the cerebrospinal fluid, as well as in the plasma, also in healthy subjects. However, in AD there is an accumulation of Aβ, especially of Aβ (1-42), which is more hydrophobic than Aβ (1-40), and therefore more prone to aggregate. The increased level of Aβ might either be the result of insufficient clearance, or enhanced production of the peptide. Genetic studies of patients with the familiar form of AD have identified several mutations in both APP and the presenilin genes, that either enhance the processing of APP to form Aβ (1-42), alter the peptide conformation, thereby augmenting aggregation, or, as recently described, cause Aβ to be more resistant to proteolytic degradation [152]. However, the majority of AD patients, i.e. those with sporadic disease, do not have these mutations. Instead many of them carry a genetic variant of the apolipoprotein E gene, ApoE4. ApoE4 alters, by unknown molecular mechanisms, the age at onset (5-20 years earlier) and therefore the lifetime risk of AD. ApoE4 alone does not cause AD, and is therefore regarded as a risk factor.
Figure 4 Generation of Aβ from its precursor, APP. APP is first cleaved by β-secretase (or -secretase precluding formation of Aβ), and then α-secretase, forming a 40-42 amino acid residue peptide. The cationic HHQK motif, presumably mediating binding to heparan sulfate, is shown in bold.

Several in vitro studies have demonstrated a cytotoxic effect of various forms of Aβ, including both Aβ monomers and fibrils (reviewed in [153]). However, according to recent studies the most toxic Aβ constituents are soluble Aβ oligomers (protofibrils) [154, 155]. The protofibrils are believed to form membrane channels, or pores that allow penetration of substances (e.g. metal ions), thereby causing neuronal cell death [156-158]. Since formation of membrane pores also has been demonstrated for other amyloid proteins [159-161], this might be a general mechanism of amyloid-induced cytotoxicity. Intriguingly, Aβ oligomers, but not monomers or fibrils, have also been shown to inhibit long-term potentiation (LTP) in the hippocampus, which is associated with memory function [154]. Moreover, synaptic loss, and other measures of neurological dysfunctions, correlates more with soluble Aβ than fibrillar amyloid load, indicating that Aβ oligomers are involved in early events of AD pathology [153, 162]. The pathological role of Aβ fibrils would then be to function as a reservoir of Aβ species, that is in equilibrium with the smaller neurotoxic components.

However, these findings do not exclude the other proposed roles of Aβ species in AD, in activating microglia [163, 164], thereby causing oxidative stress, inducing apoptosis [165], or simply blocking cell-cell-, or cell-ECM interactions [166].
Heparan sulfate in amyloidosis

HSPGs are found in all kinds of amyloid deposits in vivo. Also free HS chains are found in the lesions, although it is not known how they end up in the deposits. However, several studies have demonstrated that HS, as well as other GAGs, interact with amyloidotic peptides in vitro, thus enhancing their fibrillization, and stabilizing already formed aggregates [119, 133, 167-169]. The amino acid motif responsible for binding of HS has been characterized for several of the amyloid precursor proteins, or their products, for example apo-serum amyloid A, [170], pro-islet amyloid polypeptide (amylin) [171], APP [172, 173], Aβ [174], and cellular prion protein (PrP C) [175]. Furthermore, HS analogues have been shown to inhibit HS-induced fibrillization of for example Aβ in vitro, and to interfere with the accumulation of splenic AA-amyloid in vivo, in a mouse model [176-178]. Altogether, these findings clearly suggest that HS is involved in the process of amyloid formation. However, the physiological consequences of this involvement depend in part on the role of the amyloid aggregates per se. For example, soluble HS, added to cell culture, has been shown to inhibit Aβ-induced neurotoxicity [179, 180], possibly by preventing binding of Aβ to the cell surface. Thus, HS-induced protein aggregation could be a mechanism to sequester toxic peptide components. On the other hand, membrane-bound HSPGs might have the opposite function, i.e. mediating binding of the amyloidogenic peptides to the cell surface, and thereby having a pathogenic role. Furthermore, studies on the prion disease have shown that HS might be involved in the cellular production of cytotoxic amyloid components (reviewed in [168]). In prion disease the normal cellular prion protein (PrP C) is refolded into a β-sheet rich conformation, forming a highly toxic and transmittable form of the protein (PrP Sc). The mechanism of PrP C-PrP Sc conversion is not known, but recent studies have shown that HS can stimulate the cell-free conversion of PrP to a protease-resistant folding variant [181], and cell-bound HSPGs the cellular production of PrP Sc [182]. Moreover a heparitinase-sensitive fraction of a cell extract was shown to promote the reconstitution of infectivity to Me2SO-dispersed prion rods [183], and HSPGs have been identified as co-receptors for binding of PrP Sc to its laminin receptor (LRP/LR) [184]. To date, no structural information is available regarding the HS species alleged mediating the conversion of PrP C to PrP Sc. However, two independent studies, one showing the specific staining by the HS antibody 10E4 of the prion lesions in the brains of scrapie-infected mice [185], the other demonstrating the recognition by this antibody of an unmodified tetrasaccharide motif containing a GlcNH2 unit [186], have lead to the suggestion that free amino groups might be involved in the pathology of prion disease. The further characterization of this epitope, described in Paper IV of this thesis, is therefore of major interest.
Heparan sulfate in Alzheimer’s disease

A large number of different PGs are expressed in the central nervous system, including all classes of HSPGs (reviewed in [187]). It is therefore not surprising that various HSPGs have been found associated with the amyloid plaques in AD, as well as with neurofibrillary tangles, and blood vessels affected with amyloid angiopathy [119]. However, immunostaining of AD brains with a variety of antibodies, recognizing the core protein of a specific HSPG subtype, has shown that some HSPGs are more abundant than others in a certain AD lesion (Table 3). Collagen XVIII is for example only associated with classic senile plaques and vessels, while agrin is found in all lesions, including the diffuse plaque, believed to be an immature form of the senile plaque since it is mainly composed of non-fibrillar Aβ. This could indicate that different HSPGs have distinct roles in the AD pathogenesis.

Table 3: Distribution of heparan sulfate proteoglycans in Alzheimer’s disease brain. Table adopted from J. van Horssen et al. Lancet Neurol. 2003 [119].

<table>
<thead>
<tr>
<th>HSPG</th>
<th>Normal vessels</th>
<th>CAA&lt;sup&gt;a&lt;/sup&gt; in AD</th>
<th>Cerebellar DP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cerebellar SP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cerebral DP</th>
<th>Cerebral SP</th>
<th>Neurofibrillar tangles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen  XVIII</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Agrin</td>
<td>+++</td>
<td>-/+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Perlecan</td>
<td>+++</td>
<td>-&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Syndecan 1,3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Syndecan 2</td>
<td>-</td>
<td>+/+</td>
<td>-</td>
<td>++</td>
<td>+/+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Syndecan 3</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+++</td>
<td>+/+</td>
<td>+/+</td>
<td>++</td>
</tr>
<tr>
<td>Glypican 1</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>HS GAGs</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup>CAA, cerebral amyloid angiopathy. <sup>b</sup>DP, diffuse plaque. <sup>c</sup>SP, senile plaque. <sup>d</sup>van Horssen and co-workers could not localize perlecan to any of the AD lesions. However, previous studies by Alan Snow and co-workers have demonstrated the presence of perlecan in amyloid-laden vessels, senile plaques, as well as neurofibrillar tangles. For references, see [119].

