EXPRESSION AND FUNCTION OF PROTEOGLYCANS IN THE NERVOUS SYSTEM

by

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B.S., Chemistry
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Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of

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in Biology

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Abstract

Proteoglycans (PGs) have important functions in a variety of tissues; little is known, however, about PG expression and function in the nervous system. This thesis assesses possible functions of PGs in the nervous system by examining the neural expression of individual PG core proteins (glypican, cerebroglycan, and decorin). Glypican mRNA and protein expression was demonstrated in the nervous system. In situ hybridization experiments showed that the expression of glypican in the nervous system is regulated during development, and is expressed in a different pattern than is the glypican homolog cerebroglycan. Immunohistochemistry demonstrated that both glypican and cerebroglycan are expressed on axons, and that cerebroglycan is polarized to axons in neurons of the dentate gyrus. The pattern of glypican expression during development suggests that it is involved in specific functions in the nervous system; furthermore, the differences in expression between glypican and cerebroglycan suggest different roles for the two core proteins in the nervous system.

The expression and binding properties of the chondroitin sulfate PG decorin were also studied. Decorin was shown to be expressed in the floor plate of the developing mouse spinal cord, and was shown to bind netrin-1, a floor-plate derived chemotropic factor for axon growth; the decorin core protein was suggested to be involved in this interaction. The affinity of netrin-1 for heparin was similar to that of netrin-1 for decorin. These data indicate that decorin, and some heparan sulfate PGS, may interact with netrin-1 in the developing spinal cord. The expression of decorin in the floor plate at a time when netrin-1 may be promoting axon growth suggests that decorin may play a role in nervous system development.

Thesis Advisor: Dr. Arthur D. Lander
Title: Associate Professor of Biology and Brain and Cognitive Sciences
Dedication

This thesis is dedicated to the memory of Joshua Lapin
Acknowledgments

This work would not have been possible without the support and guidance of my thesis advisor, Arthur Lander. I have learned a great deal from working with Arthur, and have enjoyed the excitement and enthusiasm he brings to science.

I would also like to thank the other members of my thesis committee, Frank Solomon, Bob Rosenberg, James San Antonio, and Tyler Jacks, for their helpful suggestions and comments. Ralph Sanderson, Renato Iozzo, Marc Tessier-Lavigne, Michael Galko, and Brigid Hogan have collaborated with me on various aspects of this work, and have been generous with their materials and their time. In addition, I would like to thank Ann Graybiel, Sonal Jhaveri, and Mriganka Sur for helpful advice on anatomy, Clive Wilson, Jerry Yin, John J. Schwartz, and Nicholas Shworak, for advice on molecular biology and biochemistry, and Rosario Moratalla, whose advice and help on in situ hybridization has been invaluable.

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Finally, I wish to thank my partner, Kristen Fredricks, who has made it all worthwhile.
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Chapter 1: Proteoglycan expression and function in the nervous system.
1. Introduction.

During brain development, cells must respond to a wide range of environmental cues in order to proliferate, differentiate, migrate to correct locales in the nervous system, and extend axons to appropriate targets, often over long distances. *In vivo* and *in vitro* studies have shown these processes to be dependent on a number of proteins.

The proteins underlying these processes are diverse, and fall into several classes. These classes include extracellular matrix molecules, growth factors, cell adhesion molecules, and proteases and their inhibitors. Proteins within each of these classes interact with distinct receptors: extracellular matrix molecules interact with integrins, growth factors with receptor tyrosine kinases, cell adhesion molecules with other cell adhesion molecules, and proteases with protease inhibitors. Nevertheless, many of these proteins possess a common property: they can bind to linear polysaccharide polymers known as glycosaminoglycans (GAGs), and, most frequently, to a type of GAG known as heparin or heparan sulfate (Table 1) (for a review of heparin- or GAG-binding proteins, see Jackson et al., 1991; Lander, 1994). For this reason, proteoglycans (PGs), covalent complexes of protein cores and GAGs, have the potential to participate in any process that involves one of these molecules, and hence, in development of the brain.

PGs have in fact been shown to have a range of functions (for review, see Lander, 1993). PGs can mediate cellular responses to growth factors and other proteins by acting as coreceptors. In other words, they can act as proteins whose binding to ligand is important in the interaction of that ligand with distinct high affinity receptors in order to produce a physiological response (Bernfield et al., 1992). PGs can be thought of as coreceptors for ligands such as fibroblast growth factors (FGFs); they can also act as coreceptors in reactions of proteases with protease inhibitors, or in the homophilic binding of NCAM. As components of cell surfaces, PGs can also directly mediate cell adhesion, cell migration, and neurite outgrowth. As components of extracellular matrix, they can participate in matrix organization,
Extracellular matrix molecules
netrin-1/netrin-2
laminin
fibronectin
thrombospondin
tenascin
vitronectin
p30
amyloid β-protein
HB-GAM

Cell adhesion molecules
NCAM
L1
myelin-associated glycoprotein

Growth factors
FGF 1-9
PDGF
TGF-β
heregulins

Proteases and protease inhibitors
protease nexin I
thrombin
tissue plasminogen activator
urokinase plasminogen activator

Proteins involved in lipoprotein metabolism
Apo-E

Table 1. Some glycosaminoglycan-binding proteins of the nervous system.
and also provide cues for cell adhesion, cell migration, and neurite outgrowth.

It is thus apparent that PGs have the potential to play important roles in nervous system development. Many PGs have been cloned, and studies of the expression of individual PGs are now possible. It is apparent that most if not all of the major families of PGs are represented in the brain. In fact, some PGs exhibit remarkable spatial and temporal patterns of expression, suggesting specific roles in nervous system development and function. Further elucidation of the expression of individual PGs in the brain, and the relationship of that expression to other PGs and GAG-binding proteins, is necessary in order to deduce the functional roles of PGs. Furthermore, as many of the functions of PGs can be related to GAG structure, it is also important to understand the way in which individual neural PGs are processed, and, in addition, the ways in which the nervous system regulates GAG structure. Functional studies of PGs in in vitro assays have supported the idea that PGs are important for nervous system development and function, and further studies will be important for understanding their roles in vivo.
2. **Proteoglycan structure.**

PGs are proteins which possess covalently attached GAGs. Structural variability exists both at the level of the GAG and at the level of the protein core; the variability at both levels play a part in the diverse functions that PGs can have in nervous system development. In this section, I shall first review GAG structure and the role of GAG structure in protein-GAG interactions; I shall then discuss some general features of PG core proteins.

**Glycosaminoglycan (GAG) structure.** GAGs are linear polysaccharide chains that are composed of repeating disaccharide units. These disaccharide units consist of alternating amino sugars and hexuronic acids (or, in the case of keratan sulfate, alternating amino sugars and galactose residues). The polysaccharide, consisting of unbranched repeats of these disaccharides, is attached to the serine of a PG core protein by a xylose-galactose-galactose adaptor (with the xylose, sometimes phosphorylated, attached to the serine via an O-glycosidic bond). The exception to this is keratan sulfate, which, as discussed below, can be linked to proteins via N- or O-linked sugars. Once formed, the GAG is chemically modified by a series of deacetylations, N- and O-sulfations, and epimerizations. Depending on the identity of the monosaccharides of the disaccharide repeat, a GAG is defined as a heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, or hyaluronic acid (Fig. 1).

**Heparin/heparan sulfate (HS)** is defined as \([D\text{-glucuronic acid-}\beta(1\rightarrow4)-D\text{-N-acetylglucosamine-}\alpha(1\rightarrow4)]_n\), and is usually composed of 30-200 disaccharide units (for a review of heparin/heparan sulfate structure, see Gallagher et al., 1992; Lindahl, 1987). Once synthesized, HS chains can then be modified by N-deacetylation followed by N-sulfation, O-sulfation, and epimerization of glucuronic acid to iduronic acid. These modifications, however, are not evenly distributed. N-sulfated hexosamines occur in clusters, so that there
Figure 1. Glycosaminoglycan structure. $R = -H$ or $-SO_3$. $R' = -Ac$ (acetyl group) or $-SO_3$. 
Heparin/Heparan Sulfate

Chondroitin Sulfate/Dermatan Sulfate

Keratan Sulfate

Hyaluronic Acid
are clusters of GlcNSO$_3$ followed by stretches of GlcNAc$^1$. Both epimerization and O-sulfation are associated with N-sulfation, so both IdoA and IdoA(2S) are found in these clusters; GlcNAC(6S) is also associated GlcNSO$_3$. Hence, while there are potentially thousands of sequences possible in HS based on the types of modifications that can occur on monosaccharides, in reality the clustering of these modifications results in constraints on sequences present in HS.

Heparin is a highly sulfated version of HS synthesized by mast cells. Unlike HS, GlcNSO$_3$ is predominant (composing over 80% of hexosamines in the chain), and is present in clusters. Heparin is also heavily O-sulfated, (containing at least one O-sulfate per disaccharide) and has a higher percentage of di- and trisulfated disaccharides than does HS. The high IdoA content of heparin confers conformational flexibility on heparin, and may help explain why more proteins bind to heparin than to other GAGs (Casu et al., 1988).

**Chondroitin sulfate/dermatan sulfate (CS/DS)** is defined as [D-glucuronic acid-$\beta$(1$\rightarrow$3)-D-N-acetylgalactosamine-$\beta$(1$\rightarrow$3)]$_n$. Posttranslational modifications include 4- or 6-O-sulfation of N-acetylgalactosamine, 2-O-sulfation of uronic acid, and epimerization of the C-5 carbon of GlcA to form IdoA. DS is composed of the same disaccharide as CS, but DS has a high iduronate content. DS is defined empirically-a GAG is classified as DS if it can be degraded by chondroitinase ABC (which cleaves at GlcA or IdoA) but not chondroitinase AC (which cleaves at GlcA). CS can be degraded by both enzymes. This high IdoA content of DS should make it a more flexible polymer than CS (Casu et al., 1988). GAGs of this class may also have a domain structure, as some work suggests that IdoA(2S) and GalNAc(4S) occur in clusters (Maimone and Tollefson, 1990).

---

$^1$Monosaccharides are abbreviated as follows: GlcA, glucuronic acid; IdoA, iduronic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; GalNSO$_3$, N-sulfated galactosamine; GlcNAC, N-acetylglucosamine; GlcNSO$_3$, N-sulfated glucosamine. O-sulfations are designated in parentheses. For instance, iduronic acid sulfated on C-2 is abbreviated as IdoA(2S).
Keratan sulfate (KS) is defined as \([\text{D-galactose-} \beta(1\rightarrow3)-\text{D-N-acetylgalactosamine-} \beta(1\rightarrow3)]_n\) (for review, see Hounsell, 1989; Stuhlsatz et al., 1989). KS is synthesized on preexisting N- or O-linked sugars, and thus, unlike HS or CS which are only attached to serine residues, KS can be potentially be attached to any amino acid glycosylated with complex-type carbohydrates. KS is only modified by O-sulfation, which can occur only the C-6 of Gal and GalNAc. Furthermore, O-sulfation occurs in domains, so that, in order from the linkage region, a nonsulphated stretch of disaccharides is found, followed by a monosulfated stretch and then by a disulfated stretch. These domains vary in length depending on the source of KS.

Hyaluronic acid (HA) is defined as \([\text{D-glucuronic acid-} \beta(1\rightarrow3)-\text{D-N-acetylglucosamine-} \beta(1\rightarrow4)]_n\). It is found unattached to protein, and is unmodified after synthesis. Biophysical studies suggest that hyaluronic acid is a relatively rigid structure, as a result of intramolecular hydrogen bonding that can occur because hyaluronic acid is unmodified (Scott, 1989).

**GAG structure and GAG-protein interactions.** Traditionally, it has been assumed that most protein-GAG interactions have been relatively nonselective. Affinity was thought to be the result of nonspecific electrostatic interactions between highly negatively charged GAGs and clusters of basic amino acids in GAG binding proteins. While this may be true for certain proteins (see, for example, Olson et al., 1991), much recent evidence makes clear that most GAG-binding proteins bind different and specific carbohydrate sequences in GAGs. Consequently, the modifications of the basic polymeric structure of GAGs are crucial in determining which protein-binding sequences will be present in particular GAGs, and hence the functional abilities of that GAG.

Several lines of evidence support the idea that proteins bind to specific sequences within GAGs. Within a sample of HS derived from a given source, it is often possible to find a fraction of chains that bind with high affinity to a given GAG-binding protein and a fraction with low or no affinity. This is usually accomplished by fractionating the GAG on the basis of affinity. Consequently,
separate fractions of heparin have been isolated that have high
affinity for proteins such as antithrombin III, collagen, laminin, and
fibronectin (Lam et al., 1976; Lee and Lander, 1991; San Antonio et
al., 1993). Furthermore, heparin fractions which bind one protein
selectively do not necessarily bind a different protein selectively,
implying that proteins recognize distinct structural features of
heparin (San Antonio et al., 1993). For many proteins, high affinity
binding does not depend on chain length or levels of sulfation (as an
indicator of charge) (San Antonio, et al., 1993)². For example, HS
with low affinity for collagen is sulfated to a similar extent as HS
with high affinity for collagen (Sanderson et al., 1994). This low
affinity HS, however, contains less N-sulfate and 2-O-sulfate and
more 6-O-sulfate than high affinity HS. Thus, collagen may
recognize a specific structural feature in HS that involves N-sulfate
and 2-O-sulfate.

The binding sites of GAG-derived oligosaccharides with high
affinity for certain GAG-binding proteins have been sequenced, and
in some cases the contributions of certain modifications to high
affinity binding have been determined. Antithrombin III (AT)
binds to a specific pentasaccharide sequence present in this
subfraction of heparin chains. This pentasaccharide consists of the
sequence GlcNAc(6S)-GlcA-GlcNSO₃(3S6S)-IdoA(2S)-GlcNSO₃(6S)
(Fig. 2A) (Atha et al., 1984; Lindahl et al., 1984; Atha et al., 1985).
While some structural variability within this sequence can be
tolerated, it has been shown that certain modifications are crucial
for high affinity AT binding. These include the 6-O-sulfate of
residue 1 and the 3-O-sulfate of residue 3. The 3-O-sulfate of
residue 3 is a rare modification in heparin, and accounts for the
small fraction of chains that possess the antithrombin-binding
pentasaccharide sequence. Removal of the 3-O-sulfate drastically
reduces affinity for antithrombin.

Recently, binding sequences in heparin for FGF-1 (aFGF) and
FGF-2 (bFGF) have been proposed (Fig. 2B,C). Fragments of HS that

²High affinity binding may correlate with these parameters, however, as
longer or more highly modified chains are more likely to possess certain
binding sequences.
Figure 2. Protein binding sequences in glycosaminoglycans. 

A, Binding sequence in HS for antithrombin III (Atha et al., 1984; Lindahl et al., 1984; Atha et al., 1985). The modifications important for binding are in boxes. 


C, The structure of an HS disaccharide required for FGF-1 binding (Mach et al., 1993). 

D, The structure of a DS disaccharide required for heparin cofactor II binding (Maimone and Tollefson, 1990). The structures illustrated in B-D are found in the context of longer sequences of the minimum length necessary for binding.
A) Antithrombin III

B) FGF-2

C) FGF-1

D) Heparin Cofactor II
bind with high affinity to FGF-2 have a high content of IdoA(2S)-GlcNSO₃ (Habuchi et al., 1992; Turnbull et al., 1992). Fragments with weaker affinities had lower percentages of this disaccharide, even though they had the same levels of O-sulfation. The minimum size of the FGF-2 binding sequence seems to be 5 sugars (Maccarana et al., 1993). Consistent with these data, de-6-O-sulfated heparin bound FGF-2, while de-2-O-sulfated heparin did not (Guimond et al., 1993). The minimum heparin sequence needed to bind FGF-1 is a tetrasaccharide that contains with IdoA(2S)-GlcNSO₃(6S) (Mach et al., 1993). Unlike FGF-2, FGF-1 requires both 6-O- and 2-O- sulfate, while FGF-4 requires neither (Guimond et al., 1993). Thus, there seems to be distinct structural requirements within heparin for binding to FGF-1 and FGF-2, as well as other members of the FGF family.

GAG binding sequences for other proteins have been characterized. For example, heparin cofactor II has been shown to recognize a hexasaccharide in dermatan sulfate with the sequence IdoA(2S)-GalNAc(4S) (Maimone and Tollefson, 1990) (Fig. 2D). High affinity for collagen type I is associated with the presence of 4-O-sulfate (Sanderson et al., 1994).

Thus, the fact that a protein can bind a GAG does not mean that a protein will bind to that GAG with a high affinity. High affinity binding will depend on the presence of a particular binding site within that GAG, and the numbers of binding sites found within that GAG. A cell may regulate the types and numbers of particular binding sites within its GAGs by adjusting the levels of modification (such as sulfation or epimerization) of those GAGs. As a result of such regulation, the affinities of GAGs for particular GAG-binding proteins can depend on the synthetic source of the GAG. For example, HS isolated from epithelial cells has a higher affinity for collagen type I than does HS isolated from myeloma cells, and even different myeloma cells synthesize HS with different affinities for collagen type I (Sanderson et al., 1994). There is also heterogeneity in GAGs from a single source. Heparin, for instance, can be fractionated on the basis of affinity, as described above (Lee and Lander, 1991; San Antonio et al., 1993). Endothelial cells synthesize
both anticoagulantly active and inactive HS; the HS with high affinity for AT accounts for this activity (Marcum and Rosenberg, 1989).