As in the case with other types of amyloidoses, HS promotes fibrillization of Aβ peptides (Fig. 5; see [119, 168] for reviews). Both soluble HS and HSPGs, such as agrin and perlecan, bind to Aβ, enhance its fibrillization, and stabilize the formed Aβ aggregates [188-190]. The enhancement of Aβ fibril formation can be competed out with low-molecular-weight anionic sulfonates and sulfates [176], or low-molecular-weight heparin [191]. The binding of HS to Aβ is believed to involve interaction of both N-and O-sulfate groups [189], perhaps also carboxyl groups, in HS, with a cationic HHQK motif in the peptide (Fig. 4), although non-ionic interactions might also take part [174]. HSPGs moreover bind the Aβ precursor, APP, using both its protein core and the HS chains for interaction [192]. Interestingly,
HS was recently demonstrated to be a regulator of APP processing [193]. However, in contrast to the HS involved in prion disease, HS binding of either the β-secretase BACE1 (making it inaccessible to APP), or of APP itself, inhibits the formation of cytotoxic Aβ components (Fig. 5). Thus, HSPG present on the cell surface, or intracellularly, might have a neuroprotective function by regulating APP processing.

What other roles could HS have in AD? HSPGs have been shown to protect Aβ aggregates against proteolytic degradation [194], and as mentioned in the previous section, soluble HS can inhibit Aβ-induced neurotoxicity, possibly by converting the toxic Aβ oligomers into fibrils (Fig. 5). Furthermore, soluble HS, as well as heparinase III treatment of cells, was shown to inhibit Aβ-mediated activation of microglia [195, 196], indicating that HSPGs on the cell surface of microglia could regulate the uptake and degradation of Aβ peptides or aggregates (Fig. 5). Moreover, it has been demonstrated that HS is involved in the formation of intracellular neurofibrillar tangles, possible by promoting both phosphorylation and conformational changes of the tau protein (see reviews above). HS might further modulate various neuroprotective pathways by interacting with factors promoting neuronal survival, for example FGF-2 [197]. FGF-2 has been shown to attenuate Aβ-induced neurotoxicity [198, 199], possibly by preventing oxidative stress and mitochondrial dysfunction in synaptosomes [200]. The mechanism behind the synapto-protective action of FGF-2 could conceivably involve receptor binding and initiation of protein phosphorylation events, that result in release of neurotransmitters [200]. Other studies have demonstrated that FGF-2 can, by initiation of MAPK signal transduction pathways [201], regulate the expression of a variety of neuroprotective gene products, for example antioxidant enzymes and APP [200, 202, 203]. Since HS has been shown to regulate many other functions of FGF-2, by enhancing binding of the growth factor to its receptor, HS might be involved in FGF-2-mediated neuroprotection as well, although no direct evidence of such specific role of HS has yet been published. However, HSPGs (mainly syndecan-2) are indeed found in the synapses of hippocampal neurons, where they regulate the formation of dendritic spines (reviewed in [204, 205]) and possibly also LTP, by interacting with modulators of synaptic plasticity such as neuronal cell adhesion molecule, heparin-binding growth associated molecule, and FGF-2 [206, 207].
Figure 5 Some proposed roles of heparan sulfate in Alzheimer’s disease. HSPGs (exemplified by glypican in the fig., but may also be other HSPGs) may inhibit processing of APP by binding to β-secretase (BACE) located either on the cell surface, or in intracellular compartments. HS/HSPGs may also bind to released Aβ peptides in either of these locations, or extracellularly, and enhance Aβ fibrillization. In the extracellular space, HS/HSPGs stabilize the formed aggregates (senile plaques). HSPGs expressed by microglia may act as receptors or co-receptors for binding of Aβ fibrils/aggregates, resulting in uptake and degradation of Aβ. The activated microglia produce reactive oxygen species (ROS) and other cytotoxic components. Furthermore, HSPGs present on the cell surface of neurons may mediate binding of neurotoxic Aβ protofibrils. See the text for more details.
Present Investigation

Aims of study

This thesis work has been focused on two main subjects:

- the role(s) of HS in AD (paper I, III, and unpublished data)
- N-unsubstituted GlcN residues in HS (paper, II, III and IV)

Heparan sulfate in Alzheimer’s disease

A considerable amount of data supports an involvement of HS in AD (see "Background" section). HS thus is present in all lesions characteristic of AD, such as amyloid plaques and neurofibrillar tangles. HS has been shown to enhance fibrillization of Aβ peptide, and to protect the Aβ aggregates from proteolytic degradation. Moreover, HS may promote Aβ-induced neurotoxicity, but also neuroprotective pathways, for example those mediated by FGF-2. Since many of the putative roles of HS in AD seem to involve interaction with Aβ, detailed information about the HS structures that bind to Aβ would be valuable, especially in a therapeutic perspective. In the “AD project” we therefore undertook structural studies of HS sequences binding to different forms of Aβ, as well as a functional study to test the effect of Aβ on HS-dependent FGF-2 cell signaling. More specifically, the aims of this project were:

- to investigate the binding properties of human cerebral HS to Aβ(1-40) monomers and fibrils
- to compare the structural preferences of Aβ and FGF-2 in HS binding
- to assess the possible inhibitory effect of Aβ protofibrils on HS-dependent FGF-2 signal transduction in NIH 3T3 fibroblasts

31
N-Unsubstituted glucosamine residues in heparan sulfate

The presence of GlcNH$_2$ residues in HS sequences mediating herpes simplex virus infection [90], and the indirect association of GlcNH$_2$ with prion disease (recognized by the 10E4 antibody [186], which specifically stains the amyloid lesions in the brains of scrapie-infected mice [185]), has awoken a great interest in GlcNH$_2$ units the last years. However, it is still not known how these rare units are formed, and no detailed structural information has been published on GlcNH$_2$-containing HS sequences in general. Therefore, the main purpose of the “GlcNH$_2$ project” was to conduct a thorough structural analysis of free amino group-containing HS sequences found in native tissues. In addition, since GlcNH$_2$ residues have been associated with prion disease we wanted to investigate if there are any correlations between such residues and AD. The specific, and additional, aims of the “GlcNH$_2$ project” were:

- to determine the content and location of GlcNH$_2$ residues in HS from various sources
- to compare GlcNH$_2$-containing HS sequences from human control and AD cerebral cortex
- to investigate the role of GlcNH$_2$ residues in the binding of HS to Aβ(1-40) fibrils
- to further characterize the HS epitope(s) recognized by the 10E4 antibody

Results

Common binding sites for β-amyloid fibrils and fibroblast growth factor-2 in heparan sulfate from human cerebral cortex (Paper I)

In this study HS structures that bind to Aβ monomers or fibrils were characterized with regard to size and sulfation. HS binding to Aβ versus FGF-2 was then compared.

The HS was prepared from autopsy specimens of cerebral cortex from an AD patient and an age-matched control. HS chains and excised NS-domains were then $^3$H-labeled. Fibrillar Aβ was prepared by incubating synthetic Aβ(1-40) monomers in phosphate-buffered saline, pH 6.5. Binding assays with fibrillar Aβ was performed at physiological pH (7.4), while binding of
A monomers requires a lower pH [208], and was therefore assessed at pH 5.5.