The existence of heterogeneous populations of GAGs in the brain, which contains a multitude of cell types, is not unexpected. Such heterogeneity has been borne out by biochemical fractionation of HS from brain (Horner, 1991). In addition, immunostaining of nervous system tissues with antibodies that recognize specific structures on GAGs indicate that GAGs are nonuniformly distributed in the brain (Fujita et al., 1989; Watanabe et al., 1989; David et al., 1992b; Wang and Denburg, 1992; Fuxe et al., 1994; McAdams and McLoon, 1995). GAG structure may be used to control the function of proteins, such as FGFs, protease inhibitors, and extracellular matrix molecules, in the brain. It may also be that the nervous system uses GAG modification to allow cells in heterogeneous populations to respond differently to particular signals, as different cells in such a population may produce different GAGs which could have different binding specificities.

Core protein structure. The core proteins to which GAGs are attached are encoded by a variety of genes (Table 2). It is apparent that there is no obvious domain or amino acid sequence that specifies a PG, beyond those sequence determinants that direct GAG attachment. I will discuss what is known about signals in PG core proteins for attachment of HS and CS chains. All PGs (with the exception of KSPGs) must contain these signals. The structure of individual core proteins shall be discussed later in relation to PG core protein families and their expression in the nervous system.

GAG Acceptor Sites. The determinants in a proteoglycan core protein that initiate GAG synthesis have only been partially defined. GAGs are attached on serines that are followed by adjacent glycines; these sequences often occur in clusters. Nevertheless, other signals must be present, as many proteins that possess such sequences do not have attached GAGs, and not even all the serines in a PG core protein that are found in such sequences are glycanated. The
<table>
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<th>core protein size (kD) obs.</th>
<th>pred.</th>
<th>GAG</th>
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<tbody>
<tr>
<td><strong>Aggregating PGs</strong></td>
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<td>221</td>
<td>CS/KS</td>
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<td><strong>Transmembrane PGs</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Phosphacan</td>
<td>300-400</td>
<td>173/185</td>
<td>CS (KS)</td>
</tr>
<tr>
<td>NG2</td>
<td>300</td>
<td>250</td>
<td>CS</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>69</td>
<td>33</td>
<td>HS/CS</td>
</tr>
<tr>
<td>Syndecan-2</td>
<td>49</td>
<td>23</td>
<td>HS</td>
</tr>
<tr>
<td>Syndecan-3</td>
<td>120</td>
<td>35</td>
<td>HS/CS</td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>30-35</td>
<td>22</td>
<td>HS/CS</td>
</tr>
<tr>
<td>Drosophila syndecan</td>
<td>90</td>
<td>39</td>
<td>HS</td>
</tr>
<tr>
<td><strong>GPI-linked PGs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glypican</td>
<td>64</td>
<td>62</td>
<td>HS</td>
</tr>
<tr>
<td>Cerebroglycan</td>
<td>57</td>
<td>59</td>
<td>HS</td>
</tr>
<tr>
<td>OCI-5</td>
<td>-</td>
<td>69</td>
<td>-</td>
</tr>
<tr>
<td>K-glypican</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. **PG core protein families.** The observed Mr's (by SDS/PAGE), the calculated Mr's (based on primary sequence), and the type of GAG substitution are listed. Dashes indicate when this information has not been determined or reported.
consensus sequence ser-gly-xaa-gly with nearby acidic residues can act as an acceptor site, at least for chondroitin sulfate (Bourdon et al., 1987), although other sequences can be glycanated as well. Additionally, less closely situated acidic clusters have been shown to affect heparan sulfate synthesis in sequences derived from the PG betaglycan (Zhang and Esko, 1994). Tertiary structure is likely to important as well. Modeling of the GAG acceptor region of decorin shows that the ser-gly sequence may be part of a β-turn; amino acids that affect acceptor efficiency in peptides have little effect in the context of the entire protein (Mann et al., 1990).

It is apparent that, in addition to containing specific signals to specify GAG attachment, there must also be elements that specify the type of GAG attached, as some PGs exist as purely HS or CSPGs, even though they are synthesized from the same source. It has been reported that syndecan-1, a hybrid PG possessing both HS and CS, contains sites that are preferentially glycanated with HS and CS in the N-terminal domain of the protein, while the C-terminal domain seems to exclusively contain CS (Kokenyesi and Bernfield, 1994). However, mutational analysis of syndecan-4 indicates that all potential GAG attachment sites can accept either heparan sulfate or chondroitin sulfate (Shworak et al., 1994a; Shworak et al., 1994b). This discrepancy may be the result of differences in sequence between the two core proteins. Furthermore, mutagenesis of one GAG attachment site could change the preferences of other GAG attachment sites. It also may be that overexpression of the syndecan-4 protein alters the preferences of GAG attachment sites; overexpression is known to affect other aspects of GAG synthesis, such as the pattern of covalent modifications on HS (Shworak et al., 1994b).

Some studies suggest that nearby hydrophobic and possibly aromatic amino acids play a role in heparan sulfate specificity. β-D-xylosides, xylose analogs which can act as GAG acceptors, usually prime CS but not HS synthesis. Xylosides can act as heparan sulfate acceptors, however, if they have certain hydrophobic aglycone structures attached (Fritz et al., 1994; Lugemwa and Esko, 1991). Consistent with this, tryptophan-537 of betaglycan has been shown
to be important for heparan sulfate synthesis at an adjacent attachment site; mutation of this residue in a fragment of betaglycan abolishes heparan sulfate attachment to serine-535 (Zhang and Esko, 1994). Interestingly, a phenylalanine sits next to the N-terminal ser-gly cluster in syndecan-1 that is able to act as an acceptor for HS (Kokenyesi and Bernfield, 1994). These studies have lead to the suggestion that the GlcNAc transferase that initiates HS synthesis may have a pocket that confers a preference for hydrophobic regions. The GalNAc transferase that initiates CS synthesis may not have this pocket, and thus not require hydrophobic determinants. It should be noted however, that many HSPGs do not possess obvious hydrophobic determinants adjacent to their potential GAG attachment sites (e.g., glypican).
3. Expression of proteoglycan core proteins in the nervous system.

With the exception of their ability to bear GAGs, it is obvious that no feature unites PG core proteins into a single group (Table 2). PGs can exist extracellularly as matrix-associated, peripheral membrane, or integral membrane proteins, and can also be found intracellularly in vesicles. Some PGs bear HS, CS, or KS, and some PGs exist as hybrids, able to bear more than one type of GAG. Additionally, some core proteins are known as "part-time" PGs, as they are only found to be glycanated in some instances.

This diversity is preserved in the nervous system, where it has been found that at least 25 distinct PG core proteins are expressed, often in a developmentally regulated fashion (Herndon and Lander, 1990). Here, I discuss the some of the major PGs found in the nervous system, and address their structures, patterns of expression, and possible functions.

Large Aggregating CS/KSPGs. This class of PGs are referred to as large, aggregating PGs as they were initially isolated from cartilage and bone as aggregates of PG, HA, and link protein\textsuperscript{3}. They are modified with CS and, in some cases, KS. These aggregates are thought to provide structural support; the large size and extensive glycanation of the PG, complexed to the negatively charged hyaluronate, can provide hydration, resist compressional forces, and allow diffusion of ions. Studies of the expression of these PGs in the brain, however, suggest a broader role.

To date, four distinct core proteins of this class have been cloned. These are aggrecan, versican, neurocan, and brevican (Doege et al., 1987; Zimmermann and Ruoslahti, 1989; Doege et al., 1991; Rauch et al., 1992; Yamada et al., 1994)\textsuperscript{4}. Sequencing reveals a general, multidomain structure that is conserved between all family

\textsuperscript{3}Link protein is a HA-binding protein that stabilizes the interaction between aggregating PGs and HA.

\textsuperscript{4}The core for the chicken aggregating PG PG-M found in condensing limb bud mesenchyme has been cloned and is likely to be the chicken homolog of versican (Shinomura et al., 1993). Like versican, it exhibits alternative splicing of its GAG attachment domain.
members, although there is much variation between individual species. The N-terminal sequence consists of a globular domain that contains an IgG-like domain and a HA-binding domain that has homology to link protein. As expected, this region can bind HA. Aggrecan contains two sets of link protein-related domains, while other members contain a single set. This domain is followed by a GAG-attachment domain containing multiple potential attachment sites for CS and, in the case of aggrecan, KS. The C-terminal domain contains an EGF-like repeat, a lectin-like domain, and a complement regulatory protein-like domain (this C-terminal region as a whole is referred to as a LEC-CAM domain). Some of these C-terminal features are alternatively spliced in aggrecan (Doege et al., 1991).

Variability exists between members of this family, especially in the GAG attachment domain. In aggrecan, there are predicted to be roughly 30 KS attachment sites and over 100 CS attachment sites (Doege et al., 1987; Doege et al., 1991). Versican contains 12-15 potential CS attachment sites (Zimmermann and Ruoslahti, 1989), neurocan has a much shorter GAG attachment domain and contains only 7 potential CS attachment sites (Rauch et al., 1992), while brevican, which possesses the shortest GAG attachment site, contains only 3 potential CS attachment sites (Yamada et al., 1994). In addition, the GAG attachment domain of versican is alternatively spliced (Dours-Zimmermann and Zimmermann, 1994). The variability in the GAG attachment domain between family members, as well as that introduced by alternative splicing, suggests that the degree of glycanation is important for the function of these PGs.

There may also be proteolytic processing of the core proteins; a 60 kD protein from adult brain is likely to be an N-terminal fragment of versican (Perides et al., 1991; Perides et al., 1989; Perides et al., 1992), and C-terminal fragments of neurocan and brevican have been isolated from brain (Oohira et al., 1994; Rauch et al., 1992; Yamada et al., 1994).

The identification of many forms of these aggregating PGs in the brain is further evidence that there is in fact a definable ECM in
the nervous system\(^5\). The forms of these PGs do not include just those described above, but also shorter fragments of these PGs, PGs which bear antigenic relationship to previously cloned PGs, and seemingly novel PGs. Furthermore, the distinct patterns of expression of these PGs suggests that members of this family may be required for different roles in nervous system development and function.

Several lines of evidence suggest that both aggrecan and versican are expressed in the nervous system. A CSPG purified from human brain under denaturing conditions behaves identically to versican both immunologically and biochemically (Perides et al., 1992). Glia of the central and peripheral nervous systems are stained by an anti-versican monoclonal antibody. In addition, the 60 kD protein, glial-hyaluronate binding protein (GHAP), has also been purified from brain, and amino acid sequence indicates that it is likely to be an N-terminal fragment of versican (Perides et al., 1991; Perides et al., 1989). Studies in developing chick brain found two PGs, one seemingly related to aggrecan (S103L) (Krueger et al., 1992). This PG is aggrecan-like based on the possession of a hyaluronic acid binding region, and its expression in cartilage as well as in brain. S103L from brain, however, does not possess KS chains, whereas S103L from cartilage, like aggrecan, does. Its expression is regulated during development, and S103L not present in the adult brain.

Neurocan was purified and cloned from brain based on its crossreactivity with the monoclonal antibody 1D1 (Rauch et al., 1991; Rauch et al., 1992). Like other members of this family, neurocan aggregates with HA. Neurocan is reported to be expressed by neurons (Margolis and Margolis, 1994). Antibodies stain the cerebellum (Rauch et al., 1991) and the developing cerebral cortex, where neurocan is found in the subplate (Miller et al., 1992; Oohira et al., 1994). At later stages, neurocan staining demarcates the

\(^5\)It has been previously suggested that there is little or no organized matrix in the brain. More recent evidence suggests, however, that both extracellular matrix molecules and some organized matrix are found in the central nervous system (for discussion, see Sanes [1989]).

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boundaries of barrel fields in layer IV of the cerebral cortex (but is not found in the barrel fields themselves until later) (Oohira et al., 1994). Interestingly, neurocan is reported to interact with tenascin (Grumet et al., 1994), which too is found in the borders of barrel fields. Tenascin expression also overlaps with neurocan in the cerebellum (Grumet et al., 1994), as does the expression of the cell adhesion molecules N-CAM and Ng-CAM. Neurocan inhibits neurite outgrowth and cell adhesion on these molecules in vitro (Grumet et al., 1993; Friedlander et al., 1994).

Another CSPG, brevican, has recently been cloned from bovine brain, and is similar to other members of the aggrecan family (Yamada et al., 1994). Interestingly, both glycanated and nonglycanated core proteins were identified in brain extracts, indicating that brevican can exist as a "part-time" PG (although there is no evidence that nonglycanated brevican is extracellular). Brevican is expressed in brain, and by glia but not neurons in culture.

The Cat-301 CSPG, present in the adult brain, is thought to be related to aggrecan (Fryer et al., 1992). It is found in cartilage, associates with HA, is substituted with both KS and CS, and exhibits a high buoyant density in CsCl buoyant density gradient centrifugation. In brain, however, Cat-301 exists in a somewhat different form than in cartilage, as brain Cat-301 exhibits a lower buoyant density than expected and contains no KS. As the chick PG S103L, which is also thought to be related to aggrecan, does not possess KS in brain (Krueger et al., 1992), it may be that in general aggrecan(s) in the brain do not contain KS.

The expression of Cat-301 in the nervous system is remarkably restricted (Hockfield et al., 1990). Appearing late in development and present in the adult, Cat-301 is associated with subsets of neurons. These subsets include spinal motoneurons and neurons (both in the thalamus and visual cortex) involved in the pathway of the visual system associated with processing motion. In addition, its expression is activity-dependent; ablation of neuronal activity during critical periods in development prevents Cat-301 expression (Hockfield et al., 1990; Kalb and Hockfield, 1990). It may
be that, as a structural component, Cat-301 is involved in stabilizing mature synaptic structures. The association of this antigen with specific neural circuits, however, suggests that it may play more than simply a structural role, and that there is some specificity in the ECM surrounding synapses.

Another CSPG with similar biochemical properties to aggrecan and its family members is pgT1 (Iwata and Carlson, 1993; Iwata et al., 1993). pgT1 was purified from rat based on its crossreactivity with an antibody raised against PG1000, a large PG of elasmobranch electric organ (Carlson et al., 1986; Carlson and Wight, 1987; Iwata and Carlson, 1991). Like aggrecan, pgT1 forms aggregates with HA, and these aggregates have morphology to aggrecan-HA aggregates. However, the relationship between aggrecan or versican to pgT1 is unclear, as antibodies raised to a 15 amino acid epitope conserved in aggrecan and versican do not recognize pgT1. pgT1 is widely expressed in the nervous system--both white and gray matter are stained using the T1 antibody.

A unique CS/DS hybrid epitope has been reported in mouse brain, and has been used to isolate a single core protein, designated the DSD-1 PG (Faissner et al., 1994). The biochemical data, in particular the size, presence of CS chains, and extractability with saline, suggest that the DSD-1 core may belong to the aggrecan family. This core is expressed by glia in vitro (Faissner et al., 1994), and immunohistochemistry suggests that it is present in the boundaries of barrel fields in the somatosensory cortex (Steindler et al., 1995).

**Small, interstitial proteoglycans.** This family of PGs include two CS/DSPGs, decorin (Day et al., 1987; Krusius and Ruoslahti, 1986; Li et al., 1992; Scholzen et al., 1994) and biglycan (Fisher et al., 1989; Neame et al., 1989), and two KSPGs, fibromodulin (Oldberg et al., 1989) and lumican (Blochberger et al., 1992a). Decorin, biglycan, and fibromodulin are all abundant in tissues containing collagen, such as tendon, bone, and/or cartilage, while lumican has been characterized as "the" KSPG of cornea, although decorin is reported
to contain KS chains in the adult chicken cornea (Blochberger et al., 1992b).

The PGs of this family are relatively small, having core protein sizes of 38-60 kD (see Table 2). Cloning and sequencing reveal that members of this family have a common structure. The CS attachment sites of decorin and biglycan are in the first few N-terminal amino acids--decorin has one (serine-4) and biglycan has two (serine-5 and serine-11). The N-terminal domain also consists of four cysteines participating in two intrachain disulfide bonds, while the C-terminal domain contains two cysteines that participate in an intrachain disulfide bond. The central domain consists of a leucine-rich repeat of the consensus sequence L-X-X-L-X-L-X-X-N-X-L/I; these repeats, which vary in number between family members, comprise 60% or more of the amino acid sequences of these PGs. Leucine-rich repeats are likely to mediate protein-protein interactions (Blochberger et al., 1992b; Kobe and Deisenhofer, 1994), and have been implicated in the binding of decorin to a variety of molecules (Schmidt et al., 1991; Spiro et al., 1994).

Decorin is typically expressed in regions containing collagen, and in fact its name derives from the observation that in electron micrographs this PG can be seen to "decorate" collagen fibrils. With the exception of biglycan, PGs of this family bind to fibrillar collagens. Decorin has a Kd of 16 nM and fibromodulin a Kd of 35 nM for types I and II collagen in solid-phase binding assays (Hedbom and Heinegård, 1989; Hedbom and Heinegård, 1993); this interaction is mediated by the core protein and not the GAG chain. The binding sites on collagen for these PGs are different (Scott and Haigh, 1988; Hedbom and Heinegård, 1993). Decorin has been localized to the d and e bands and fibromodulin to the a and c bands in the D period of collagen fibrils from tendon and cornea (Hedlund et al., 1994; Pringle and Dodd, 1990; Scott and Haigh, 1988). This binding decrease the rate of collagen fibril formation and the size of the fibrils formed, and is thus thought to allow the formation of small, regular arrays of collagen fibrils (Hedbom and Heinegård, 1989; Vogel et al., 1984). This function also contributes to the
transparency of the cornea, where decorin and lumican are expressed. Decorin has also been reported to bind to type VI collagen (Bidanset et al., 1992; Spiro et al., 1994). Some data suggests that the disulfide-bonded globular regions affect fibrillogenesis, while it is the leucine-rich repeat region that mediates collagen binding (Spiro et al., 1994).