Incubation of increasing amounts of radiolabeled HS chains with A, followed by quantification of bound HS species using a nitrocellulose filter trapping procedure, showed that both AD and control HS samples, as well as [³H]heparin, bound in a dose-dependent, saturable manner. The minimal A-binding domain was identified using either size-fractionated NS-domains, or heparin oligomers. It appeared that a hexasaccharide is sufficient for binding to both A fibrils and monomers (data not shown for monomers).

A -binding HS structures were then characterized by various means. NS hexamers and octamers were separated into A-bound and unbound fractions by nitrocellulose filter trapping, or affinity chromatography on an A fibril column. The bound and unbound fractions were subjected to deaminative cleavage at pH 1.5, followed by NaB³H₄-reduction, and the resulting radiolabeled disaccharide species analyzed by anion-exchange chromatography. The major disaccharide product was for all samples IdoA2S-aManR, corresponding to an IdoA2S-GlcNS sequence in the native HS chain. However, no consistent difference in composition between A-bound and unbound fractions was found, possibly indicating that positioning rather than the total amount of sulfate groups is important in the interaction with A fibrils. We then tested the ability of selectively desulfated, unlabeled, heparins to compete out the binding of native [³H]heparin to A. In assays with A fibrils efficient inhibition was achieved with native and 6-O-desulfated heparin, while N-or 2-O-sulfated heparin had no or little effect. In contrast, binding to A monomers was only inhibited by native heparin.

The conclusion from these experiments was that binding of HS to A fibrils requires N- and 2-O-sulfation only, while the interaction with A monomers involves N-, 2-O- and 6-O-sulfation. Direct binding studies with radiolabeled selectively desulfated heparin 12-mers confirmed this finding (data not shown). Thus, the fibrillar and non-fibrillar forms of A might be recognized by distinct HS domains.

Since the structural requirements of A fibrils matched those described for FGF-2, dependent on GlcNS and IdoA2S residues only [209-211], and known for its neuroprotective properties, we wanted to investigate the possibility that the two proteins share a common binding site in HS. Therefore, NS octamers were affinity chromatographed on A fibril and FGF-2 columns, and the unbound and bound HS pools were then tested for binding to the converse protein ligand. Analysis of A -fractionated octasaccharides on the FGF-2 column showed that the A-bound fraction was quantitatively bound to the growth factor, while the A-unbound fraction was partially bound. The opposite procedure, applying FGF-2-fractionated saccharide to the A column, showed that a major part of the FGF-2-bound octamers was also retained by A, while the unbound fraction
had no Aβ affinity. These data demonstrated that the HS sequences binding to Aβ indeed represent a major subpopulation of FGF-2 binding domains, suggesting that Aβ and FGF-2 may compete for binding to the same HS binding sites.

Effect of Aβ protofibrils on heparan sulfate dependent fibroblast growth factor-2 cell signaling? (unpublished data)

The presence of common binding sites for Aβ and FGF-2 in human brain HS (demonstrated in Paper I) suggests that neurotoxic and neuroprotective signals might converge by competing for the same binding sites on the HS chain. Several findings by other research groups also point to an interplay between Aβ and FGF-2 actions (see "Background"). However, if there is a direct physical interplay between Aβ and FGF-2 is not known. Therefore, in collaboration with the research groups of Prof. Lena Claesson-Welsh (Dept. Genetics and Pathology, Vascular Biology unit, Uppsala University, Sweden) and Prof. Lars Lannfelt (Dept. Public Health and Caring Sciences, Uppsala University, Sweden) we undertook a study to test if Aβ can compete out the binding of FGF-2 to cell surface HSPGs, and thereby inhibit HS-dependent FGF-2 signaling (C. Westling, A-S. Johansson, M. Cross, C. Lee, L. Lannfelt, L. Claesson-Welsh, and U. Lindahl – unpubl.) (Fig. 6).

Figure 6 Hypothetical mechanism of Aβ-induced neurotoxicity. Aβ protofibrils compete with FGF-2 for binding to cell surface HS, resulting in inhibition of FGF-2-induced MAPK-signaling, and thereby reduced expression of neuroprotective gene products. See text for more details.
NIH 3T3 fibroblasts, like neurons, express HSPGs, the FGF receptor 1, and the proteins in the mitogen-activated protein kinase (MAPK) signal transduction pathway, and were used as model system. The MAPK signaling pathway has been shown to mediate at least some of the neuroprotective effects of FGF-2, and includes a series of protein phosphorylation reactions (reviewed in [105]). Since the most cytotoxic form of Aβ appears to be the Aβ protofibril we decided to use the Aβ(1-42)Arc peptide, which carries the arctic mutation (E693G in APP) and therefore forms protofibrils at a much higher rate and in larger quantities than wild-type (wt) Aβ [212].

Briefly, NIH 3T3 cells were seeded at 8 x 10^4 cells per well of 12-well plates in DMEM containing 10% newborn calf serum and grown at 37°C overnight. The medium was then changed to low-serum (0.5%)-containing DMEM and cells were incubated for 24 h. About 30 min before the FGF-2 activation assay, a 100 µM stock solution of Aβ(1-42)Arc peptide was prepared by dissolving the peptide in 10 mM NaOH, and then adding phosphate-buffered saline (PBS; pH 7.4), after rigorous vortexing, to a final NaOH conc. of 5 mM. The peptide was then diluted in NaOH-PBS buffer to different concentrations, centrifuged (17,900 g for 5 min at 16°C) and kept on ice before addition to cells. According to gel chromatography analysis (method described in[212]), 95% of the Arctic peptide (in the stock solution) is converted to protofibrils under these conditions. The FGF-2 activation assay was performed by first adding 50 µl Aβ (0.1 nM-10 µM in a final volume of 500 µl), or NaOH-PBS buffer, to the cells, and then immediately 50 µl FGF-2 (human recombinant; final conc. 0.06 nM), lysophosphatidic acid (LPA; control of MAPK signal activation; final conc. 10 µM), or medium. The cells were stimulated for 10 min at 37°C, and thereafter rinsed with ice-cold PBS, and lysed by sonication following addition of Laemmli buffer (4% SDS, 0.25 M Tris/HCl, pH 8.8, 0.5 M sucrose, 5 mM EDTA, 0.01% Bromophenol Blue, 4% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) and transfer to Eppendorf tubes. The samples were boiled for 5 min, resolved by SDS-PAGE on 10% polyacrylamide gels, and electrophoretically transferred to nitrocellulose filter membranes. The membranes were then blocked for 2 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% BSA, before incubation overnight with a phosphospecific antibody against p42/44 MAPK (Thr202/Tyr204) (i.e. read-out of FGF-2-induced cell signaling) at 4°C. The membranes were washed with TBS/Tween and incubated for 1 h with a secondary antibody coupled to peroxidase. After washing of membranes, bound antibody was visualized using ECL detection (Amersham Biosciences). To control the protein load for each sample, membranes were stripped, and reprobed with an anti-actin antibody.

The addition of Aβ(1-42)Arc protofibrils to NIH 3T3 cells did not have any detectable effect on FGF-2-induced phosphorylation of the MAPK proteins (two isoforms, 42 and 44 kDa, resp.) (Fig. 7). Even at the highest
Aβ concentration (10 µM) FGF-2 (1/170,000 of the Aβ conc.) was able to induce MAPK phosphorylation to the same extent as FGFs in the absence of Aβ. Addition of Aβ(1-40)wt monomers instead of protofibrils gave the same result (data not shown). Since Aβ alone did not induce MAPK phosphorylation, we concluded that neither Aβ(1-42)Arc protofibrils, nor Aβ(1-40)wt monomers, are able to prevent FGF-2-induced MAPK signaling in NIH 3T3 cells. These results tentatively argue against a direct inhibitory effect of Aβ on neuroprotective FGF-2 actions (see also “Discussion”).

<table>
<thead>
<tr>
<th>Aβ protofibril (µM)</th>
<th>-</th>
<th>0.0001</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>10</th>
<th>-</th>
</tr>
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<tbody>
<tr>
<td>FGF-2 (0.06 nM)</td>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LPA (10 µM)</td>
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</tr>
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</table>

Figure 7 Aβ protofibrils have no effect on FGF-2-induced MAPK-signaling.
NIH 3T3 fibroblasts were stimulated with FGF-2 in the presence of various concentrations of Aβ(1-42)Arc protofibrils for 10 min. Cells were thereafter lysed by sonication in Laemmli buffer, and the samples resolved by SDS-PAGE. The samples were then electrophoretically transferred to nitrocellulose filter membranes and blotted for active MAPK using a phospho-specific (p42/44) MAPK antibody. Blots were stripped and reprobed with an anti-actin antibody. LPA, lysophosphatidic acid (control of MAPK signal activation).

Location of N-unsubstituted glucosamine residues in heparan sulfate (Paper II)

In paper II we describe the content and location of GlcNH2 residues in HS from three different sources: human aorta and kidney, and porcine intestinal mucosa.

To determine the content of free amino groups, each HS sample was subjected to deaminative cleavage with HNO2 at pH 3.9. At this pH the HS chain is specifically cleaved at the sites of GlcNH2 units, that are thereby converted into reducible 2,5-anhydromannose residues (Fig. 2; Paper II). Subsequent NaB3H4-reduction of the cleavage products introduced a radiolabel that could be used as a measure of GlcNH2 residues. By quantification of the total incorporation of 3H-label, and processing of data using a heparin disaccharide standard, we showed that the proportion of GlcNH2 residues varied between HS samples. Human aortic HS has the highest content (4% of total disaccharide units), followed by renal (2%), and porcine intestinal (0.7%) HS. Estimates of the molecular mass of HS from
aorta and intestine allowed us to calculate the average number of GlcNH₂ residues per polysaccharide chain: ~4 in aortic and ~0.5 in intestinal HS.

Since an excess of HNO₂-reagent has been claimed to cause some cleavage at N-sulfated residues as well [213], control samples of HS were chemically N-acetylated and subjected to HNO₂-pH 3.9 treatment. The conversion of GlcNH₂ into GlcNAc units precluded deaminative cleavage, thus confirming the specificity of the HNO₂-pH 3.9 reaction under our conditions.

The location of GlcNH₂ residues along the HS chain was investigated by gel chromatography before and after HNO₂-pH 3.9-treatment. Both unlabeled cleavage products, detected by colorimetric HexA analysis, and end-group-labeled products were chromatographed. Deaminative cleavage of porcine intestinal HS resulted only in minimal change compared to untreated control, i.e. unlabeled cleavage products were of similar size as the intact HS chains, suggesting that any GlcNH₂ unit present should be located close to either end of the polysaccharide. The gel filtration profile of ³H-labeled deamination products supported this finding, pointing to the presence of two or more clustered free amino groups toward the carbohydrate-protein linkage region in a small proportion of the HS chains (residual HS chains then lacking GlcNH₂ residues, since the HS sample contains on average 0.5 GlcNH₂ units per chain). In contrast, HNO₂-pH 3.9 treatment of aortic HS resulted in a marked depolymerization of the HS chains, indicating the presence of at least one free amino group per HS chain, located either toward the carbohydrate-protein linkage region, or in more peripheral parts of the polysaccharide.

The HS sequences located upstream and downstream of the GlcNH₂ residues (i.e. toward the non-reducing and reducing end, respectively) were then structurally characterized (summarized in Fig. 9; Paper II). Upstream sequences were defined following deamination at the sites of GlcNH₂ residues (HNO₂-pH 3.9), and radiolabeling of cleavage products. The resulting [HexA-GlcNAc/NS]₀⁻HexA-[³H]aManᵦ products were fractionated into three size classes, ≥6-mer, 4-mer and 2-mer, by Sephadex G-15 gel chromatography. The 2-mer fraction, originating from -GlcNH₂-HexA-GlcNH₂- motifs in the native HS chain, was subjected to disaccharide compositional analysis, while the 4-mer and ≥6-mer fractions, and individual 6-12-mers, were further subjected to HNO₂-pH 1.5 treatment (cleavage at N-sulfated GlcN units), and the products analyzed for size, HexA identity and possible O-sulfation.

To study downstream sequences we first subjected HS samples to HNO₂-pH 1.5/NaB₃H₄ treatment (radiolabeling at the sites of GlcNS residues), and isolated the resulting [HexA-GlcNH₂/NAc]₀⁻HexA-[³H]aManᵦ products. Oligomers ≥4-mer were fractionated into 4- to ≥18-mers, and each size class thereafter separately treated with HNO₂ at pH 3.9 in order to release the HS structures located downstream of any GlcNH₂ unit, i.e. found in -GlcNH₂-
[HexA-GlcNAc]$_n$-HexA-GlcNS- motifs in the intact HS chain. The cleavage products were quantified and identified.

Taken together, the analysis of upstream and downstream sequences showed that most of the GlcNH$_2$ residues in aortic HS are flanked on both sides by one or more N-acetylated disaccharide unit. About 10% of the free amino groups are found immediately adjacent to each other, 25% are joined by fully N-acetylated stretches of variable length, and 30% of upstream, as well as downstream sequences, contain one single N-acetylated disaccharide unit between the GlcNH$_2$ reference point and the nearest N-sulfated unit. Only 20-30% of the upstream and downstream sequences are linked to the GlcNH$_2$ residue via an N-sulfated disaccharide unit. Analysis of renal HS gave similar results, although that sample showed a higher proportion (20%) of clustered GlcNH$_2$ residues. We therefore conclude that the GlcNH$_2$ residues are mainly located in the less modified NA/NS-domains, consisting of alternating N-acetylated and N-sulfated GlcN units, or in the N-acetylated domains in HS, completely lacking O-sulfation and IdoA residues. However, a few GlcNH$_2$ residues might be found in the highly modified NS domains as well, and even be 6-O-sulfated, as shown by the analysis of -GlcNS-HexA-GlcNH$_2$- sequences.

Identification of the HexA units located immediately adjacent to the GlcNH$_2$ residue showed that the upstream HexA found in -GlcNAc-HexA-GlcNH$_2$- sequences is invariably a GlcA, which is in accordance with the biosynthetic "rule" that HexA epimerization requires N-sulfation of the neighboring upstream GlcN unit. Thus, the presence of an adjacent downstream GlcNH$_2$ unit does not change this constraint. The majority of HexAs found in -GlcNS-HexA-GlcNH$_2$- position were also identified as GlcAs, although IdoA units could be detected as well. In contrast, the nearest downstream HexA neighbor was invariably GlcA, including that found in -GlcNH$_2$-HexA-GlcNS- sequences. This indicates that the GlcNH$_2$ residue has never been N-sulfated, suggesting that the mechanism of GlcNH$_2$ formation involves incomplete action (N-sulfation) of the NDST enzyme, see “Discussion”.