Decorin has also been reported to bind to fibronectin \( (K_d = 20\ nM) \) (Lewandowska et al., 1987; Schmidt et al., 1991; Winnemöller et al., 1991) and thrombospondin \( (K_d = 5\ nM) \) (Winnemöller et al., 1992) in solid phase assays. In both cases this binding is mediated by the core protein and can inhibit \textit{in vitro} cell attachment to those molecules. Decorin can bind to TGF-\( \beta \) via its core protein, and this binding can inhibit TGF-\( \beta \) function \textit{in vitro} (Yamaguchi et al., 1990; Yamaguchi and Ruoslahti, 1988). Decorin can also bind to netrin-1 and may modulate its neurite-outgrowth activity (see Chapter 4 of this thesis).

Very little is known about the expression of these PGs in the nervous system. Given the widespread association of decorin and other family members with collagen, one might expect not to find them present in the brain (it is unclear to what extent collagen fibrils are present in the nervous system; see footnote 5). Indeed, no lumican message has been detected in the brain (Blochberger et al., 1992a). However, decorin has been detected in the mouse, in the floor plate of the developing spinal cord and in the region of the pons in the adult (Scholzen, et al., 1994), and has been reported also in the human brain (Voss et al., 1986). Decorin has also been reported in association with amyloid plaques related to Alzheimer's disease (Snow et al., 1992). \textit{In situ} hybridization in the adult rat nervous system suggests that decorin is expressed by Schwann cells in the PNS, and by neurons in the CNS (Hanemann et al., 1993).

\textbf{NG2.} NG2 is a large, membrane-associated CSPG with a core protein of about 300 kD (Stallcup et al., 1983). It is widespread and developmentally regulated in the nervous system, where it is associated with glial progenitor cells (Levine and Card, 1987; Levine and Stallcup, 1987; Stallcup and Beasley, 1987). NG2 is up-
regulated in response to brain injury (Levine, 1994), which may represent a proliferation of glial precursors. NG2 is also expressed in a variety of tissues outside the nervous system.

NG2 does not fall into any known class of PG, or any other protein. It is a transmembrane protein, with a large extracellular domain and a relatively small cytoplasmic domain (Nishiyama et al., 1991). The extracellular domain consists of a GAG attachment domain bounded by two cysteine-containing regions, some of which are disulfide-bonded. The GAG attachment domain possesses nine potential GAG attachment sites, but not all of them seem to be used.

Recently, the NG2 core protein has been shown to inhibit neurite outgrowth by cerebellar granule cells on substrates consisting of laminin or L1; furthermore, these neurites will avoid regions of a substrate possessing NG2 (Dou and Levine, 1994). As NG2 is expressed in the deep regions of the cerebellar molecular layer during development, it has been suggested that one role of NG2 in the developing cerebellum is to repel developing axons of granule neurons, hence keeping them parallel (Dou and Levine, 1994). This inhibitory effect would also explain the inability of axons to grow in damaged regions, as these would be regions with increased NG2 expression (Levine, 1994).

**RPTPβ/Phosphacan.** One CSPG identified in brain by the use of monoclonal antibodies (Rauch et al., 1991; Yamada et al., 1994) has recently been cloned and sequenced. The cDNA of the 3F8 epitope from rat brain (Maurel et al., 1994) and that of a CSPG recognized by a polyclonal antiserum (Shitara et al., 1994) encode a sequence identical to that of a brain-specific receptor-type protein tyrosine phosphatase, RPTPβ (Krueger and Saito, 1992; Levy et al., 1993). RPTPβ possesses an extracellular domain with homology to carbonic anhydrase (although this domain is not thought to be enzymatically active), a transmembrane domain, and two C-terminal phosphatase domains. Three alternatively spliced transcripts of RPTPβ exist in rat--two are transmembrane and one seems to be extracellular. The 3F8 proteoglycan (designated phosphacan) appears to be an extracellular, soluble, alternatively spliced variant of RPTPβ (Maurel
et al., 1994), while the clone from human may encode a transmembrane variant (Shitara et al., 1994). There does not appear to be alternate splicing of the human gene.

RPTPβ is brain-specific, and is expressed by glia (Canoll et al., 1993; Milev et al., 1994). Within the brain, RPTPβ is widespread, being found on Bergmann glia of the developing and adult cerebellum, radial glia of the cerebral cortex, the roof plate of spinal cord, and several nerve fibers (where it is probably associated with glia, and, in the periphery, with Schwann cells) (Canoll et al., 1993). The 3F8 antigen has also been reported in the neocortical subplate during development, becoming widespread as the cortex matures (Miller et al., 1992). The relationship between these two studies remains unclear. Interestingly, some phosphacan seems to be substituted with both CS and KS chains (Maurel et al., 1994; Rauch et al., 1991).

In vitro assays using purified phosphacan suggest that it interacts with certain cell adhesion molecules (Ng-CAM, NILE/L1, and N-CAM) and extracellular matrix molecules (tenascin) (Grumet et al., 1993; Grumet et al., 1994; Milev et al., 1994). Most of the observed binding seems to be mediated by the core protein. Phosphacan can inhibit cell adhesion and neurite outgrowth on these substrates, possibly, in the case of homophilic molecules such as N-CAM, by binding to cell-surface N-CAM (Grumet et al., 1993). This inhibition seems also to be due to the core protein and not to the CS or KS GAG chains. As phosphacan expression in the brain overlaps with that of some of these molecules, there is the possibility that phosphacan may modulate their effects in vivo.

Synaptic vesicle PGs (SV2). One component of synaptic vesicles is a KSPG designated SV2 (Scranton et al., 1993). SV2 was identified and characterized in elasmobranch electric organ (Carlson and Kelly, 1983). SV2 is a transmembrane protein that has 12 predicted transmembrane domains and possesses homology to both bacterial sugar transporters and mammalian neurotransmitter transporters (Bajjalieh et al., 1992; Feany et al., 1992). In rat there are two SV2 isoforms, with different patterns of expression in the brain.
(Bajjalieh et al., 1994). It may act as an acetylcholine transporter in synaptic vesicles, as it is associated with the vesamicol receptor (Bahr et al., 1992). Furthermore, the negatively charged GAG of synaptic vesicle PGs could create a physical environment that can regulate release of neurotransmitters and other components.

SV2 expression seems to be widespread throughout the brain (Bajjalieh et al., 1994; Scranton et al., 1993). The KS chains attached to it seem to be regulated, as certain KS epitopes, such as SV1, are specific to electric organ synaptic vesicles (Scranton et al., 1993). Furthermore, the KS in synaptic vesicle seems to have an unusual structure—the inability to significantly degrade synaptic vesicle KS with keratanase suggests that there may be other sugars, such as fucose or sialic acid, covalently linked to the KS of SV2 in synaptic vesicles (Scranton et al., 1993).

**Basement membrane PGs.**

i) **Perlecan.** Perlecan is a large, multidomain HSPG that is typically associated with laminin and other components in the basement membranes of all tissues (for review, see Iozzo et al., 1994). While it has not been reported in the nervous system tissues (it has only been found in the basement membranes of capillaries within the brain [Kato et al., 1988; Lin, 1990]), it is present in the basal lamina surrounding the neural tube and along which peripheral nerves grow, and in the endoneurium surrounding mature peripheral nerves (Halfter, 1993; Halfter and Schurer, 1994). In this role, it is likely to help play a number of functions, including organization of the extracellular matrix, localization of growth factors, and support of axon growth. The ability of cells to adhere via integrins to the perlecan core protein (Hayashi et al., 1992) suggests that perlecan may directly mediate interactions with cells.

ii) **Agrin.** The neuromuscular junction is a specialized synaptic structure possessing an organized basal lamina and clusters of acetylcholinesterase and acetylcholine receptors (AChRs) (McMahon, 1990). One component of this structure is agrin; agrin was purified based on its ability to induce clustering of acetylcholine
receptors, and is thus likely to play a key role in organizing the neuromuscular junction.

Recently, it has been demonstrated that agrin is an HSPG (Tsen et al., 1995); the core protein of an HSPG originally identified by the monoclonal antibody 6D2 was cloned, and was found to have a sequence identical to that of agrin\(^6\) (Halfter, 1993; Tsen et al., 1995; Tsim et al., 1992). The HSPG form of agrin has gone undetected up to this point because a fraction of agrin isolated from brain is a smaller molecular weight and does not seem to contain HS (Tsen et al., 1995). It is unclear whether this smaller molecular weight form of agrin is a truly unglycanated form, or whether it is a degradation product. The existence of this agrin form may explain the fact that the PG form of agrin has gone undetected up to this point.

It is likely that HS, either on agrin or on other PGs, plays a role in organizing the neuromuscular junction. Exogenous HS can inhibit AChR clustering, presumably by disrupting the binding of agrin to other proteins (Hirano and Kidokoro, 1989; Wallace, 1990). Myotubes deficient in HS synthesis exhibit defects in the clustering of acetylcholine receptors, either spontaneously or in response to purified agrin (Ferns et al., 1993; Gordon et al., 1993). However, other PGs (possibly perlecan), at least as components of the basal lamina, are present at the neuromuscular junction (Anderson and Fambrough, 1983; Bayne et al., 1984). Hence, it is unclear whether the HS involved in AChR clustering is required on agrin or on another protein. Furthermore, it is not known if HS is involved directly in AChR clustering or more generally in organization of the basal lamina.

It is likely that AChR clustering is not agrin's sole function. Alternatively spliced variants of agrin exist (Ferns and Hall, 1992), with some variants having either reduced or no AChR clustering activity (Ferns et al., 1993; Ruegg et al., 1992). No evidence exists that the potential GAG attachment sites are spliced, and they are not encoded in the exons encoding enhanced clustering activity.

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\(^6\)The agrin gene possesses several EGF-like repeats, several domains with homology to serine trypsin inhibitors, and a laminin-like domain (Rupp et al., 1991; Tsim et al., 1992).
Expression studies of agrin, which indicate that agrin is widespread and found in areas not containing neuromuscular junctions, also suggest other functions. During development, agrin is expressed by neurons and Schwann cells during development in peripheral nerves and ganglia (Halfter, 1993; Thomas et al., 1993), and is also found in association with ventricular zones and, as expected, with spinal motoneurons (Ma et al., 1994; Rupp et al., 1991). In the adult nervous system, agrin expression, which is mostly neuronal, is widespread (Ma et al., 1994; O’Connor et al., 1994). Agrin is also present in muscle, and in basement membranes outside the nervous system. Thus, agrin may also stabilize other synapses, or may contribute to matrix structure. Its expression in nerve during development suggests that it may play a role in axon outgrowth.

iii) 6C4/1B11. A 180 kD HSPG core protein from chick (total MW 250 kD) recognized by the 6C4/1B11 monoclonal antibody is distinct from perlecan and agrin. Like perlecan, it is found only in basal lamina and not in brain parenchyma (Halfter and Schurer, 1994). This HSPG is expressed in the basal lamina surrounding nerves; in vitro, however, it appears to be inhibitory for neurite outgrowth, as neurites do not grow on 6C4/1B11 when spotted on nitrocellulose alone or with extracts of basal lamina.

Syndecan. The syndecans are a family of four transmembrane HS or HS/CSPGs (for review, see Bernfield et al., 1992). Their members include syndecan-1 (Cizmeci-Smith et al., 1992; Kiefer et al., 1990; Kojima et al., 1992; Mali et al., 1990; Saunders et al., 1989), fibroglycan/syndecan-2 (David et al., 1993; Marynen et al., 1989; Pierce et al., 1992), N-syndecan/syndecan-3 (Carey et al., 1992; Gould et al., 1992), and ryudocan/amphiglycan/syndecan-4 (Baciu et al., 1994; David et al., 1992a; Kojima et al., 1992). These proteins are characterized by an extracellular domain with several GAG attachment sites (syndecan-1 and syndecan-3 have longer extracellular domains with more of these sites near the N-terminus), a dibasic sequence in the extracellular domain proximal to the membrane that is a site predicted for proteolysis, a transmembrane domain, and a cytoplasmic domain that contains several tyrosine
residues (of which only one fits the consensus sequence for tyrosine phosphorylation). The transmembrane and cytoplasmic domains are conserved both between members and between species, but the extracellular domains are divergent (although the protease sites and the GAG attachment sites are conserved). This suggests that the transmembrane and cytoplasmic domains are likely to serve a similar function in all cases, such as binding to the cytoskeleton and/or participating in focal adhesions (Carey et al., 1994; Woods and Couchman, 1992; Woods and Couchman, 1994).

Of all members of the syndecan family, syndecan-3 is the most abundant in the nervous system (Kim et al., 1994). Syndecan-3 expression is developmentally regulated, being highest in the neonatal rat brain, and is widespread throughout the nervous system (Carey et al., 1992). Syndecan-3 is expressed by neurons in culture, and is found on the cell body as well as on neurites (Raulo et al., 1994). Syndecan-3 purified from brain has been reported to bind FGF-2 and HB-GAM but not other heparin-binding proteins (Chernousov and Carey, 1993; Raulo et al., 1994); as these studies were carried out using solid phase binding assays, however, they may not reflect the binding abilities of these proteins in solution.

It is unclear if other members of the syndecan family are found in the nervous system. Both syndecan-2 and syndecan-4 messages have been detected in brain RNA (Kim et al., 1994; Pierce et al., 1992). However, antibodies to syndecan-2 thus far have yielded no staining of nervous tissue (David et al., 1993), suggesting that the syndecan-2 message detected may be due to contaminating endothelia (which express high levels of syndecan-2) from blood vessels and meninges. Antibodies to syndecan-4 have been reported to stain both central and peripheral nervous system (Baciu et al., 1993). Syndecan-1 does not seem to be expressed in the nervous system, although a mouse syndecan probe hybridizes to a 4.5 kb transcript from cerebrum (Saunders, et al., 1989).

Recently, a cDNA with homology to the syndecans has been cloned from Drosophila, and has been shown biochemically to encode an HSPG (Spring et al., 1994). As expected, this homology is highest in the transmembrane and cytoplasmic domains. Analysis
suggests that this gene is related to the evolutionary ancestor of the four syndecan genes present in vertebrates. Interestingly, *Drosophila* syndecan is expressed in both the central and peripheral nervous systems in developing embryos. Its expression correlates with that of FGF receptors in the *Drosophila* nervous system, further suggesting that syndecan in the nervous system may act as a coreceptor for FGF.

**Glypican.** Recently, many cell surface HSPGs have been identified which associate with membranes via a glycosylphosphatidylinositol (GPI) lipid linkage. These include HSPGs from hepatocytes (Ishihara et al., 1987), ovarian granulosa cells (Yanagishita and McQuillan, 1989), Schwann cells (Carey and Stahl, 1990), fibroblasts (David et al., 1990), melanoma cells (Drake et al., 1992), and rat brain (Herndon and Lander, 1990). The GPI-linked HSPG from human fibroblasts was cloned, and named glypican (David et al., 1990). Since that time, three other homologues have been identified—cerebroglycan (Stipp et al., 1994), OCI-5 (Filmus et al., 1988), and K-glypican (Watanabe and Yamaguchi, 1994).

These PGs seem to be, on the basis of sequence, a completely novel class of proteins, as they contain no functional domains found in other proteins or any homology to any other protein. All members possess both putative N-terminal signal peptides and short hydrophobic C-terminal stretches which form part of the glypiation signal (i.e., the signal for attachment of the GPI-linkage). While the number of potential GAG attachment sites vary between members, they are all characterized by a cluster of serine-glycine repeats (of varying size) roughly 40 amino acids upstream of the predicted site of glypiation. They also contain other scattered Ser-Gly sequences further upstream. The 14 extracellular cysteines, which are conserved between family members, suggest that the glypican is highly disulfide bonded and has a compact tertiary structure. Indeed, the apparent molecular weights of glypican and cerebroglycan increase upon reduction as measured by electrophoresis (Herndon and Lander, 1990).
All glypican family members are expressed in the brain; their patterns of neural expression, however, seem to be unique. Glypican has been purified from rat brain (Litwack et al., 1994a; this thesis), and the characterization of the neural expression of glypican is the subject of this some of this work (for further discussion, see Chapters 2 and 3 of this thesis; also, Karthikeyan et al., 1994; Litwack et al., 1994a). In addition to the results presented here, glypican has also been reported to be expressed in Schwann cells (Carey et al., 1993); this shall be discussed in Chapter 3 of this thesis.

Another core protein, cerebroglycan, has been purified from rat brain. In situ hybridization experiments indicate that it is nervous system specific, and is expressed by newly postmitotic neurons as they begin migration or axon outgrowth (Stipp et al., 1994). Its message, at least during early development, is expressed in a complementary fashion to that of glypican (Lander, 1993; Chapter 3, this thesis). OCI-5 was originally cloned from intestinal cells, but OCI-5 message has been detected in the developing nervous system (Watanabe and Yamaguchi, 1994). K-glypican, named because of its expression in kidney, is also expressed in developing brain; unlike glypican, however, the messages for both OCI-5 and K-glypican decrease as the brain matures (Watanabe and Yamaguchi, 1994).

The function of these PGs in the nervous system is unknown. The spatial and temporal pattern of cerebroglycan expression suggests that it may play a role in axon growth and cell migration (Stipp et al., 1994); the localization of cerebroglycan to axons (see Chapter 3 of this thesis) is consistent with this proposed role. The high levels of glypican in the ventricular zones of the early developing nervous system (Litwack, this thesis) at a time when there is extensive proliferation and differentiation of cells imply that it may act as growth factor receptors, mediating proliferation of neuroepithelia and perhaps differentiation into neurons or glia. In addition to ventricular zone expression, I shall show in this thesis (Chapter 3) that glypican is widespread during later stages of
development, suggesting that glypican may be involved in a common function at those stages.

As a result of the GPI-linkage found on all glypican family members, these HSPGs may confer different responses of cells in response to heparin-binding proteins than transmembrane PGs (for review of GPI-linked proteins, see Cross [1990]). The GPI-linkage is thought to confer higher lateral mobilities on membrane proteins, as the attached protein can potentially diffuse through the membrane near the rates of lipid (Chan et al., 1991).

Transmembrane proteins are likely to be associated with intracellular components via their cytoplasmic domains, and thus may directly signal the cytoplasm or associate with the cytoskeleton. GPI-linked proteins, however, are thought to often be associated with specialized membrane structures known as cavaeolae, and are often copurified with cavaeolar markers (Lisanti et al., 1994). Such structures could provide a mechanism for GPI-linked proteins to directly signal cells, as they could colocalize these proteins with intracellular components. Intracellular tyrosine kinases have been found to coimmunoprecipitate with GPI-linked proteins, suggesting that they may be associated in cells (Rudd et al., 1993; Stefanova et al., 1991; Thomas and Samelson, 1992). Such interactions could occur via association of these proteins in membrane domains. Alternatively, an "adaptor" protein, which spans the membrane and binds to both extracellular and intracellular proteins, could mediate such interactions (no such protein has yet been identified).

GPI-linked proteins are also likely to be processed differently than transmembrane proteins. They can be shed from the cell surface by phospholipase C or potentially by a protease, whereas syndecan presumably must be shed by a protease (Bernfield, et al., 1992). Some groups have suggested that GPI-linked PGs, once shed by the cell, remain on the cell surface as peripheral membrane (Carey and Evans, 1989; Ishihara, et al., 1987). GPI-anchored PGs have also been reported to be metabolized by different intracellular pathways than transmembrane PGs (Yanagishita, 1992).

The GPI-linkage may sometimes lead to differential sorting in cells. In epithelial cells, GPI-linked proteins are often sorted to the
apical surface as opposed to the basolateral surface (Lisanti et al., 1989). The observation that some GPI-linked proteins in neurons are sorted to axons lead to the proposal that the axon of a neuron is equivalent to the apical surface of an epithelial cell, and the dendrites and cell body to the basolateral surface with respect to sorting of GPI-linked proteins (Dotti et al., 1991; Dotti and Simons, 1990). These studies were performed on neurons in vitro, however; more extensive observation in vivo demonstrates in fact that GPI-linked proteins are often not sorted to axons (Morris and Grosveld, 1989). The compartment in which a GPI-linked protein is found may depend on the neuron in which it is being expressed, the time during development when it is expressed, and also on the specific GPI-linked protein being expressed (Faivre-Sarrailh et al., 1992; Xue et al., 1990; Xue et al., 1991). Nevertheless, while generalizations cannot be made about the relationship between GPI-linkage and sorting, it is clear from these studies that GPI-linked proteins are not always evenly distributed in neurons. Studies in this thesis will demonstrate that glypican expression is consistent with axonal polarization, and that cerebroglycan is polarized to axons in granule cells of the hippocampus (see Chapter 3 of this thesis). The GPI-linkage may allow a PG to be localized in cellular compartments differently than if it were a transmembrane PG. The expression of such PGs may represent one mechanism for cells to specifically localize HS on the cell surface.
4. Functions of proteoglycans in developmental processes.

PGs have been shown to modulate many processes important in nervous system development, including cell adhesion, cell migration, axon outgrowth, extracellular matrix organization, cell proliferation, and differentiation. The modulation of these processes can be ascribed partly to the GAG portion of the PG. The binding of proteins to GAGs is often directly important for function despite the existence of many non-PG signal-transducing receptors with high affinity for those proteins. Functional studies have led to the concept of PGs (especially cell surface PGs) as coreceptors, receptors whose binding to ligand is important in the interaction of that ligand with distinct high affinity receptors (Bernfield et al., 1992). The role of PGs as coreceptors shall be illustrated below by examining the functions of PGs, and particularly GAGs, in modulating the actions of protease inhibitors, growth factors, and cell adhesion molecules.

In addition, PG core proteins can also directly interact with these developmentally important proteins, or on their own can directly modulate developmental events. Hence the existence of multiple families of PG core proteins, with multiple members of each family. Also, many cores are highly conserved between species. In many cases, cores have already been shown to directly bind other proteins. I will also note in this discussion the function of some PG core proteins in developmental processes.7

Proteases and Protease Inhibitors.

(i) Antithrombin III (AT). The anticoagulant properties of heparin are well known. Heparin possesses this anticoagulant activity as a result of its ability to bind AT, a protease inhibitor of the serpin family8. This binding event accelerates the rate of inactivation of thrombin and other proteases by AT. In reality, only a fraction of heparin or HS chains possess AT binding activity; these

7The evidence suggesting that heparin can regulate cell proliferation is not discussed here (see Marcum et al., 1987; Karnovsky et al., 1989).
8Serpin is an abbreviation of serine protease inhibitor.
chains account for the anticoagulant activity in those samples (Marcum and Rosenberg, 1989).\(^9\)

The binding of AT by heparin increases rates of inactivation by two basic mechanisms. In the case of inactivation of some proteases, such as factor Xa, the conformational change induced in AT upon binding to heparin is sufficient to account for this acceleration (Evans et al., 1992). In this case, the AT-binding pentasaccharide enhances inactivation to the same extent as full-length heparin. In the case of proteases such as thrombin, heparin also increases rates of inactivation by participating in a thrombin-AT-heparin ternary complex (Evans et al., 1992; Olson and Björk, 1991; Pratt et al., 1992). This process, known as surface approximation, can take place because thrombin binds to heparin (Olson et al., 1991). Consistent with the mechanism of surface approximation, the rate of thrombin inactivation is dependent on the size of the heparin fragment--heparin fragments longer than but containing the pentasaccharide are required for maximal acceleration (Evans et al., 1992; Pratt et al., 1992). Furthermore, the enhancement of inactivation rate is abolished in the presence of high concentrations of heparin, a result consistent with the binding of multiple factors on a single heparin chain. However, part of the effect of heparin on AT inhibition of thrombin can additionally be attributed to the conformational change of AT induced by heparin, as the pentasaccharide sequence alone enhances the inactivation rate, although not as much as do longer fragments (Evans et al., 1992). It is likely that heparin’s effect on thrombin inactivation by AT results from both surface approximation and the allosteric effects of heparin binding on AT.

(ii) **Protease nexin-1 (PN-1).** PN-1, which is identical to glia-derived nexin, is a protease inhibitor of the serpin family. PN-1 is abundant in the nervous system, where it is expressed by both glia and neurons (Mansuy et al., 1993; Reinhard et al., 1994). Widespread during development, it is retained in a number of adult cells.\(^9\)

\(^9\)Such anticoagulant activity can be found on endothelial surfaces *in vivo*, and can accelerate thrombin inactivation by AT (Marcum and Rosenberg, 1989).
brain regions; it has also been localized to the neuromuscular junction (Festoff et al., 1991).

The inactivation of proteases such as thrombin by PN-1 is important in the regulation of neurite outgrowth, cell migration, and astrocyte morphology in vitro (Cavanaugh et al., 1990; Lindner et al., 1986; Monard, 1988). As the rate of inactivation of proteases by PN-1 can be accelerated by heparin or HS (Evans et al., 1991; Farrell and Cunningham, 1987)\(^\text{10}\) and to a lesser extent by CS (Farrell and Cunningham, 1987), it is likely that nervous system HS and HSPGs are important for the regulation of PN-1's effects. The interaction of PN-1 with cell surface HS and inactivation by PN-1 can be accelerated by cell surfaces (Farrell and Cunningham, 1986) implies that cell surface HSPGs such as glypican or syndecan may be involved. In addition, PN-1 can be found in extracellular matrix (Halfter et al., 1990). Thus, HSPGs of basal lamina may also play a role in regulating PN-1.

**Fibroblast growth factors (FGFs).** HSPGs are binding proteins, distinct from the high affinity tyrosine kinase receptors FGFRs 1-4, for members of the FGF family. They can bind FGFs because of the high affinity the FGFs have for heparin and HS (FGFs have also been referred to as the heparin-binding growth factors).

The affinity of FGFs for HS has been well characterized. In many in vivo and in vitro situations FGF-2 has been shown to associate with HS. HS can act as a reservoir for FGF-2 in the extracellular matrix (Ishai-Michaeli et al., 1990), or as low affinity binding sites on the surfaces of cells (Moscatelli, 1987). HSPGs released from cell surfaces or the ECM are often found to be complexed with FGF-2 (Bashkin et al., 1992; Brunner et al., 1991). As a result, it is likely that HS acts as a reservoir for FGF-2, and in fact little FGF-2 is found in the conditioned medium of FGF-2-expressing cells (Vlodavsky et al., 1987).

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\(^{10}\)Evans et al. (1991) suggest that heparin accelerates PN-1 inactivation of thrombin by surface approximation.
This binding has many consequences important for FGF function. Heparin can affect the diffusion rate of FGF-2 (Flaumenhaft et al., 1990), and can protect both FGF-1 and FGF-2 from proteolytic degradation (Saksela et al., 1988). Heparin-FGF binding has also been shown to be required for FGFs to signal through their high affinity receptor tyrosine kinases. This requirement has been demonstrated for FGF-2 signalling of cells (Olwin and Rapraeger, 1992; Rapraeger et al., 1991; Yayon et al., 1991) and for interaction with FGFR1 in solution (Ornitz and Leder, 1992). This requirement has also been demonstrated for FGF-1 signalling through FGFR1 and FGFR3 (Olwin and Rapraeger, 1992; Ornitz and Leder, 1992) and also for FGF-4 (K-FGF) signalling (Olwin and Rapraeger, 1992). Hence, cell surface HSPGs have been proposed to be coreceptors for FGFs (Bernfield et al., 1992).

Heparin can function as a coreceptor in one of several ways. Heparin can support the oligomerization of FGF-2 (Ornitz et al., 1992) and FGF-1 (Spivak-Kroizman et al., 1994), thus assisting in dimerization of FGF receptors. Heparin is also likely to participate in the formation of a FGF-FGFR-heparin ternary complex and thus stabilize the binding of FGF to its receptor. In support of this, heparin has been shown to bind to a domain of FGFR1, and a mutations in this domain affect both FGF-2 binding and heparin binding (Kan et al., 1993)\textsuperscript{11}. It thus seems likely that some sort of heparin-bFGF-FGFR1 complex is formed\textsuperscript{12}. Kinetic studies suggest that the role of heparin in this case is to stabilize the complex by decreasing its dissociation rate (Moscatelli, 1992; Nugent and Edelman, 1992).

Studies using oligosaccharides derived from heparin or heparan sulfate are consistent the model of ternary complex formation. An oligosaccharide consisting of five sugars is the minimum length necessary to bind to FGF-2 (Maccarana et al., 1993), while the minimum length of about 8-12 saccharides is necessary for FGF-2 binding to its receptor and for mitogenic

\textsuperscript{11} Spivak-Kroizman et al. (1994)\textsuperscript{a} report that heparin does not bind FGFR2.
\textsuperscript{12} For an opposing view, see Roghani et al. [1994].
activity in response (Aviezer et al., 1994; Guimond et al., 1993; Ishihara et al., 1993; Ornitz et al., 1992; Walker, 1994). Shorter oligosaccharides can inhibit the binding of FGF-2 to its receptor (Tyrell et al., 1993). Furthermore, selectively de-6-O-sulfated heparin still binds to FGF-2 but blocks its mitogenic effect, implying that a second site on heparin, not present in the FGF-2 binding sequence is required for function. While consistent, these studies do not rule out the possibility that the regions present in the longer, activating heparin sequences, are also binding to different domains in the same FGF-2 molecule or to different FGF-2 molecules (causing oligomerization). These studies imply that there is a second binding site on heparin, whether for FGF-2 or FGFR, that is necessary for activity.\(^{13}\)

HSPGs are likely to be involved in FGF signalling in the nervous system. FGFs and their high affinity receptors are widely expressed throughout the nervous system, both during development and in adulthood (Elde et al., 1991; Fu et al., 1991; Gómez-Pinilla and Cotman, 1992; Gómez-Pinilla et al., 1992; Gonzalez et al., 1990; Hughes et al., 1993; Peters et al., 1993; Stock et al., 1992; Wanaka et al., 1991; Wilcox and Unnerstall, 1991; Woodward et al., 1992). FGFs can affect neuronal proliferation, survival, and repair (Gómez-Pinilla et al., 1992; Hughes et al., 1993; Walicke, 1988), and can also promote mitogenesis and differentiation of neuroepithelia (Bartlett et al., 1988; Murphy et al., 1990). Interestingly, a HSPG has been characterized from neuroepithelia which has a high affinity for FGF-2, but at a later stage developmental age switches to having an apparent high affinity for FGF-1 (Nurcombe et al., 1993)--the timing of this suggested switch correlates with the expression of these FGFs in neuroepithelia.

Cell adhesion, migration, and axon outgrowth. The interaction of cells with each other and to the extracellular matrix is important in regulating processes such as cell migration and axon

\(^{13}\)Ornitz et al. (1995) report, however, that nonsulfated di- and trisaccharides of heparin can activate signaling by FGF-1 and FGF-2, presumably by oligomerization.
growth, and influences the morphogenesis of the nervous system (Hynes and Lander, 1992). PGs probably play a role here, as many of these interactions are mediated by proteins that can bind GAGs. Such proteins include NCAM, which is involved in cell-cell adhesion, and extracellular matrix molecules such as laminin or fibronectin, which can bind integrins associated with cell membranes. These proteins are widely distributed in the central and/or peripheral nervous system during development.

PGs are likely to act as coreceptors in modulating cell-cell or cell-substratum adhesion. HS is important in the homophilic binding of NCAM, as exogenous heparin and antibodies to the heparin-binding domain of NCAM inhibit NCAM-mediated cell adhesion; peptides corresponding to the heparin-binding domain of NCAM mutations in this domain also inhibit adhesion via NCAM (Cole et al., 1986; Reyes et al., 1990). Attachment of retinal cells to thrombospondin can be blocked by the addition of heparin (Neugebauer et al., 1991). Likewise, CHO cells deficient in HS and CS adhere to fibronectin but poorly or not at all to the heparin-binding domain of fibronectin (LeBaron et al., 1988). Even on intact fibronectin, these cells do not form stress fibers or focal adhesions as judged by localization of F-actin. This suggests that even when adhesion is occurring in the absence of GAG, that adhesion is somehow different or incomplete. These results also imply that measurement of cell adhesion may be a poor way to assess the roles of GAGs, as GAGs may be involved in responses that cannot be detected by simply measuring the initial strength of cell adhesion.

In addition to acting as coreceptors, PGs can also directly mediate adhesion. For instance, syndecan-1 directly mediates the adhesion of myeloma cells to collagen type I (Sanderson et al., 1992), and prevents migration of those cells into collagen gels (Liebersbach and Sanderson, 1994) The ability of different myeloma cell lines to adhere to collagen correlates with the affinity of their HS for collagen. Melanoma and neuroblastoma cells can adhere to a heparin-binding peptide derived from fibronectin; this adhesion is heparin-dependent, and is likely to be mediated through
a GPI-linked HSPG (Drake et al., 1992; Haugen et al., 1992a; McCarthy et al., 1990).

Other studies have found that PGs can have inhibitory effects on cell adhesion in vitro. While the effects of PGs on cell adhesion mentioned above are likely due to cell surface PGs, the inhibitory effects discussed below are usually due to the effects of PGs on the substrates used. In some cases what is observed is likely to involve the steric effects of the GAG. The inhibition of rat yolk sac tumor cell attachment to fibronectin or collagen type I by a CSPG (presumably serglycin) depends on the ability of that CSPG to bind to the substrate (Brennan et al., 1983). Likewise, cell adhesion of fibroblasts to substrates of fibronectin or other ECM molecules is inhibited by substrate-bound PG-M, but not the core protein (Yamagata et al., 1989). The inhibitory effect is due to the presence of substrate-bound CS, as the effect could be reproduced with BSA-conjugated CS but not soluble CS. A brain KSPG, when added to substrates of laminin or NCAM, also inhibits cell adhesion in a KS-specific manner (Cole and McCabe, 1991). That these effects are steric is likely, as the inhibitions reported are generally seen with large added concentrations of PG (furthermore, these studies usually do not measure the amount of protein actually absorbed to substrate). Further supporting the idea of steric hindrance is the finding that integrins can bind to the core protein of perlecan, but this binding is reduced with the intact PG (Hayashi et al., 1992). It is also possible that inhibition is due to competition of GAG for binding with cell surface GAG-binding receptors; however, no such mechanism has ever been demonstrated.