N-Unsubstituted glucosamine residues in heparan sulfate from human cerebral cortex – Relation to Alzheimer’s disease? (Paper III)

The possible connection between GlcNH$_2$ and prion disease (GlcNH$_2$ is found in an HS epitope that is recognized by the prion lesion-staining antibody 10E4) made us wonder if GlcNH$_2$ units have any role(s) in other amyloid disorders, such as AD. Positive 10E4-staining has been demonstrated also for senile plaques in AD brain [190]. In Paper III, we...
therefore compared the content and location of GlcNH₂ units in cerebral HS from an AD patient and one control subject. We also investigated the role of GlcNH₂ units in binding of cerebral HS to fibrillar Aβ(1-40).

To determine the content of GlcNH₂ residues, we applied the same method as used before (Paper II). The two HS samples were subjected to deaminative cleavage at pH 3.9, resulting in specific attack on N-unsubstituted GlcN units, followed by ³H-labeling of the cleavage products. HS from AD cerebral cortex generated about twice as much radiolabeled products as the control sample, corresponding to a GlcNH₂ content of 4% and 2.5% (of total disaccharide units) in AD and control HS, respectively. Estimates of the molecular weights (both HS samples 45 x 10³) were used to calculate the average number of GlcNH₂ residues per polysaccharide chain: 4 in AD HS and 2.5 in control HS.

The structural analyses performed in this study were confined to sequences located upstream of the GlcNH₂ residues (Fig. 4; Paper III). As in Paper II, the sequences were excised and end-labeled by HNO₂-pH 3.9/NaB³H₄ treatment, and separated into 2-mer, 4-mer and ≥6-mer pools by gel chromatography. The proportions of cleavage products were about the same for the two HS samples: 5-10% of 2-mers, 30-40% of 4-mers, and 60% of ≥6-mers. The 2-mer fraction, representing -GlcNH₂-HexA-GlcNH₂- sequences in the intact HS chain, was subjected to disaccharide compositional analysis. In both AD and control samples the major 2-mer component was unsulfated GlcA-[³H]aManR. The 4-mer fraction was treated with HNO₂ at pH 1.5 in order to differentiate GlcNH₂-HexA-GlcNS-HexA-GlcNH₂- and GlcNH₂-HexA-GlcNAc-HexA-GlcNH₂- sequences, present in the native HS chain. Analysis by gel chromatography showed that only a minor fraction (~5% and 10% in control and AD samples, resp.) of the 4-mer was cleaved, demonstrating a much higher prevalence of the latter sequence. HNO₂-pH 1.5 treatment of the ≥6-mer fraction also showed a predominance of GlcNAc over GlcNS as the nearest upstream GlcN neighbor. Although N-sulfated GlcN units could be localized to various internal positions of the ≥6-mers, a significant proportion of extended sequences (>14-mer) were still intact after pH 1.5 deamination of either sample. Since extended N-acetylated sequences are typically found adjacent to the polysaccharide-protein linkage region [214], this finding suggests that most of the GlcNH₂ residues in cerebral HS are located toward the reducing-terminal domain of the HS chain.

Due to shortage of material we could not use colorimetric HexA analysis for detection of unlabeled cleavage products following HNO₂-pH 3.9 deamination. Instead HS chains were radiolabeled using a new labeling strategy based on limited 6-O-³⁵S-sulfation of HS chains using recombinant 6-O-sulfotransferase. In contrast to other labeling techniques, such as N-[³H]acetylation, this method leaves the GlcNH₂ units intact. Gel chromatography of ³⁵S-labeled HS chains before and after HNO₂-pH 3.9
showed only a minor decrease in size of the intact $[^{35}S]$HS, thus demonstrating that most of the GlcNH$_2$ units are indeed found in a region close to the reducing-terminal domain. As in the other structural analyses performed, no major difference between AD and control samples could be detected. Thus, we concluded that AD and control cerebral HS have similar GlcNH$_2$ distribution patterns.

To assess the role of GlcNH$_2$ residues in binding of HS to A fibrils, both AD and control cerebral HS were $^{35}$S-labeled, using recombinant 6-O-sulfotransferase, and thereafter tested in binding to fibrillar A (1-40). Quantification of bound HS species, using the nitrocellulose filter trapping technique, showed that AD and control samples bound to A in similar dose-dependent and saturable manner. Conversion of GlcNH$_2$ into GlcNAc residues by chemical N-acetylation had no significant effect on A fibril binding. We therefore concluded that GlcNH$_2$ residues are not important to the interaction between cerebral HS and A fibrils.

A tetrasaccharide determinant for heparan sulfate antibody 10E4 engineered from the polysaccharide of Escherichia coli serotype K5 (Paper IV)

This project was performed in collaboration with the research group of Prof. Ten Feizi (Imperial College School of Medicine, Harrow, UK). In an earlier study, Feizi and co-workers identified a GlcNH$_2$-containing HS epitope, $\Delta$UA-GlcNH$_2$-HexA-GlcNAc, that is recognized by the monoclonal antibody 10E4 [186]. Both the GlcNH$_2$ and the neighboring upstream 4,5 unsaturated HexA unit (induced by lyase cleavage) were shown to be important for antigenicity. Since the 10E4 antibody has been shown to specifically stain the prion lesions in the brains of scrapie-infected mice, as well as the senile plaques found in human AD brain, additional information about the structural requirements for HS-10E4 binding would be of great interest. In paper IV we describe the continued work on characterizing the 10E4-binding epitope(s).

First, a low-sulfated fraction of porcine intestinal mucosa HS (HS-1) was subjected to deaminative cleavage at pH 3.9, in order to destroy GlcNH$_2$ residues. HNO$_2$-pH 3.9 treated and untreated control HS were then immobilized in microwells and tested for 10E4 antigenicity. The 10E4 antibody bound well to the control HS, while binding to the deaminated HS was reduced sixfold, but not abolished. Nitrous acid treated HS-1 was consistently less active than the untreated polysaccharide as an inhibitor of the binding of 10E4 to immobilized HS-1, requiring approximately a three times higher concentration for equivalent inhibition. These results indicate that, in addition to the HS-epitope(s) that are destroyed by deamination, HS-
1 contains a minor proportion of 10E4-binding sequences that lack GlcNH₂.
In contrast, binding of another HS-antibody, JM-403, known to be critically
dependent on GlcNH₂ residues for HS recognition [43], was completely
abolished by the HNO₂-pH 3.9 treatment.

We next investigated binding of 10E4 and JM-403 to immobilized
partially N-deacetylated K5 polysaccharide (composed of repeating
[GlcA 1,4-GlcNAcα1,4] disaccharide units in the native form). In the case
of 10E4 no binding signals were detected at all, neither could N-deacetylated
K5 inhibit binding of 10E4 to microwells with immobilized HS-1. JM-403,
on the other hand, bound to the N-deacetylated K5 very strongly, and the
modified K5 polysaccharide was a potent inhibitor of JM-403-HS-1
interaction.

Since the 10E4-positive GlcNH₂-containing tetrasaccharide, described in
the previous study, was identified using the neoglycolipid technology, we
wanted to test if oligomers of N-deacetylated K5 could be recognized by the
10E4 antibody, using this technique. Therefore, the polysaccharide was first
partially fragmented, either by heparin lyase III treatment, or by restricted
HNO₂-pH 3.9 deamination. Resultant tetrasaccharides (subfractionated and
analyzed by mass spectrometry) were then converted into fluorescent
neoglycolipids and resolved by high-performance TLC. Overlay with 10E4
antibody identified a 10E4-positive tetrasaccharide, ΔUA-GlcNH₂-GlcA-
GlcNAc, generated by lyase-treatment and identical with the previously
described HS-1 tetrasaccharide. However, neither of the tetrasaccharides
obtained by HNO₂-pH 3.9 treatment, including GlcA-GlcNH₂-GlcA-aManR,
showed any positive 10E4 staining. Since the lyase-derived tetrasaccharide
ΔUA-GlcNAc-GlcA-GlcNAc also was negative, we concluded that the
binding of 10E4 to the ΔUA-GlcNH₂-GlcA-GlcNAc sequence is dependent
on the presence of both a GlcNH₂ residue and a HexA unit that is
unsaturated (ΔUA), and hence is artificial. However, since HNO₂-pH 3.9
treatment of HS-1 resulted in a substantial decrease in 10E4 binding, this
finding does not rule out the possible involvement of GlcNH₂ in 10E4
recognition of the native HS epitope(s), see “Discussion”.

Conclusions

• The minimal A-binding site in human cerebral cortex HS is found
  in NS-domains of 6-mer size.

• Binding of A monomers requires N-, 2-O- and 6-O-sulfation of
  the HS, while binding of A fibrils depends on N- and 2-O-
sulfation only.
• The interaction between HS and Aβ fibrils does not involve N-unsubstituted GlcN units in HS.

• HS domains binding to Aβ fibrils also bind to FGF-2, indicating common binding sites for the two ligands in cerebral cortex HS.

• Aβ protofibrils do not interfere with FGF-2-induced MAPK signaling in NIH 3T3 fibroblasts.

• The content of N-unsubstituted GlcN residues in HS varies between different tissues and is generally low (found in 0.7-4% of total disaccharides).

• AD may be associated with an increased number of free amino groups in cerebral HS, although we cannot exclude that the higher GlcNH₂ content observed in HS from AD cortex, compared to the control, simply reflects inter-individual variation. A larger number of samples needs to be analyzed.

• GlcNH₂ residues are mainly found in poorly modified sequences toward the polysaccharide-protein linkage region, but can also be located in NA- or NA/NS-domains in more peripheral parts of the HS chain. GlcNH₂ is rarely found in NS-domains.

• The observed binding between the 10E4 antibody and the saccharide sequence ΔUA-GlcNH₂-GlcA-GlcNAc, dependent on GlcNH₂, requires the upstream HexA residue to be 4,5 unsaturated, and thus is artificial.

• The role of GlcNH₂ in 10E4-recognition of the native HS epitope(s) is unclear.
Discussion

Heparan sulfate in Alzheimer’s disease

A substantial amount of experimental data supports the idea of HS as an important player in AD pathology. However, the relation of HS to the disease is complex. Being ubiquitously expressed the polysaccharide might participate in various pathophysiological events characteristic of the disease, but also in counteracting neuroprotective pathways. For this reason it is easy to get confused by the sometimes contradictory findings described in the AD literature, some implicating an AD-promoting role, others a neuroprotective role, of HS, depending on which experimental system (cell-free, cell culture, or in vivo system) that was used, and if the HS under study was soluble or cell bound. In order to correctly rationalize the experimental data into a specific role (or roles) for HS, it is important to be aware of all potential effects of HS.

Many of the putative roles of HS in AD appear to involve direct interaction with Aβ peptide. In our study (Paper I) the requirements for Aβ binding was investigated in more detail. Binding was tested in vitro to two forms of Aβ: monomers and fibrils. Binding to Aβ monomers is known to occur only at low pH (≤5), due to the requirement for protonated His^{13} and His^{14} residues in the peptide [208], while binding to fibrils readily occurs at physiological pH (7.4). Incubation of HS and peptide under these different pH conditions might therefore reflect two separate in vivo situations: interaction between HS and Aβ monomers in the acidic milieu of intracellular compartments (e.g. endosomes), possibly initiating Aβ fibrillization, and interaction between HS and Aβ fibrils in the neutral extracellular space, resulting in enhancement of Aβ fibrillization, and stabilization of formed Aβ aggregates. Our analyses showed that the minimal binding site interacting with Aβ fibrils is found in N-sulfated hexasaccharide domains containing critical 2-O-sulfated IdoA residues, while binding to monomers requires, in addition, 6-O-sulfation on the GlcN residues. Thus, the low-pH interaction with Aβ monomers can be distinguished from the interaction with Aβ fibrils by the requirement for 6-
O-sulfate groups. If this observed difference in structural requirements plays any role in AD pathology is not known. Compositional analysis of HS from human control and AD cerebral cortex did not reveal any significant difference in O-sulfation [65]. On the other hand, if AD lesions contain HS chains with unique sulfate patterns, those HS structures would easily have escaped detection since the samples included all HS populations present in cerebral cortex. Unfortunately, due to restricted supply of human AD brain material we have not been able to isolate and analyze the structure of HS(s) found specifically in plaques or other AD lesions.

The dependence on 2-O-sulfation, but not 6-O-sulfation, in binding to HS has also been demonstrated for FGF-2 [209-211]. Since FGF-2 is known to have neuroprotective functions, we investigated the possibility that Aβ fibrils and FGF-2 share a common binding site in HS. Indeed, we found a subpopulation of HS sequences that bound to both protein ligands (Paper I), indicating that neuroprotective and neurodegenerative pathways might converge via binding to the same HS sequence, possibly resulting in competition between the opposing pathways. One potential site of competition between Aβ and FGF-2 in vivo could be at the cell surface of neurons. Hypothetically, Aβ might inhibit the HS-enhanced binding of FGF-2 to its receptor, and thereby prevent the induction of intracellular signaling cascades important for neuronal survival. Using NIH 3T3 fibroblasts as a model system, we tested the effect of Aβ protofibrils, implicated as the most toxic Aβ components, on FGF-2 receptor binding and induction of the MAPK signaling pathway (unpublished data). Although this pathway is known to be activated by FGF-2, resulting in increased transcription of genes encoding various neuroprotective proteins [200], we failed to detect any inhibitory effect on FGF-2-induced MAPK phosphorylation following addition of Aβ protofibrils to the cell culture. There could be several explanations for the Aβ insensitivity of this system. First, FGF-2 is able to induce FGF receptor activation also in HS-deficient cells, although less efficiently than in cells expressing HSPGs [215]. If, in our system, the basal HS-independent FGF-2 signaling induces a strong MAPK phosphorylation per se, moderate changes induced by Aβ might not be detected. Treatment of cells with heparitinase, or with chlorate to inhibit HS sulfation [216], before addition of Aβ and FGF-2 would in this case have been informative, but was not performed. Second, Aβ protofibrils could affect so far unidentified signaling cascades, not involving MAPK, that are induced by FGF-2 and result in transcriptional activation. Finally, as shown in Paper I, FGF-2 may interact with HS sequences that do not bind to Aβ. In addition, we cannot exclude that Aβ-HS interaction is weaker than the FGF-2-HS interaction, and Aβ is thus not able to compete out binding of FGF-2 to HS.