Often, the inhibitory effects of PGs on cell adhesion are due to the specific effects of individual core proteins as opposed to the actions of GAGs. Inhibitory effects of neurocan and phosphacan on NCAM- and NgCAM-mediated cell-cell adhesion, as well as cell-substratum adhesion to and neurite outgrowth on those proteins as well as tenascin, have been observed (Friedlander et al., 1994; Grumet et al., 1993; Grumet et al., 1994; Milev et al., 1994). These effects are generally still observed, however, when PG core proteins (prepared by enzymatic degradation of attached CS) are utilized in
these assays. As with GAGs, these effects may also be due to the steric effects of these cores, perhaps by the masking of functional domains on the proteins used as substrates in these assays.

Less is known regarding the involvement of GAGs in axon growth and guidance. Much of the focus of research involves the reported inhibitory effects of CS and KS for axons. Some have hypothesized that CS and KS are inherently inhibitory for axons, and that these GAGs are used in brain to form barriers. This idea is based on the localization of CS and KS to apparent barriers in vivo (such as the roof plate of the spinal cord) and on the inhibitory effects of some CS or KS on neurite outgrowth in vitro (Brittis et al., 1992; Geisert and Bidanset, 1993; Snow et al., 1992; Snow et al., 1991). One mechanism proposed for such inhibition involves the interaction of CS or KS with a cell surface receptor for CS or KS. No such receptor has yet been identified.

The effects of these GAGs on neurite outgrowth, as with their effects on cell adhesion, could be explained by steric hindrance on outgrowth-promoting substrates. For instance, an HSPG has been purified from Schwannoma cells which inhibits neurite outgrowth on laminin; in order for this inhibition to occur the PG must be complexed to laminin on the substrate and HS must be attached (Muir et al., 1989). While other interpretations are possible (such as conformational effects of binding on laminin), these experiments are consistent with a model of steric hindrance. In fact, as with cell adhesion, other studies demonstrating inhibition of neurite outgrowth have often used high relative concentrations of inhibitory PG or GAG.

In some cases, however, the inhibition of neurite outgrowth cannot be ascribed simply to a steric effect. The KSPG ABAKAN is an inhibitor of cell adhesion and neurite outgrowth on laminin; this inhibitory effect is masked by the addition of an antibody that binds KS (Geisert and Bidanset, 1993). This result is difficult to explain by a simple steric mechanism, as the GAG chain is still present on the substrate, and in fact has additional size due to the presence of the attached antibody. It may be that, in this case, KS is interacting with a cell surface KS-binding protein important for cell adhesion.
neurite outgrowth. However, other effects, such as changes in the substrate as a result of antibody binding, are also possible.

Further examination suggests that CS and KS are not in general inhibitory molecules. While present in apparent axonal barriers, CS and KS are also found in regions where axons grow, such as the neocortical subplate or optic nerve, and at times when axons are growing (Bicknese et al., 1994; McAdams and McLoon, 1995). In addition, the CSPG DSD-1 has been reported to support, via its GAG, neurite outgrowth in vitro (Faissner et al., 1994); neurites grow on substrates of DSD-1 CSPG but fail to due so when the CS has been enzymatically removed.

Often, inhibitory effects on neurite outgrowth are due not to GAG but to the action of individual core protein. CSPGs purified from brain inhibit PC12 cell neurite outgrowth, even when the CS has been enzymatically removed (Oohira et al., 1991). Likewise, it has been reported that neurocan and phosphacan core proteins inhibit neurite outgrowth on NCAM, NgCAM, and tenascin (Friedlander et al., 1994; Grumet et al., 1993). Individual core proteins can have effects on cell migrations as well—the hyaluronate-binding domain of a cartilage PG, presumably aggrecan, has been suggested to inhibit neural crest cell migration on fibronectin (Perris and Johansson, 1990).

Little is known about the role of HS in promoting axon growth and guidance. The few studies done, however, suggest that HS can influence these processes. Spinal cord neurons extend neurites on heparin-binding peptides derived from fibronectin, and this outgrowth can be reduced by soluble heparin (Haugen et al., 1992b). Neurite outgrowth on HB-GAM is inhibited by heparitinase or added heparin (Raulo et al., 1994). In addition, HS has been shown in insects to be important for axon guidance, as exogenous heparin or treatment with heparitinase causes axons to grow randomly or along incorrect routes (Wang and Denburg, 1992). That HS is likely to play a role is supported by the observation that many of the proteins which promote axon growth and guidance bind to heparin.
5. Conclusion.

PG structure and function can be regulated at many different levels by cells. Cells can selectively regulate expression of PG cores. They can also regulate the structure of the GAG that is attached. In this way, cells may create an environment, both at the cell surface and in the extracellular matrix, which with they and other cells can interact.

It is apparent that this regulation at both levels occurs in the nervous system. Most if not all of the major PG core protein families are represented in the brain. The expression of these core proteins, however, is both temporally and spatially regulated. GAG structure is also temporally and spatially regulated in the nervous system, and is likely to reflect in synthetic potentials of the various cell types composing the nervous system.

This regulation is likely to reflect function. The GAG-binding proteins to which cells respond will depend on the way in which those cells regulate GAG synthesis, and hence which high affinity binding sites they produce on their GAGs. The subsequent responses to those selected GAG binding proteins may depend on the particular core proteins expressed by those cells; such responses could thus be controlled by regulation of core protein expression. In addition, the extracellular matrix will also be shaped by the regulation of GAG synthesis and core protein expression. PG expression thus is likely to reflect function; studying PG expression during neural development will give insight into those functions.

This thesis addresses aspects of PG expression and function. In Chapter 2, I present data showing that a particular PG, glypican, is expressed in the rat brain, and that its expression is spatially regulated in the adult nervous system. Chapter 3 addresses the expression of glypican during neural development. In this chapter, I also discuss the localization of both glypican and cerebroglycan.

\[\text{There is little evidence that core proteins direct the synthesis of specific GAG structures beyond the specification of HS or CS chains. While some groups have reported binding differences between core proteins from the same source to certain GAG binding proteins (Aviezer et al., 1994; Mertens et al., 1992), these differences have not been linked to differences in GAG structure.}\]
protein products. In Chapter 4, I present data regarding the expression of the small matrix CSPG decorin during spinal cord development, and show that it binds netrin-1, a factor that promotes axon growth and guidance. Appendix 1 discusses preliminary results regarding the processing of glypican in myeloma cells and its implications for mechanisms of GAG synthesis.
Chapter 2: Neuronal expression of glypican, a cell surface glycosylphosphatidylinositol-linked heparan sulfate proteoglycan, in the adult rat nervous system.
Introduction.

The interaction of cells with their environment is important for the growth and function of the nervous system. These interactions control processes such as cell adhesion, migration, proliferation, axon growth and guidance, and responsiveness to growth factors. Proteoglycans (PGs), proteins that contain one or more covalently attached glycosaminoglycan (GAG) chains, are thought to be involved in many such interactions. PGs are found both in the extracellular matrix and on cell surfaces. Through their GAG chains PGs have the ability to bind a number of important nervous system proteins, including extracellular matrix proteins (such as laminin, fibronectin, thrombospondin, and agrin), cell-surface molecules (such as NCAM, myelin-associated glycoprotein, the amyloid-β-protein precursor), synaptic enzymes (acetylcholinesterase) and growth factors of the fibroblast growth factor (FGF) family (for reviews, see Jackson et al. [1991]; Lander and Calof, [1993]). In some cases it has been demonstrated that GAGs—in particular GAGs of the heparan sulfate (HS) class—play an essential role in the functions of these proteins. For example, cell-surface HSPGs are apparently required for basic FGF (bFGF) to bind to cells and exert its effects (Yayon et al., 1991; Rapraeger et al., 1991; Kan et al., 1993), as well as for NCAM-mediated cell adhesion (Cole et al., 1986; Reyes et al., 1990), and agrin-mediated clustering of muscle acetylcholine receptors (Gordon et al., 1993).

Currently, it is not known which PGs in the nervous system mediate these and other biological activities, nor where such PGs are expressed. Up to 25 PG core proteins have been reported in the developing and adult rat brain (Herndon and Lander, 1990), including molecules bearing both HS and chondroitin sulfate (CS) GAGs. Those PGs for which information exists on regional distribution in the nervous system are mainly CSPGs: these include the Cat-301 antigen (Hockfield et al., 1990), neurocan (Rauch et al., 1992), versican (Perides et al., 1992), and the T1 antigen (Iwata and Carlson, 1993)—all of which appear to be extracellular matrix-associated—and one cell surface CSPG, NG2 (Stallcup et al., 1983; Levine and Card, 1987; Nishiyama et al., 1991). In addition, immunological studies have identified distinct CS epitopes on
subsets of CNS neurons (Watanabe et al., 1989; Fujita et al., 1989; Maeda et al., 1992).

In contrast, much less is known about the expression of PGs in the brain that bear HS chains. One, known as N-syndecan or syndecan-3 (for nomenclature, see Bernfield et al. [1992]), is an integral membrane PG expressed mainly during perinatal development, and appears to be associated with many cell bodies (Carey et al., 1992). An HSPG has also been purified from mouse neuroepithelium (Nurcombe et al., 1993). Other brain HSPGs have been characterized biochemically, but their distributions have not been elucidated (Margolis et al., 1975a; Margolis et al., 1975b; Klinger et al., 1985; Herndon and Lander, 1990).

Two of the major HSPGs of the rat brain, known as M12 and M13 (Herndon and Lander, 1990) associate with isolated membranes, and exhibit detergent-partitioning properties indicative of integral membrane proteins. These PGs lose their detergent-partitioning properties when treated with phosphoinositide-specific phospholipase C, indicating that they associate with membranes via a covalent glycosylphosphatidyl-inositol (GPI) lipid linkage (Herndon and Lander, 1990). Several other HSPGs have been reported to possess GPI-anchors, including HSPGs isolated from rat ovarian granulosa cells (Yanagashita and McQuillan, 1989), mouse melanoma cells (Drake et al, 1992), rat Schwann cells (Carey and Stahl, 1990), and human lung fibroblasts (David et al., 1990). The latter molecule was cloned and given the name glypican (David et al, 1990).

In the present study we describe the purification and cDNA cloning of M12 from rat brain and show that it is the rat homologue of glypican. In situ hybridization studies are presented that indicate that M12/glypican is regionally expressed in the adult brain. Specifically, glypican is found to be expressed at high levels by restricted populations of projection neurons. Some of these data have been presented previously in abstract form (Litwack et al., 1992).
Materials and Methods.

Purification of HSPG M12. A membrane fraction was isolated from 46.4 g wet weight of neonatal rat brains (roughly 165 animals), and a detergent extract of this fraction was prepared (Herndon and Lander, 1990). All further steps were done at 4°C except where noted. PGs were purified by loading the detergent extract onto a column (172 ml) of DEAE-Sephacel, washing sequentially in 50 mM Tris-HCl (pH 8.0 at 4°C), 0.1% Triton X-100, and protease inhibitors as in Herndon and Lander (1990) containing a) 0.15 M NaCl (starting buffer), b) 0.25 M NaCl, c) 0.25 M NaCl, 6 M urea, and d) 0.25 M NaCl, 6 M urea, with 50mM formate (pH 3.5) replacing the Tris-HCl. The pH of the column was then restored to pH 8.0 with starting buffer, and the PGs were eluted with starting buffer containing 0.75 M NaCl. This PG-enriched fraction was concentrated and exchanged into starting buffer containing 0.15 M NaCl using a Centriprep-10 (Amicon), and was then digested with 0.09 units/ml chondroitinase ABC (Sigma) for 2 hours at 37°C. This material was loaded on a 0.2 ml DEAE-Sephacel column, washed with starting buffer (without protease inhibitors) and then 0.2 M NaCl, 6 M urea, 100 mM sodium acetate (pH 3.5), 0.1% Triton X-100. The column was washed with 25 mM ammonium acetate (pH 7.0), 0.1% Triton X-100 and eluted with a 20 ml 0.15 M - 0.75 M NaCl gradient in this buffer. The fractions from 0.3 M to 0.75 M NaCl were pooled, and this PG-enriched fraction, now depleted of chondroitin sulfate PGs; was concentrated and exchanged into 25 mM ammonium acetate (pH 7.0), 0.1% Triton X-100, 0.15 M NaCl in a Centricon-10. This sample was made 25 mM in Tris-HCl (pH 7.1 at 37°C) and digested with 9 μg/ml heparitinase (prepared as in Herndon and Lander [1990]) for 3 hours at 37°C. The sample was concentrated in a Centricon-10, subjected to electrophoresis in a 9% SDS-PAGE gel (Laemmlli, 1970) and electroblotted to nitrocellulose (Schleicher & Schuell). HSPG core proteins were visualized by amido black staining (Schaffner and Weissman, 1973). M12 and M13 were excised, and were simultaneously eluted from the filters and digested with trypsin as in Tempst et al. (1990). The tryptic peptides were separated by reverse-phase HPLC (Tempst et al., 1990) and microsequenced by automated Edman degradation (Biopolymers Lab, MIT).
Cloning and sequencing of rat glypican. To obtain a human glypican probe, cDNA was synthesized from 0.5 μg of human foreskin fibroblast total RNA (purified as described by Chomczynski and Sacchi [1987]) using MMLV-reverse transcriptase (Gibco BRL). This cDNA was then amplified by PCR using 120 pmol each of oligonucleotides GLA (5'-ggttcggaaagtggctcaggtc-3') and GLB (5'-ggtttggtgatctggttggccag-3'). These primers correspond respectively to bases 920 to 941 (in the sense orientation) and 1539 to 1518 (in the antisense orientation) of human glypican (David et al., 1990). PCR was performed for 30 cycles: 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1.5 minutes. The resulting product was digested with XhoI and KpnI (human glypican contains an XhoI site at base 690 and a KpnI site at base 1494), isolated by agarose gel electrophoresis, and cloned into pBluescript (Stratagene). The clone was verified by partial sequencing.

A Lambda-Gem 2 cDNA library (gift of Dr. John Wagner, Cornell University Medical School) constructed from A126 cells (a PC12 rat pheochromacytoma cell variant) was screened with the human glypican fragment labelled by the random primer method. Twelve positive clones were obtained from an initial screen of 400,000 plaques. The inserts from positive phage were subcloned into the EcoR1 site of pBluescript and both strands of the largest, a 3 kb clone designated 41a, were sequenced by the dideoxynucleotide method using Sequenase (USB) or the fmol DNA Sequencing System (Promega). Sequencing primers were synthesized based on internal sequence (Biopolymers Lab, MIT), or restriction fragments were subcloned into pBluescript and sequenced using T3 and T7 primers. Clone 41a contains 3000 base pairs (bp) of the 3' end of rat glypican.

In order to obtain the 5' fragment of rat glypican, a primer-extended cDNA library was constructed from 5 μg of PC12 poly(A)+ RNA using the Time-Saver kit (Pharmacia). This poly(A)+ RNA was isolated over oligo-dT cellulose using the FastTrack kit (Invitrogen). The oligonucleotide RG6 (5'-ccctgcacaaaggatcgt-3', situated 213 base pairs downstream of the 5' end of clone 41a) was used to prime first-strand synthesis. The final cDNA product was ligated into the EcoR1 site of pBluescript and transformed into XL1-Blue cells.
clone of about 950 bp, designated 7a3, was obtained by colony hybridization with probe 4EX (see below). Both strands of 7a3 were sequenced as above, and assembled with 41a based on their overlapping sequence. The assembled cDNA contains an open reading frame that predicts the glypican amino acid sequence.

In order to obtain glypican cDNA fragments directly from rat brain, PCR was performed as above from 0.5 μg rat brain total RNA using the following sets of oligonucleotides: a) RG15 (5'-tttgttgtctcgcctctc-3') and RG12 (5'-acgtcgctcaggctaaag-3'); b) RG11 (5'-cactgcgcgtcatcactgg-3') and RG10 (5'-tgctcaaaggctgccttg-3'); c) RG11 and RG4 (5'-ctcatcactgacaagttc-3'); and d) RG5 (5'-aggatgcccagtgtgcagc-3') and RG7b (5'-tgcccaagacagtccttt-3'). The PCR products were purified on a 2% Nusieve (FMC) agarose gel, and sequenced with Taq polymerase using the fmol sequencing kit (Promega) and oligonucleotides end-labelled with 32P-ATP.

**Rat glypican probes.** Probe 4X1 is a 162 bp EcoR1-XhoI fragment from clone 41a inserted into pBluescript. Antisense RNA probes were made by digesting the plasmid with EcoR1 and transcribing with T7 RNA polymerase (Stratagene) in the presence of 35S-UTP; sense RNA probes were synthesized by digesting with XhoI and transcribing with T3 RNA polymerase (Stratagene). Probe 4P1 is a 567 bp PstI fragment subcloned from 41a into pBluescript. Antisense RNA probes were made by digesting with EcoR1 and transcribing with T7 RNA polymerase. Probe 4P2 is a 332 bp PstI fragment subcloned from 41a into pBluescript. Antisense RNA probes were made by digesting with BamH1 and transcribing with T7 RNA polymerase. A rat glyceraldehyde-3-phosphate dehydrogenase DNA probe was a gift of Dr. Timothy Hayes (N.I.H.). Probes for Northern blots were labelled with 32P-dCTP using the Random Primer Labelling Kit (USB), and denatured at 100°C for 7 minutes before use.