The lack of Aβ-induced inhibition of the FGF-2 induced MAPK pathway does not rule out an inhibiting effect of Aβ protofibrils on other HS-mediated functions. The mechanism(s) behind Aβ-induced oxidative stress
and mitochondrial dysfunction in synaptosomes are still unknown [200], but could perhaps involve competition between Aβ and FGF-2, or other proteins such as nerve growth factor and brain-derived neurotrophic factor (reviewed in [107, 217]), for binding to a common site in HS. However, the nerve growth factor does not appear to be dependent on HS for its activity, and any possible relation between the brain-derived neurotrophic factor and HS has yet not been described. The regulatory role of HS in LTP is then perhaps a more likely target for competition between Aβ and HS ligands. Aβ protofibrils inhibits LTP [154], but the mechanism behind the inhibition is not known. Several of the molecules that are responsible for synaptic plasticity, including the neuronal cell adhesion molecule (NCAM), heparin-binding growth associated molecule (HB-GAM), and FGFs, are either dependent or modulated by HS [206, 207]. Therefore, a possible explanation for the Aβ-induced inhibition of LTP could be that Aβ competes out the binding of either of these ligands to HS.

N-Unsubstituted glucosamine residues in native heparan sulfate

With the recognition of N-unsubstituted GlcN units as true structural components of native HS about ten years ago [43, 44], and more recent data associating these units with pathophysiological phenomena such as prion disease and herpes simplex invasion, the GlcNH2 residue has evoked great interest in the HS research field. However, since the unit is rare and therefore not easily detected, the structural information regarding GlcNH2-containing sequences has been scarce. In papers II and III we therefore undertook comprehensive structural studies of GlcNH2-containing HS sequences found in native tissues. Quantification based on HNO2-pH 3.9/NaB3H4 treatment of HS samples indicated GlcNH2 proportions of 0.7-4% of total GlcN, corresponding to an average number of ~0.5-4 GlcNH2 residues per HS chain. This is in fair agreement with the values (1.2-7.5%) determined earlier for various bovine and porcine tissues, using the o-phthaldialdehyde assay [45].

Size exclusion chromatography of HS chains before and after HNO2-pH 3.9 treatment indicated that most of the GlcNH2 residues are located toward the carbohydrate-protein linkage region. Such a location has also been suggested for GlcNH2 residues found in HS chains attached to glypican-1 [127]. However, in aortic HS, with the highest GlcNH2-content, free amino groups were also found in more peripheral positions of the chain.

Structural analysis of HS sequences located upstream and downstream, respectively, of the GlcNH2 residues, pointed to an enrichment of free amino

45
groups in NA/NS domains, and in N-acetylated domains, consistent with a location toward the polysaccharide-protein linkage region. This finding supports in part the previously proposed occurrence of GlcNH$_2$ residues in transitions zones between modified (N-sulfated) and unmodified (N-acetylated) saccharide regions [43, 45, 127]. However our data does not support the notion that GlcNH$_2$ residues are mainly found downstream of highly modified, IdoA-rich sequences, and upstream of less modified saccharide regions [45]. Only a minor proportion of the GlcNH$_2$ residues was flanked by N-sulfated disaccharide units, indicating that free amino groups are scarce in NS-domains. Notably, though, some of these GlcNH$_2$ residues were found in -IdoA2S-GlcNH$_2$- sequences, that are implicated as acceptor sites for the 3-O-ST 3A and 3-OST 5 enzymes [35].

The mechanism of GlcNH$_2$ formation is unclear. The invariant presence of an unmodified GlcA residues as the closest downstream GlcNH$_2$ neighbor (Paper II), could indicate that the GlcNH$_2$ unit has never been N-sulfated, and thus is formed in an N-deacetylation reaction (epimerization of GlcA into IdoA requires N-sulfation of the upstream GlcN neighbor). The incomplete action of the NDST enzyme during biosynthesis would then be the most likely explanation. Notably, one of the NDST isoforms (NDST-3) has a much higher relative N-deacetylase activity compared to the other isoforms [72]. However, it cannot be excluded that N-acetyl groups are selectively removed at a later stage, either by a so far unidentified N-deacetylase enzyme, or by one of the NDST isoforms [78]. The action of an endosulfamidase, removing N-sulfate groups from HexA-GlcNS-GlcA sequences, might also be possible. However, while the existence of HS-acting 6-O-endosulfates (Sulf1 enzymes) has been proven [75-77], no HS endosulfamidase has yet been discovered.

Any role(s) for free amino groups in Alzheimer’s or other amyloid diseases?

The functional aspect of GlcNH$_2$ residues is still an unexplored area. Apart from the potential involvement in HSV-1 infection [90], and possibly an indirect role in glypican-1-mediated ligand transport [128], there are yet no examples of biological functions ascribed to GlcNH$_2$-containing HS epitopes. However, the identification of GlcNH$_2$ in an HS epitope recognized by the 10E4 antibody [186], previously shown to specifically stain scrapie lesions in murine brain [185], could point to a third, intriguing, role of free amino groups, that is in prion (and related amyloid) disease. In paper IV we
therefore continued the characterization of the HS epitope recognized by 10E4. Using partially N-deacetylated K5 polysaccharide we could isolate a 10E4-positive, heparinase III-derived, tetrasaccharide, \( \Delta UA-\text{GlcNH}_2-\text{GlcA-GlcNAc} \), identical to the earlier identified saccharide sequence [186]. Surprisingly, though, a corresponding tetrasaccharide, GlcA-GlcNH2-GlcA-aManR, produced by HNO2-pH 3.9 treatment of N-deacetylated K5, had no 10E4 antigenicity. The most likely explanation for this finding is that the combination of a 4,5 unsaturated HexA unit (\( \Delta UA \)) and a neighboring GlcNH2 residue forms an artificial epitope that is recognized by the 10E4 antibody. This could also explain the lack of antigenicity of partially N-deacetylated full-length K5. However, binding of 10E4 to full length HS-1 occurred readily, and was significantly reduced by HNO2-pH 3.9 treatment, showing that the GlcNH2 residue still might be a component of at least some of the "true" 10E4-binding epitopes in native HS (epitopes lacking GlcNH2 probably also exist, since pH 3.9-deamination did not abolish 10E4 binding). Since HS-1, in contrast to the 10E4-negative N-deacetylated K5 saccharide, is sulfated, such HS epitopes probably include one or more N-and O-sulfate groups in addition to the GlcNH2 unit(s). Previous findings actually indicate that N-sulfated structures are prerequisite to 10E4 recognition [56]. Moreover, the antibody reacts poorly with heparin [56], pointing to a location of 10E4-binding epitopes in less modified (NA/NS-) domains of the HS chain, that should contain one or more GlcNH2 residues (Paper I and III). However, although present in native antigenic HS epitopes, the GlcNH2 unit(s) must not necessarily contribute to 10E4-HS binding per se. The observed HNO2-pH 3.9-effect could rather reflect that GlcNH2 units are required for the correct positioning of the residual epitope structures. Further work is needed to clarify the role(s) of GlcNH2 in 10E4 recognition.