**Northern blotting.** Total RNA, purified as described by Chomczynski and Sacchi (1987), was separated by electrophoresis in a 1.2% agarose/6.4% formaldehyde gel and transferred to nitrocellulose by capillary blotting. The filter was air dried for at least one hour, and
then vacuum baked for 2 hours at 80°C. Prehybridization was carried out at 42°C overnight in 50% formamide, 5X saline-sodium phosphate-EDTA (SSPE), 5X Denhardt’s, 0.1% SDS, 0.2 mg/ml yeast RNA, and 0.1 mg/ml salmon sperm DNA. Hybridization was carried out at 42°C for two days in 50% formamide, 5X SSPE, 1X Denhardt’s, 0.1% SDS, and 0.1 mg/ml yeast RNA. Filters were washed once at 42°C in 50% formamide, 5X SSPE, 0.5% SDS for 30 minutes, twice at 42°C in 1X SSPE, 0.5% SDS for 15 minutes each, and twice at 65°C in 0.1X SSPE, 0.5% SDS for 30 minutes each. Filters were exposed to Kodak XAR-5 film at -80°C and/or imaged using a phosphorimager (Molecular Dynamics, Sunnyvale CA).

In situ Hybridization. Brains from 6-7 week old female Sprague-Dawley rats were dissected and quick-frozen in isopentane chilled on dry ice. 10 μm cryostat sections were thawed onto gelatin-coated slides or Probe-On Plus slides (Fisher). Sections were stored at -80°C until use.

In situ hybridization was performed as described by Simmons et al. (1989). Briefly, sections were pretreated by fixation with 4% paraformaldehyde in PBS for 5 minutes, washed in 2x saline-sodium citrate (SSC), and treated with acetic anhydride to block positive charges. Sections were then washed again in 2x SSC, dehydrated stepwise in ascending alcohol concentrations, delipidated (in some instances) in chloroform for 10 minutes, washed again in 100% ethanol, and dried for at least one hour.

Sections were incubated overnight at 55-60°C in 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1x Denhardt’s solution, 1 mM EDTA, 10 mM Tris (pH 8.0), 0.1 mg/ml yeast tRNA, 200 mM DTT, 1% sarcosyl, and RNA probes at a concentration of 5x10^6-1x10^7 cpm/ml.

Sections were washed several times in 4x SSC, treated with RNase A for 30-60 minutes, washed in descending salt concentrations, and incubated in 0.1X SSC at 60°C for 30 minutes. Sections were then dehydrated in ethanol and dried. Sections were exposed to Hyperfilm-ßMax (Amersham) or XAR-5 film (Kodak) for 10 to 14 days at -80°C. Sections were dipped in NTB-2 diluted 1:1 in water at -80°C for 15-30 days, developed in D-19 (Kodak),
counterstained with either cresyl violet or bisbenzimid (Hoechst 33258) (Sigma), and studied with dark-field optics on a Zeiss Axiophot or WILD microscope. Hybridized sections were compared in some cases with nearby sections stained with cresyl violet, and with sections illustrated in Paxinos and Watson (1986) and Swanson (1992).

Production of glypican polyclonal antibodies. The EcoR1 fragment of glypican clone 41a was cloned into the EcoR1 site of pMalc2 (New England Biolabs), producing a construct that encoded a fusion protein of glypican with the bacterial maltose-binding protein. The construct was sequenced to verify that the junction was in-frame. The fusion protein was induced by IPTG, and isolated by affinity chromatography on an amylose column (New England Biolabs). The fusion protein was then dialyzed into 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 25 mM octyl-β-D-glucopyranoside. Antibodies were raised at Pine Acre Rabbitry and Farms (Norton, MA). Rabbits were injected intradermally with 0.5 mg of fusion protein in complete Freund’s adjuvant and boosted subcutaneously with 0.5 mg fusion protein in incomplete Freund’s adjuvant. After two boosts, sera were collected and antibodies were purified over fusion protein coupled to Affi-Gel 10 (Bio-Rad).

Immunoprecipitation. Embryonic day 18 and adult rat brain membrane PGs were prepared and radioiodinated as described by Herndon and Lander (1990) and boiled in 0.1% SDS to disrupt aggregates of PGs that are present (cf. Herndon and Lander, 1990); Triton X-100 was then added to a final concentration of 1%. This material was incubated overnight at 4°C with 5 µg of affinity purified anti-glypican antibodies, and then absorbed to protein A-Sepharose (Sigma) for 1 hour at 4°C. The protein A-Sepharose was then washed sequentially in 0.1 M Tris (pH 7.5), 2mM EDTA, 0.5% 3-[(3-cholamidopropyl)dimethylammonio-]-1-propanesulfate (CHAPS) containing a) 150 mM NaCl, b) 0.5 M NaCl, and c) 150 mM NaCl; each wash was done at 4°C for 30 minutes. Antibody-antigen complexes were dissociated by boiling the Sepharose in 0.5% SDS and diluting into 1% Triton X-100, 50 mM Tris, 15 mM phosphoric acid (pH 7.3),
and protease inhibitors. Aliquots were digested with heparitinase at a concentration of 2 μg/ml at 43°C for 3 hours. Undigested and heparitinase digested samples were separated by electrophoresis on a 10% SDS-PAGE gel. The gel was dried and exposed to Kodak XAR-5 film at -80°C.

**Western Blotting.** Membrane and soluble fractions of rat brain were prepared and 20 μg aliquots were digested with heparitinase, chondroitinase ABC, or simultaneously with both enzymes (Herndon and Lander, 1990). Samples were subjected to electrophoresis in a 7% SDS/PAGE gel (Laemmli, 1970), and electroblotted to nitrocellulose (Schleicher and Schuell). The nitrocellulose was incubated at room temperature for one hour in 20% goat serum (Sigma) in TBST (100 mM Tris-HCl [pH 8 at 4°C], 150 mM NaCl, 0.1% Tween-20), and then for one hour in 2.5 mg/ml 343-1 in 5% goat serum/TBST. After washing several times in 5% goat serum/TBST, the nitrocellulose was then incubated for one hour in a 1:5000 dilution of a horseradish peroxidase-linked goat anti-rabbit IgG antibody (Amersham) in 5% goat serum/TBST. The filter was then washed several times in TBST and then developed by ECL (Amersham).
Results

To determine the identities of two rat brain PGs that had previously been shown to contain GPI anchors, HSPGs M12 (core Mr~65 kD) and M13 (Mr~55 kD) (Herndon and Lander, 1990) were purified from neonatal rat brain, and peptides derived from the two core proteins were sequenced. Four M12-derived peptides were obtained. These possessed a total of about 80% identity to regions of the human glypican protein (Table 1), suggesting that M12 is rat glypican. M13 peptides were not highly similar to human glypican, and were used to isolate a cDNA clone encoding a novel PG core protein (Stipp et al., 1994).

To obtain a rat glypican cDNA clone with which to compare the M12 peptides, a human glypican probe was synthesized via PCR. This probe detected a single 3.7 kb message in neonatal rat brain total RNA (Fig. 1A). A message of the same size was detected in human foreskin fibroblast RNA (Fig. 1A), in agreement with the previously reported human glypican message size from human lung fibroblasts (David et al., 1990). A single message of this size was also detected in PC12 cells, a rat pheochroma-cytoma cell line, and was more abundant in RNA from these cells than in RNA from neonatal rat brain (Fig. 1A). This finding is consistent with previous evidence that an M12-like proteoglycan is the major, if not only, heparan sulfate proteoglycan found in PC12 cells (Herndon et al., 1991).

A full-length rat glypican clone was obtained from PC12 cells as described in Materials and Methods. The cDNA sequence predicts a protein that is 89% identical to human glypican (the degree of identity increases to 91% if GPI-attachment sequences, which should not be present in the mature protein, are removed). The predicted protein sequence also contains sequences identical to those of the peptides derived from M12 (Fig. 2). Antibodies raised and purified against a fusion protein synthesized from part of this cDNA detected M12 in a Western blot of rat brain membranes (data not shown) and also immunoprecipitated M12 from crude mixtures of PGs obtained from both embryonic and adult rat brain (Fig. 3). Thus, we conclude that M12 is the rat form of glypican.

While this work was in progress, peptide sequences from a rat
PEPTIDE 15: AEALRPFGDAPR
human glypican: AEALRPFGDAPR

PEPTIDE 29: BXLPEVMGDGLANQXNPEVD
human glypican: RYLPEVMGDGLANQINNPEVE

PEPTIDE 15a: MELETALHDSSR
human glypican: AELETALRDSSR

PEPTIDE 26: BALQATLATQLHGIDDDHFQ
human glypican: RVLOQAMLATQLRSPDDHFQ

Table 1. Sequence of tryptic peptides of purified M12 compared with human glypican. Solid lines represent identities; dotted lines represent amino acids with similar properties. B refers to either K or R.
Figure 1. Detection of glypican message in rat brain, PC12 cells, and fibroblasts. A, Northern blot of 30 μg total RNA probed with a human glypican cDNA. Lane 1, Rat fibroblast cell line F2408 RNA; lane 2, PC12 cell RNA; lane 3, neonatal rat brain RNA; lane 4, human foreskin fibroblast RNA. B, Northern blot of 20 μg total RNA from rat brain probed with rat glypican cDNA probe 4X1. Lane E, embryonic day 18 rat brain RNA; lane N, neonatal rat brain RNA; lane A, adult rat brain RNA. The filter was stripped and reprobed for rat glyceraldehyde phosphate dehydrogenase (shown below), to demonstrate the efficiency of loading and transfer of RNA. Levels of glypican RNA relative to this control RNA are roughly equivalent at the three developmental stages. Arrows in A and B point to position of 18S and 28S ribosomal RNA bands.
Figure 2. Comparison of rat and human glypican predicted protein sequences. The rat sequence is listed on top; the human, on the bottom. Amino acids in the human sequence identical to those in the rat sequence are indicated with a dash; differences are listed. Potential glycosaminoglycan attachment sites are indicated in boldface; potential N-linked glycosylation sites are indicated by asterisks; potential glypiation site is indicated by an arrow; putative N- and C-terminal transmembrane hydrophobic stretches (corresponding respectively to putative signal and glypiation sequences) are indicated in italics; regions corresponding to microsequenced peptides derived from purified M12 are underlined. The nucleotide sequence of the full-length cDNA extends about 70 bp past the 5' end and 200 bases beyond the 3' end of the cDNA reported by Karthikeyan et al. (1992). Sequence was assembled from clones 7a3 and 41a. The 3' end of clone 7a3 corresponds to base 883 and the 5' end of 41a corresponds to base 681 of the sequence in Karthikeyan et al. (1992).
MELRARGWLLCAAALVACARGDPASKRSCSEVRQIIYGAKGFSLSVPQAEISGEHLRI^{51} (rat)
---------------------------------------- -G-----------------------------------------
CPQGYTCCTSEMEENLANSHSMELETALHDDSSRALGATLATGLHGIDDDHFEQRLINDSERTL^{122} (human)
---------------------------------------- -R-HA----R----V----M----RSF----H--------
QDAFPAGFDLYTQNTAFRDLYAELRLYYRGAHLHLEETLAEFWARRLERLFKLHPLQL{183}
-AT--------E---------A----S----------------------------------
LPDDYLDCLGKQAELRPECADDRERLRRLRATRAFVALRSFVQGGLVAGAVRVRKVAQVPLA{244}
---------------------------------------- -E----------------------------------
ECSRAVMKLVYCAHRGVPGARPCTDYCRNVLKCLANQALDAEWRNLLDSMVNLITDFP^{305}
---------------------------------------- -L-----------------------------------
GPSGAENVIGSVHWMALAEAINALQDNKDTLTAKVQGCGNPVPHGSPFPEKRRGKLAL^{366}
-T----V----T----T---------R----------------------------------Q-P----------
QEKSSTGTLEKLVEAKALRDIQDYWISLPGTLCSEKMAMSPASDDCNGISKGRYTE^{427}
R-RPPS----------------------------------L-T------MAR------
VMGDGLLANGINPEVEVDITKPDMTIRQQIMQLKIMTNRLRGRAYGNDVDQDASDDGS^{488}
---------------------------------------- -S-N----------------------------------
GSGGGCPDCCGRRVSKKSSSSTPTLHALPGLSEQEQQKTSATRPEPHYFFLLFLFTL^{549}
---D---L---L---K---R----------------------------------SC-Q-PT-L-PL-LF-A
LAAAARPWR^{558}
-IV---------
Fig. 3. Antibodies raised against glypican fusion protein recognize adult rat brain HSPG M12. $^{125}$I-labelled adult rat brain PGs (Herndon and Lander, 1990) were immunoprecipitated with 5 µg rabbit IgG (lanes 2 and 6), or 5 µg affinity purified anti-glypican serum (lanes 3 and 7), or 5 µg anti-glypican serum containing 20 mg glypican fusion protein. Lanes 1 and 5 show the starting material. Samples were either subjected to SDS-PAGE without heparitinase treatment (lanes 1-4) or treated with heparitinase before electrophoresis (lanes 5-8). Arrowhead indicates the position of the M12 core protein. Mr markers (in kD) are shown at right.
brain HSPG that were closely related to human glypican were reported (Karthikeyan et al., 1992). This report also contained a sequence for a rat glypican cDNA that contains four amino acid differences (due to single base differences) from the sequence presented here (Fig. 2): T instead of A at position 21, Y instead of N at position 311, A instead of G at position 362, and I instead of T at position 515. To address whether these differences represent sequence polymorphisms between glypican from PC12 cells and glypican from Sprague Dawley rats, we used PCR to obtain from Sprague-Dawley rat brain RNA, cDNA fragments spanning three of the four regions of sequence discrepancy. Sequenceable PCR products were obtained that span three of the four regions (positions 312, 362, and 515), and direct sequencing of these fragments gave results that agreed in all cases with the sequence reported here for PC12 cells (data not shown). Consequently, the data do not support the existence of glypican sequence polymorphisms.

A northern blot using a probe from the rat glypican cDNA confirmed that a single 3.7 kb glypican message is present in total RNA from adult rat brain, and also detected a similarly sized message in the embryonic (day 18) and neonatal rat brain (Fig. 1B). This is in agreement with expression of M12 protein, which was detected at each of these stages (Herndon and Lander, 1990).

In situ hybridization experiments were undertaken to identify the cell types that express glypican in the adult rat nervous system. High levels of glypican expression were detected in several structures in the adult rat brain. In the hippocampus, glypican was expressed in high levels in the pyramidal cell layer (Fig. 4A). Furthermore, the pyramidal cell layer in regions CA3 and CA4 expressed glypican mRNA at substantially higher levels than in regions CA1 and CA2. Some hybridization signal was also seen in the dentate gyrus, although at lower levels than in regions CA3 and CA4. High levels of expression were found throughout the dorsal thalamus (Fig. 4A-C), including the ventrolateral, ventrobasal, mediodorsal, lateral geniculate, and medial geniculate nuclei, but expression was not seen in the medial and lateral habenula nor in the ventral thalamus (e.g., the reticular nucleus and zona incerta).
Figure 4. *In situ* hybridization of adult rat brain with *rat glypican.*

**A,** Coronal forebrain section probed with 4X1, viewed in dark field. **B,** Coronal forebrain section at the level of the anterior thalamus probed simultaneously with 4X1 and 4P2, viewed in dark field. **C,** Coronal section through the posterior thalamus and anterior midbrain, probed with 4X1, viewed in dark field. **D,** Coronal section at the level of that in **A** probed with the sense (control) strand of 4X1, viewed in dark field. **E,** Coronal section through the anterior forebrain probed simultaneously with 4X1 and 4P2; hyperfilm image. **F,** Coronal section through the brainstem probed with 4X1, viewed in dark field. **G,** A section approximately 30 μm from that in **F** stained with cresyl violet. **H,** Coronal section through the brainstem probed with 4X1, viewed in dark field. **I,** Coronal section through the brainstem probed with 4X1, viewed in dark field. **J,** Section through a dorsal root ganglion probed with 4P1, viewed in darkfield. *Arrows* point to silver grains clustered over sensory neuron cell bodies. **K,** Same section as in **J** stained with cresyl violet. **L,** High-magnification view of the septum and lateral ventricle from a coronal section through the forebrain. **M,** Same section as in **L** stained with cresyl violet. *Asterisk* in **B** indicates supraoptic nucleus of the hypothalamus; *arrow* in **B** indicates the pyramidal layer of the nucleus of the lateral olfactory tract. *acc,* nucleus accumbens; *bla,* basolateral nucleus of the amygdala; *cb,* cerebellum; *ct,* cerebral cortex; *dg,* dentate gyrus; *dt,* dorsal thalamus; *e,* ependyma; *ha,* habenula; *hi,* hippocampus; *lc,* locus coeruleus; *lv,* lateral ventricle; *me5,* mesencephalic nucleus of the trigeminal (5th) nerve; *mg,* medial geniculate nucleus of the thalamus; *mo5,* motor nucleus of the trigeminal (5th) nerve; *oc,* Edinger-Westphal nucleus of the oculomotor complex; *p,* piriform cortex; *rn,* red nucleus; *s,* septal area; *sc,* superior colliculus; *sn,* substantia nigra; *st,* striatum; *s5,* sensory nucleus of the trigeminal (5th) nerve; *tu,* olfactory tubercle; *7,* nucleus of the 7th cranial nerve; *12,* nucleus of the 12th cranial nerve. In the sequence reported by Karthikeyan et al. (1992), 4X1 corresponds to bases 681-843, 4P1 corresponds to bases 1873-2440, and 4P2 corresponds to bases 2435-2767. Scale bars: **A-I,** 500 μm; **J** and **K,** 200 μm; **L** and **M,** 20 μm.
Strong hybridization was also seen to certain nuclei of the amygdala (Fig. 4A,B), notably the pyramidal layer of the nucleus of the lateral olfactory tract and the basolateral nucleus. Weaker hybridization was seen to the lateral and basomedial nuclei, but no hybridization to the anterior, medial, central, and cortical nuclei were detected. The sense strand of glypican did not hybridize to any of these or other structures (Fig. 4D). In addition, similar results were obtained using several antisense probes synthesized from different regions of glypican (see Materials and Methods) (data not shown).

Other forebrain structures tended to show somewhat less hybridization than that observed in the hippocampus, thalamus, and amygdala. These included the piriform cortex (Fig. 4E), olfactory tubercle (Fig. 4E), septal nuclei (data not shown), and the supraoptic (Fig. 4B) and paraventricular nuclei (data not shown) of the hypothalamus. Still weaker, but detectable hybridization was seen to the cerebral neocortex (Fig. 4E; see also below) and nucleus accumbens (Fig. 4E).

In the brainstem, relatively strong glypican hybridization was seen to the red nucleus (Fig. 4C), locus coeruleus (Fig. 4F,G), and several cranial nerve nuclei, including the motor nuclei of the 5th (Fig 4F,G), 7th (Fig. 4H), and 12th (Fig. 4I) nerves. Hybridization was also seen to the oculomotor complex (Fig. 4C). In the spinal cord, relatively strong hybridization was seen to the ventral horns (data not shown), and to the dorsal root ganglia (Fig. 4J,K).

Many of the brain regions where glypican hybridization was seen contain principal neurons with large, easily recognizable cell bodies. When sections containing these regions were examined at higher magnification, it could frequently be established that hybridization was localized to such neurons. For example, Figure 5A shows glypican hybridization to cells in the nucleus of the 5th cranial nerve. Figure 5B shows that clusters of silver grains are present over large, lightly stained motoneuron cell bodies, but not over smaller, more darkly stained cell bodies (i.e., glia and small neurons). Similar results, that is, hybridization to large, lightly stained cells, were obtained in other regions of the brain, such as the thalamus and hippocampus (data not shown).
Figure 5. **Neuronal expression of glypican.** A, Motor nucleus of the trigeminal nerve. *Arrows* point to clusters of silver grains. *Arrowheads* point to positions of small nuclei. Dark-field view. B, Same field as in A. *Arrows* point to large, lightly stained nuclei under the clusters of silver grains indicated in A. *Arrowheads* point to small nuclei at positions indicated in A. C, Cerebellar cortex. *Arrows* point to positions of Purkinje cells. Dark-field view. D, Same field as in C. *Arrowheads* indicate the Purkinje cells marked in C. Scale bars, 20 µm.
A characteristic of most of the neurons in regions that were found to express glypican mRNA is that they are projection neurons. In the cerebral neocortex, however, large numbers of local circuit neurons are also present, and in layer 4 account for a large majority of the neurons (Zilles et al., 1990). Interestingly, glypican hybridization was not detected in layer 4 of the cortex, but was readily detected in other cell body layers, that is, 2/3, 5, and 6 (Fig. 6A,B). In layer 2/3, hybridization was strongest in the superficial part (Fig. 6B), and could be localized to a subpopulation of large cell bodies (Fig. 6E,F). In layers 5 and 6, hybridization was present at all levels, and was associated with most, but not all, large cell bodies (Fig. 6C,D). Although cell body size and location alone are not sufficient to establish the projection patterns of cortical neurons, the data are at least consistent with the view that glypican is primarily expressed by projection neurons.

Many types of projection neurons throughout the brain expressed little or no glypican mRNA, however. These included most neurons of the caudoputamen (Fig. 4E), the medial and lateral habenula (Fig. 4A), the superior and inferior colliculi (Fig. 4C and data not shown), the substantia nigra (Fig. 4C), and the cerebellum (Fig. 4H,I). In the latter structure, individual Purkinje neurons are readily visualized, and could be clearly shown to exhibit no glypican hybridization over background (Fig. 5C,D). Although the possibility remains that levels of glypican mRNA too low to detect are present in these cells, the results emphasize the fact that levels of glypican mRNA expression throughout the brain do no merely correlate with cell body size.

In addition to being present in subsets of neurons, glypican mRNA was also found in some supporting cell (stromal) elements of the brain. For example, glypican hybridization could be seen associated with some large blood vessels, although at weaker levels than in many of the glypican-expressing neurons (data not shown). Higher but variable levels of hybridization could also be seen associated with ependymal zones (e.g., of the lateral ventricles) (Fig. 4E,L,M).
Figure 6. **Glypican expression in the cerebral cortex.** A, *Right,* coronal section at the level of the basal ganglia stained with cresyl violet. *Left,* dark-field view of coronal section 30 μm from that on right hybridized simultaneously with 4X1 and 4P1. B, Magnified views of corresponding regions from sections in A. Lines indicate approximate divisions between cortical layers. C, High-magnification view of layer 5, dark field. D, Same field as in C, stained with Hoechst 33258. E, High-magnification view of layer 2, dark field. F, Same field as in E, stained with Hoechst 33258. Solid arrows in C-F indicated clusters of silver grains, and the cell nuclei with which they are associated; open arrows indicate large cell bodies lacking clusters of silver grains. Scale bars: A, 500 μm; B, 200 μm; F, 20 μm.
Discussion

A major PG of the adult rat brain, HSPG M12, has been identified as glypican based on the following observations: 1) M12 is a GPI-anchored PG with a core protein size (~65 kD) similar to that observed for human fibroblast glypican (Herndon and Lander, 1990; David et al., 1990); 2) peptides derived from M12 have amino acid sequences identical to those predicted from a rat glypican cDNA (this is in agreement with Karthikeyan et al., 1992, who also identified peptide sequences in a rat brain HSPG core protein that corresponded to sequences in a rat glypican cDNA); and 3) antibodies raised against a rat glypican fusion protein specifically immunoprecipitate M12 from a crude mixture of brain PGs and recognize a PG core protein that comigrates with M12 on a Western blot of heparitinase-digested brain membranes (data not shown). In addition, the expression of glypican message parallels the appearance of M12 core protein: we have found that glypican message is expressed in the adult and developing rat brain, as is M12, and that glypican mRNA can be detected by in situ hybridization in many brain regions.

Neuronal expression of glypican.

The regional distribution of glypican mRNA in the adult brain, as revealed by in situ hybridization, suggests the following general features of glypican expression in the central nervous system:

*Glypican is primarily a product of neurons.* In many of the regions where glypican mRNA is expressed, neuronal cell bodies can be recognized by morphological criteria, e.g. location, nuclear size and intensity of cresyl violet staining. In such regions (e.g. hippocampus, motor nuclei, dorsal root ganglia), glypican hybridization is found directly over such cell bodies. This suggests that glial cells, which outnumber neurons in the brain, do not express glypican, although low level expression, or expression by small numbers of glia cannot be ruled out. Supporting and connective tissue elements of the brain (e.g. linings of large blood vessels, meninges, ependyma) also exhibit some glypican hybridization, although at relatively low levels compared with neuronal expression.
Glypican-expressing neurons are predominantly projection neurons. Each of the neuronal populations that expresses glypican appears to consist mainly of cells with axons that project to other brain regions, or, in the case of motoneurons, to the periphery. Even in the cerebral cortex, glypican expression is present in laminae with corticocortical or corticofugal connections (e.g., layers 2/3, 5, and 6), but absent from layer 4, which consists primarily of local circuit neurons. Since projection neurons typically have long axons, relatively high levels of glypican mRNA expression might be required in some projection neurons, if it turns out that the physiological processes that involve glypican require the molecule to be present at the axonal surface. Indeed, glypican protein can be localized in some regions of the adult brain to fiber tracts. This is consistent with axonal expression and, in some cases, axonal polarization of other GPI-anchored proteins (e.g. F3/F11 [Faivre-Sarrailh, 1992], Tag-1 [Dodd et al., 1988; Furley et al., 1990], Thy-1 [Xue et al., 1990]).

Levels of glypican mRNA expression vary substantially among different populations of neurons. Based on the autoradiographic exposure times required to detect glypican expression in different brain regions, we can estimate relative levels of glypican expression (Table 2). High level expression does not bear a simple relationship to neuronal cell body size or axon length. Indeed, some large and medium-sized projection neurons do not express detectable glypican (e.g. cerebellar Purkinje cells).

Although others have suggested that some PGs are localized to neurons that form functional or anatomical systems (Hockfield et al., 1990; Maeda et al., 1992), the types of neurons that express glypican mRNA do not correspond to any single functional or transmitter-specific subdivisions of the brain. Nevertheless, limbic structures—hippocampus, amygdala, thalamus, cingulate and piriform cortex, nucleus accumbens, and septal nuclei—are relatively prominent among glypican-expressing structures. At least one other GPI-anchored protein is known to be associated with limbic system structures (LAMP [Levitt, 1984; Zacco et al., 1990; Zhukareva and Levitt, 1993]). Also prominent among glypican-expressing neurons are neurons whose axons contribute to peripheral nerves (spinal
Table 2. **Relative levels of glypican hybridization in the adult rat nervous system.** Intensity of hybridization is graded as +++ (very strong), ++ (strong), + (moderate), +/- (weak), or - (absent), based in part on autoradiographic exposure times. For example, structures, such as CA3 and CA4 of the hippocampus were darkly exposed in <15 d, while structures such as the cerebral cortex were usually only detected in longer exposures (≥25 d), or when two probes were used simultaneously during hybridization. Structures graded as - sometimes exhibited small clusters of silver grains over rare large cell bodies. Unlisted structures were not determined.
and cranial motoneurons; dorsal root ganglion neurons).

**Functional roles of glypican in the adult rat nervous system.**

Although glypican is only one of many PGs in the mammalian brain, there are reasons to believe that whatever roles it plays there may be unique. In adult brain, glypican is a major HSPG, and it is the only HSPG that exhibits detergent partitioning properties indicative of an integral membrane protein (Herndon and Lander, 1990). This suggests that the bulk of the HS borne by integral membrane proteins in the adult brain may be found on glypican. Since glypican is only expressed by certain populations of neurons, this suggestion raises the interesting possibility that CNS glia and some neurons may be relatively or entirely deficient in cell-surface HS. If so, expression patterns of glypican might play an important role in restricting the sites of action of molecules that normally interact with cell-surface HS.

Among such molecules that are found in the adult brain, members of the FGF family of polypeptide growth factors are present in many locations where glypican-expressing neurons are found. Acidic FGF, for instance, is highly expressed in spinal and cranial motoneurons, as well as neurons of the dorsal root ganglia, thalamus, septum, and red nucleus, but differs from glypican in being largely absent from the cerebral cortex and hippocampus (Elde et al., 1991; Stock et al., 1992). However, hippocampal pyramidal neurons do express basic FGF (primarily in subfield CA2 [Gómez-Pinilla et al., 1992; Woodward et al., 1992]), and FGF-5 (in subfields CA2 and CA3 [Gómez-Pinilla and Cotman, 1992]). Basic FGF is also found in some cranial motor nuclei (Gómez-Pinilla et al., 1992), and FGF-5 is present in skeletal muscle (Hughes et al., 1993). Although the functions of these growth factors in the adult nervous system are not known, evidence suggests that they may be involved in facilitating neuronal repair and/or neuronal survival (Gómez-Pinilla et al., 1992; Hughes et al., 1993). Since the actions of growth factors of the FGF family apparently require the participation of the HS chains of HSPGs (Yayon, et al., 1991; Rapraeger et al., 1991), glypican may play an important role in the trophic and injury responses of neurons.
Glypican could also play a role in neuronal injury responses by virtue of interactions with HS-binding extracellular matrix molecules. For example, laminin, a strongly neurite outgrowth-promoting molecule, becomes exposed in the endoneurial sheaths of peripheral nerves during nerve regeneration (Bignami et al., 1984; Longo et al., 1984); both motoneurons and primary sensory neurons express glypican, which could then be involved in the recognition of and response to endoneurial laminin. Alternatively, it is known that at least one HSPG that is released by cells strongly inhibits the neurite outgrowth effects of laminin (Muir et al., 1989); neuronal glypican might be released under some circumstances and thereby interfere with regeneration. Significantly, GPI-anchored molecules, including some GPI-anchored PGs, are known to be shed by cells, presumably through the action of endogenous surface phospholipases (Ishihara et al., 1987; Carey and Evans, 1989; Low, 1989). This could account for the existence of an HSPG with biochemical properties very similar to those of glypican in the adult rat brain soluble (non-membrane associated) fraction ("S9"; Herndon and Lander, 1990).

It is possible that glypican mediates different effects depending on its cellular source. Several heparin-binding proteins bind specifically to subfractions of heparin, suggesting that these proteins recognize aspects of GAG structure that may be found only on some HS chains (Marcum and Rosenberg, 1989; Lee and Lander, 1991; Nurcombe et al., 1993; San Antonio et al., 1993). Purification of glypican from specific neural sources, followed by binding studies with heparin-binding proteins of the brain, should indicate whether heterogeneity in the HS borne by the glypican core protein influences the kinds of molecules for which glypican, in different types of neurons, may act as a receptor.
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Chapter 3: Determination of glypican expression and the localization of glypican and cerebroglycan protein during development.
Introduction.

Many processes in the development of the nervous system involve cell surface heparan sulfate proteoglycans (HSPGs). Cell surface HSPGs can participate in NCAM-mediated cell adhesion (Cole et al., 1986; Reyes et al., 1990), as well as cell-substratum adhesion and neurite outgrowth (LeBaron et al., 1988; Sanderson et al., 1992; Wang and Denburg, 1992). HS on the cell surface can also affect neurite outgrowth by regulating the inactivation of proteases by their inhibitors (Monard, 1988). Furthermore, HSPGs are required for the action of some growth factors, in particular fibroblast growth factors (FGFs) (Rapraeger et al., 1991; Yayon et al., 1991); thus HSPGs are likely to play a role in FGF-mediated cell survival, proliferation, and differentiation. Consistent with these roles, many HSPGs have been identified from brain membranes, and the expression of these PGs has been found to be developmentally regulated (Herndon and Lander, 1990).

Cell surface HS can be provided by two classes of integral membrane HSPGs, each of which define a gene family. Syndecans 1-4 are hybrid chondroitin sulfate (CS)/HSPGs (for review, see Bernfield et al., 1992). They are composed of an extracellular domain containing several potential attachment sites for HS and CS, a hydrophobic transmembrane domain, and a highly conserved cytoplasmic domain that has been suggested to interact with cytoskeletal elements. Of all members, only syndecan-3 (N-syndecan) has been shown to be expressed by nervous tissue. Regulated during development, syndecan-3 is expressed highly and widely in neonatal rat brain, with lower levels in embryonic and adult brain (Carey et al., 1992).

The other family of integral membrane HSPGs consists of glypican and its homologs. Glypican is a roughly 60-70 kD glycosylphosphatidylinositol (GPI)-linked HSPG first cloned in fibroblasts (David et al., 1990), but later shown to also be expressed in brain (Karthikeyan et al., 1992; Karthikeyan et al., 1994; Litwack et al., 1994a). In the brain, glypican has been shown to be expressed by subpopulations of projection neurons (Litwack et al., 1994a). Other family members include K-glypican and OCI-5.
(Filmus et al., 1988; Watanabe and Yamaguchi, 1994), both of which are found in brain, and cerebroglycan, which is expressed specifically in the nervous system by postmitotic neurons during periods of initial migration or axon growth (Stipp et al., 1994). Thus, while all members seem to be represented in the brain, at least some of them are expressed in distinct fashions.

Based on their modes of insertion in cell membranes, these two classes of cell surface HSPGs are likely to mediate different cellular responses upon binding heparin-binding proteins, and thus may serve different functions in the nervous system. Syndecan, for instance, possesses a cytoplasmic domain that is thought to interact with the cytoskeleton, and syndecans have in fact been found in focal adhesions (Woods and Couchman, 1992; Carey et al., 1994; Woods and Couchman, 1994). Thus, heparin-binding proteins could be linked to the cytoskeleton upon binding syndecan. Glypican, on the other hand, is inserted into the membrane via covalently attached lipids (for a review of GPI-anchored proteins and their properties, see Cross, 1990). This mode of insertion could confer higher lateral mobilities in the cell membrane with respect to transmembrane proteins (Chan et al., 1991). Such a mode of insertion could also allow glypicans to be shed from the cell surface by phospholipases C or D (as contrasted with syndecan, which must be released by proteases). The GPI-linkage might also localize glypicans to specialized domains in the cell membrane (Lisanti et al., 1994); this could be a mechanism of association of glypican with particular membrane proteins. GPI-linked proteins have also been shown to be associated with intracellular tyrosine kinases (Stefanova et al., 1991; Thomas and Samelson, 1992; Rudd et al., 1993), and might therefore be able to transduce signals. Interactions of heparin-binding proteins with different PGs may thus lead to different functional consequences.