In case the free amino group(s) actually are crucial for 10E4 antigenicity, what would this mean in correlation to prion pathology? One possibility would be that the GlcNH2 unit is involved in the binding of HS to the protein causing the disease, \( \text{PrP}^{\text{Sc}} \). To the best of my knowledge, the binding between this pathogenic form of PrP and HS has not been studied, but three independent HS binding sites in the normal, nonpathogenic, \( \text{PrP}^{\text{C}} \) have been identified [175]. The binding characteristics of those sites were not studied in detail, but binding of HS/heparin required 2-O-sulfation, and at least one of the PrP sites was shown to be independent on 6-O-sulfation. Whether GlcNH2 residues are needed for PrP binding is however not known. Likewise, no experiments have been pursued to investigate a possible role for free amino groups in the presumed HS-dependent conversion of \( \text{PrP}^{\text{C}} \) into \( \text{PrP}^{\text{Sc}} \). Interestingly, though, a heparinase III-sensitive (but not heparinase I-sensitive) activity of cellular HS in PrP\( ^{\text{Sc}} \) biogenesis was recently demonstrated [182]. Since GlcNH2 units are generally found in less modified (NA or NS/NS) HS domains, including that in the polysaccharide-protein linkage region (Paper II and III), free amino groups were most probably
present in the remaining, heparinase I-resistant, part of the HS chains, shown to mediate the cellular formation, and/or metabolism of PrP\textsuperscript{Sc}.

Do GlcNH\textsubscript{2} residues have any role(s) in other amyloid disorders, such as AD? Positive 10E4 antibody staining has been reported also for the senile plaques present in the AD brain [218], and, moreover, in our analysis of the GlcNH\textsubscript{2} content in HS from human cerebral cortex (Paper III), we noted a higher proportion of GlcNH\textsubscript{2} residues in HS from AD cortex (4% of total GlcN), compared to normal control HS (2.5%). Whether this difference reflects an AD-specific increase in GlcNH\textsubscript{2} content, or just inter-individual variation is not known; more samples need to be analyzed. In any case, the free amino groups do not appear to have any role in binding to A\textbeta. HS from AD and control cerebral cortex bound equally well to A\textbeta fibrils, and eliminating GlcNH\textsubscript{2} units by chemical N-acetylation did not affect the binding (Paper III). Thus, the presence of 2-O sulfate groups seems to be the major prerequisite for HS-A\textbeta fibril interaction (Paper I). The possible involvement of GlcNH\textsubscript{2} in HS interaction with other A\textbeta forms, such as monomers or protofibrils, were not tested. Neither is it known if GlcNH\textsubscript{2} residues are involved in binding of HS to other senile plaque components, such as apolipoprotein E, serum amyloid P-component, or APP [219]. Notably, HS binding to the \beta-secretase BACE1 (one of the APP processing enzymes), is dependent on 6-O-sulfation, but not 2-O-sulfation, and is enhanced if N-sulfate groups are exchanged for N-acetyl groups [193], indicating a role of less modified HS domains (NA/NS) in the interaction with BACE1. Considering the location of GlcNH\textsubscript{2} to such HS domains (Paper II), it is tempting to speculate as to whether GlcNH\textsubscript{2} has any role in HS-regulated APP processing by BACE1. Possibly, the HS-mediated inhibition of \beta-secretase cleavage of APP is carried out by HSPGs present within lipid rafts. The dynamic interaction between BACE1 and APP was recently demonstrated at such site [220], which also harbors syndecans [221], and most likely glypicans [21]. Since the HS chains of glypican-1 indeed have GlcNH\textsubscript{2} residues [127], it would therefore be interesting to investigate whether these units have any role in the regulation of APP processing.
Future Perspectives

Since the start of this thesis work in the late 90s, a number of new analytical tools have emerged improving both the detection and structural characterization of GAGs. Purified HS oligomers can be sequenced using direct-sequencing strategies involving partial chemical and enzymatic cleavage of the oligomers, followed by analysis of products by either electrophoresis [222], HPLC [223], or mass spectrometry [224]. Moreover, the classical methods and techniques used for purification, quantification, and compositional analysis of full-length GAGs have been rationalized, allowing more rapid and efficient screening of very small amounts of samples. For example, only 0.1 µg of saccharide is required for complete compositional analysis of HS, including determination of HS/CS proportions (Johan Ledin, pers. commun.; for method see [225]). Having access to these new or refined techniques it would be interesting to resume the studies on cerebral HS involved in AD (Paper I). For example, Aβ-binding HS structures could be sequenced, and, possibly, plaque-specific HS could be isolated and analyzed in order to see if it has any specific characteristics that differ from cerebral HS in general. In addition, rare HS structures, such as GlcNH₂, should be more easily detected in the future, as well as novel, so far not discovered, HS structures, if such exist.

The research on HS biosynthesis has been intense during the last decade. The biosynthetic enzymes have been cloned, and genetic studies of cultured cells and various organisms, including humans, have given new insights into enzyme specificities, as well as the organization of the biosynthetic apparatus. However, still not much is known about the regulation of the enzymes, their topographical distribution in the Golgi, or their relative concentration. Furthermore, the demonstration of enzyme activities modifying the HS chain ”post-biosynthetically”, such as the Sulf-1 6-O-endosulfatase, has made the mechanisms of HS biosynthesis even more elusive. How are specific HS structures generated in general? What are the mechanism(s) of GlcNH₂ formation? These and many other intriguing questions still remain to be answered.

The functional aspects of HS have been intensively explored during the last years. Genetic manipulation of model organisms, such as knock-outs of the biosynthetic enzymes or the core protein of HSPGs, have pointed out many novel functions for HS, especially during development, and have also
given some clue about HS structure-function relationships in in vivo processes. However, in order to identify and get more detailed structural information about HS motifs involved in a certain biological or pathophysiological process, other strategies must be used. One approach is to produce libraries of size-defined and structurally characterized HS oligomers, that can be used for protein-binding screening or tested in various biological assays. Although straightforward, this approach has its limitations due to the heterogenous nature of HS, which makes the establishment of such libraries both time-consuming and difficult. A better option would then be to use chemically synthesized, and thereby structurally defined, HS oligomers. To date no such oligomers are commercially available, but in the future they will most likely be, since methods for both chemical synthesis [226] and enzymatic in vitro synthesis [227] of specific HS epitopes have already been worked out. Such structure-defined HS epitopes would also be valuable in the production of novel HS-specific phage-display antibodies. This type of antibodies have been demonstrated to be a useful tool for detection of differential expression of HS structures in tissues and organs [58, 59]. For example, staining of neuromuscular junctions indicates a role of specific HS structures in myogenesis and synaptogenesis in the muscle [58]. It would therefore be interesting to know if these antibodies also can reveal distinct HS expression patterns in neuronal synapses, and if those are affected by amyloid disease such as AD.

With increased knowledge about the HS structure-function relationships, the development and application of novel HS-based therapeutics become more feasible. Provided that the candidate HS structure is specific (prerequisite to avoid side effects) it could either be used unmodified, or work as a lead for the generation of HS mimetics. In the treatment of amyloid disease, there are already examples of HS mimetics that have documented anti-amyloidotic effects. Chemically modified dextran sulfates have been shown to have anti-prion activity both in vitro and in vivo [228], and modified sugar-precursors affecting HS biosynthesis inhibit both AD-associated and inflammation-associated amyloidosis in vivo [229]. However, further refinement of these developments is needed before they can be applied as therapeutics in humans.
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52
References


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