In an effort to understand the function of HSPGs in the development of the nervous system, we have begun to characterize the expression of glypican and its homologs in the brain. Previous studies have shown that glypican is expressed by subpopulations of neurons in the adult rat nervous system (Litwack et al., 1994a).
Studies presented here characterize the expression of glypican during neural development, as well as in other developing tissues. Immunohistochemistry indicates that both glypican and cerebroglycan are expressed on axons; in some neurons, cerebroglycan is polarized to axons and not to dendrites or cell bodies. Some of this data has been presented previously in abstract form (Kumbasar et al., 1994; Litwack et al., 1994b).
Materials and Methods.

Rat glypican probes. RNA probes from rat glypican clones 4X1 and 4P1 were synthesized as previously described (Litwack et al., 1994a; Chapter 2, this thesis).

In situ hybridization. Embryos were dissected from timed pregnant Sprague-Dawley rats, with plug date considered embryonic day 0 (E0). E14 embryos were fixed overnight with 4% paraformaldehyde in PBS, and then equilibrated in sequentially in 5% and then 15% sucrose in PBS. Rats of all other embryonic ages and postnatal rats were frozen in isopentane chilled on dry ice. 20 μm cryostat sections were collected on Probe-On Plus microscope slides (Fisher). In situ hybridization experiments were performed as previously described (Litwack et al., 1994a).

Anti-peptide antibodies. A peptide designated 343 (CGNPKVNHGPHGPEEKR) was synthesized and purified by reverse-phase HPLC (Biopolymers Lab, M.I.T.). This peptide corresponds to amino acids 343-360 of rat glypican (Litwack et al., 1994a). 20 mg of keyhole limpet hemocyanin (KLH) (Pierce) was reacted with 2 mg sulfo-SMCC (Pierce) at room temperature with stirring for 30 minutes. Activated KLH was purified over a Sephadex G-25 column (Pharmacia) in 0.1 M sodium phosphate (pH6). 22 mg 343 was dissolved in the same buffer, and reacted overnight at room temperature with the activated KLH. KLH-343 complexes were purified over a Sephadex G-25 column. Antibodies were raised at Pine Acres Rabbitry and Farms (Norton, MA). Rabbits were injected intradermally with 2.5 mg KLH-343 in complete Freund's adjuvant and boosted four times intramuscularly with 2.5 mg KLH-343 in incomplete Freund's adjuvant. Antibodies were collected and purified over 343 coupled to Sulfo-Link (Pierce). This antiserum was designated 343-1.

A rabbit polyclonal antiserum was also raised as described above to a peptide designated 521 (CRPPRPPPAPPRDGL). This peptide corresponds to amino acids 521-535 of cerebroglycan. The
antiserum was designated 521-2, and was used after two intramuscular boosts.

**Western blots.** GAG lyase digestions and Western blots were performed as described in Chapter 2.

**Immunohistochemistry.** E13.5 CD-1 mouse embryos, E13 rat embryos, E19 rat heads, and P0 rat brains were dissected and fixed by overnight immersion in 4% paraformaldehyde in PBS at 4°C. Tissue was equilibrated sequentially in 5% and 15% sucrose in PBS. were treated similarly. E14 rat embryos, P21 rat brains, and adult rat brains were quick-frozen on isopentane. 20 μm cryostat sections of all samples were collected on Probe-On Plus slides (Fisher) and stored at -80°C until further use. For immunohistochemistry, sections were incubated in block (2% BSA, 100 mM Tris [pH 8 at 4°C], 150 mM NaCl, 0.3% Triton X-100). Sections were washed in TBS (100 mM Tris [pH 8 at 4°C], 150 mM NaCl) and, when sections were to be developed with horseradish peroxidase, treated twice for 30 minutes each in 0.3% H2O2 and washed again in TBS. Affinity purified 343-1 was applied at 2.5-5 μg/ml in block; affinity purified 521-2 was applied at 2.5 μg/ml. For immunofluorescence, a 1:100 dilution of Cy3 (Jackson Immunoresearch) was used as a secondary antibody, and sections were coverslipped using GelMount (Biomeda). For horseradish peroxidase, sections were incubated in biotin-conjugated goat anti-rabbit antibody (Vector) diluted 1:300 in block as a secondary antibody. Sections were washed in TBS and then incubated in ABC reagent (Vector) diluted in block. Sections were washed again in TBS and developed in 50 μg/ml DAB/0.3% H2O2/50 mM Tris. Sections were washed in H2O, dehydrated, cleared in xylenes, and coverslipped using Permount. In some cases, sections were counterstained with methyl green before dehydration and mounting.
Results.

Glypican expression in the developing nervous system. In the adult rat nervous system, glypican is expressed by subpopulations of neurons (Litwack et al., 1994a). In situ hybridization experiments were undertaken in a series of embryonic and postnatal rats in order to determine how glypican expression is regulated during development.

At E14, glypican is associated primarily with the nervous system (Fig. 1A). In particular, glypican expression is associated with ventricular zones (Fig. 1A,B,D,2), regions of proliferating neuroepithelia that give rise to both neurons and glia. Only background levels of hybridization are seen in the preplate (Fig. 2), which contains the earliest-born neurons of the cerebral cortex. At this age, the pattern of glypican expression is complementary to the expression of cerebroglycan, a member of the glypican family of lipid-linked HSPGs (Fig 1D,E; Stipp et al., 1994). Cerebroglycan expression is associated with the preplate and with other zones of differentiated neurons (Fig. 1E).

During later stages of development, glypican expression in the nervous system is widespread. In the E18 rat brain, glypican expression is associated with the ventricular zone, intermediate zone, and cortical plate of the cerebral cortex in a pattern that correlates with the distribution of cell bodies (Fig. 3A). Only the marginal zone, which contains relatively few cell bodies, has background levels of glypican hybridization.

Glypican is expressed in other regions of the nervous system, including the corpus striatum (Fig. 3A), which does not express glypican in the adult (Litwack et al., 1994a). Glypican expression is also associated with the thalamus and hippocampus (Fig. 3A).

The pattern of glypican expression in the P0 rat brain is virtually identical to that observed in the E18 rat brain (Fig. 3B); widespread expression is seen in the thalamus, corpus striatum, and cerebral cortex. The low levels of hybridization in the intermediate zone of the cerebral cortex is consistent with the lower density of cells in this area in the P0 rat. High levels of expression continue to be observed in the corpus striatum, thalamus, and all layers of the
Figure 1. Glypican expression in E14 rat. A, Hyperfilm image of a sagittal section through E14 rat, probed with 4X1. B, Darkfield view of a sagittal section through E14 rat, probed with 4X1. C, Darkfield view of a section nearby to that in B, probed with sense (control) strand of 4X1. D, Hyperfilm image of a sagittal section through E14 rat, probed with 4X1. E, Hyperfilm image of a section nearby to that in D, probed with a cerebroglycan antisense RNA probe. Abbreviations: f, forebrain; h, hindbrain; m, midbrain; t, tegmentum. Scale bars: A, 1 mm; B-C, 250 μm; D-E, 1 mm.
Figure 2. Glypican expression in the ventricular zone of E14 rat telencephalon. A, Darkfield view of a sagittal section through E14 rat forebrain, probed with 4X1. B, Same section as that in A, counterstained with Hoechst and viewed by fluorescence. C, Darkfield view of a sagittal section nearby that in A, probed with the sense (control) strand of 4X1. D, Same section as that in A, counterstained with Hoechst and viewed by fluorescence. In all panels, rostral is to the left, dorsal is up. Dashed lines indicate the approximate boundary between the ventricular zone and preplate. Abbreviations: p, preplate; v, ventricular zone. Scale bar: A-D, 100 μm.
Figure 3. **Glypican expression in the E18-P7 rat nervous system.** A, Darkfield view of a sagittal section through E18 head, probed with 4P2. B, Darkfield view of a sagittal section through P0 head, probed with 4P2. C, Darkfield view of a sagittal section through P7 brain, probed with 4X1. D, Same section as in C. E, Same section as in C. F, Darkfield view of a coronal section through P7 brain, probed with 4P2. G, Darkfield view of a coronal section through P7 brain, probed simultaneously with 4X1 and 4P2. H, Darkfield view of a coronal section through P14 brain, probed with 4P2. In A-E, anterior is to the left, dorsal is up. Abbreviations: a, nucleus accumbens; b, bone; c, cerebral cortex; cb, cerebellum; cp, cortical plate; dg, dentate gyrus; egl, external granule cell layer; h, hippocampus; hf, hair follicles; i, intermediate zone of the cerebral cortex; igl, internal granule cell layer; lv, lateral ventricle; ml, molecular layer; o, olfactory bulb; p, piriform cortex; pl, pyramidal cell layer of the hippocampus; s, striatum; t, thalamus; v, ventricular zone. Scale bars: A-E, 250 μm; F-H, 1.5 mm.
hippocampus, including proliferative cells in the hilar region of the dentate gyrus that give rise to granule neurons.

In the P7 rat brain, glypican expression continues to be widespread (Fig. 3C-G). All regions observed express glypican, including the external and internal granule cell layers of the cerebellum (Fig. 3E); as with the corpus striatum, this expression must be transient, as no glypican expression is seen in the adult rat cerebellum (Litwack et al., 1994a).

Despite the widespread expression of glypican at this age, some regional differences can be observed. In the hippocampus, the pyramidal cell layer has higher levels of expression than the dentate gyrus (Fig. 3D), similar to the adult pattern of expression in the hippocampus (Litwack et al., 1994a). In addition, the anterior thalamus has lower levels of expression than the posterior thalamus (Fig. 3D). Expression in the cerebral cortex is still relatively uniform, however, and significant glypican expression persists in the corpus striatum (Fig. 3F,G).

By P14, the pattern of expression more closely resembles the adult pattern of expression (Fig. 3H). In the hippocampus, high levels of expression are seen in the CA3 region of the pyramidal cell layer, whereas lower levels are seen in the rest of the pyramidal cell layer and in the dentate gyrus. The dorsal thalamus expresses glypican, while the ventral thalamus generally does not (although there is a small amount of hybridization in the most ventrolateral regions of the ventral thalamus). Very low levels of cortical expression are observed.

In all cases, sections hybridized to sense (control) strands of glypican showed no specific hybridization to any region of the nervous system (Fig. 1C,2C,D; data not shown).

Nonneural expression of glypican. Outside the nervous system, particularly high levels of glypican message were detected in developing bone. During endochondral ossification in an E18 rat humerus, hybridization is seen in the periosteum and also in the trabeculae (Fig. 4A-D). In a section through E18 trunk, hybridization is likewise observed in association with periosteum
Figure 4. **Expression of glypican in developing bone.** A, Section through E18 forearm probed with 4P2, viewed in dark field. B, Section adjacent to that in A, stained with hematoxylin and eosin. C, Section through E18 vertebral column probed with 4X1, viewed in dark field. D, Section 40 μm from that in C, stained with hematoxylin and eosin. E, Section through P7 limb probed with 4X1, viewed in dark field. F, Same section as in E, stained with Hoechst. G, Section through P7 limb probed with the sense (control) strand of 4X1, viewed in dark field. G, Same section as in G stained with Hoechst. The hypertrophying chondrocytes in F-G were lost during sectioning. In C-D, rostral is to the left, and dorsal is to the top. Abbreviations: c, chondrocytes; d, dorsal root ganglion; ep, epiphyseal plate; hc, hypertrophying chondrocytes; o, osteoblasts; p, periosteum; t, trabeculae. Scale bars: A-D, 250 μm; E-H, 100 μm.
and trabeculae in vertebral bodies and ribs (Fig. 4C,D). However, no expression is observed in the resting, proliferating, or hypertrophic chondrocytes (Fig. 4A-F).

The pattern of expression in developing bone is consistent with expression by populations of differentiated osteoblasts. Osteoblasts differentiate in the periosteum and then migrate into the trabeculae, both regions that express glypican. Consistent with this interpretation, vertebral bodies at different stages of development show hybridization that appears in a spatial and temporal progression that is consistent with osteoblast differentiation. Development of vertebral bodies proceeds in a rostral-to-caudal fashion. In the younger, more caudal vertebral bodies expression appears in the periosteum (Fig. 4C,D). The periosteal expression extends completely around the older, more rostral vertebral bodies. In these older vertebral bodies, expression also appears in the trabeculae. The caudal-to-rostral pattern of glypican expression parallels the development and differentiation of osteoblasts.

In situ hybridization experiments in P7 bone provide results consistent with the hypothesis that glypican is expressed by differentiated osteoblasts. In the epiphyseal plate of a long bone in P7 limb, hybridization is seen in association with cells near bony spicules (Fig. 4E,F,5), i.e., the same area where osteoblasts are located. However, osteoclasts may also be present as well in these regions, and these experiments do not distinguish between the two populations. Association of glypican with differentiated osteoblasts is also observed in intramembranous bone formation (which, for example is the mechanism of formation of skull bones) (Fig. 3A).

Hybridization to the sense strand only shows signal associated with the bony spicules in the trabeculae (Fig. 4G,H,5C,D), an artifact seen in all hybridization experiments performed on bone. In these controls no hybridization is seen over cell bodies.

At this stage hybridization is also seen in the bone marrow in clusters (Fig. 6C,D). Glypican could be expressed by subpopulations of hematopoietic cells or stromal cells of the marrow. In P7 limb, glypican expression is also associated with hair follicles (Fig. 6A,B);
Figure 5. **High magnification view of epiphyseal plate.** A, Darkfield view of a section through epiphyseal plate in P7 limb, probed with 4X1. B, Same section as in A, stained with Hoechst. C, Darkfield view of a section nearby to that in A, probed with sense (control) strand. D, Same section as in C, stained with Hoechst. Scale bar: A - D, 25 μm.
Figure 6. **Expression of glypican in hair follicles and bone marrow.**

A, Darkfield view of hair follicles in P7 limb, probed with 4X1.  
B, Same section as in A, stained with Hoechst.  
C, Darkfield view of bone marrow in long bone of P7 limb, probed with 4X1.  
D, Same section as in C, stained with Hoechst.  
Scale bar: A–D, 100 μm.
expression of glypican in hair follicles was also noted in E18 rat head (Fig. 3A). While some variation in the levels of glypican expression between individual hair follicles can be observed, comparison with a Hoechst-stained section indicates that these variations are likely due to the region of the particular hair follicle present in the section (Fig. 6A,B). The expression of glypican in hair follicles is consistent with results previously obtained via immunostaining (David et al., 1992a).

Localization of glypican to nerve fibers. In order to determine the distribution of the glypican protein product, an antisera was raised to a glypican-derived peptide. This antisera was then purified by affinity chromatography using the peptide antigen; these affinity-purified antibodies, designated 343-1, were used in all experiments described below.

By Western blotting of PO rat brain membranes treated with heparitinase, 343-1 recognizes a single band of about 66 kD (Fig. 7A)\(^1\). No higher molecular weight smear, typical of intact PG, was detected in untreated brain membranes; it is likely that the intact PG, which contains a high level of negative charge due to attached GAG, failed to bind to the relatively hydrophobic nitrocellulose membrane (Rapraeger et al., 1985). 343-1 also recognizes a protein of the correct molecular weight in extracts of myeloma cells expressing a rat glypican transgene, but not in control myeloma cells (Appendix 1).

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\(^1\)No core protein was observed by western blot when PO membranes were treated with chondroitinase ABC, but the intensity of the glypican signal was higher when membranes were treated with chondroitinase and heparitinase together than when membranes were treated with heparitinase alone. This could reflect a population of glypican that possesses CS as well as HS. However, no CS has previously been observed on glypican. Furthermore, comparisons of relative intensities between treatments are sometimes misleading. For instance, in adult rat brain soluble fraction, the intensity of the immunoreactive band is higher with heparitinase treatment alone than with heparitinase and chondroitinase treatment (Fig. 8). It would be necessary to purify GAGs from glypican that had been purified from brain in order to definitively say that some glypican possesses CS chains.
Figure 7. **Western blot of P0 rat brain membranes.** A. Western blot of 18 μg of P0 membranes using 343-1. B. Western blot as in A using 521-2. Lane 1, heparitinase- and chondroitinase-treated; lane 2, chondroitinase-treated; lane 3, heparitinase-treated; lane 4, untreated.
No specific bands corresponding to intact glypican were observed in Western blots of adult membranes using 343-1. A broad smear of about 47-58 kD was observed in adult soluble fractions that had been treated with heparitinase (Fig. 8A). The apparent Mr of this protein increased to 62 kD upon reduction, a property that has been observed with glypican (Fig. 8B). This band is likely to represent a proteolytic fragment of glypican. It is unclear whether this is an endogenous proteolytic product or whether proteolysis occurred during purification. The immunoreactive band in the soluble fraction was observed with two separate purifications of soluble fraction from adult brain (data not shown).

Immunostaining using 343-1 generally gave results consistent with the data obtained from in situ hybridization experiments. In the E14 rat, for example, high levels of glypican mRNA message are expressed in the ventricular zone of the developing nervous system (Fig. 1,2). Likewise, high levels of staining with 343-1 were observed in the ventricular zone (Fig. 9A), while, as described below, no specific pattern of staining was observed with control antibodies (data not shown). 343-1 staining near the pial surface appeared to be at or just slightly above background, consistent with the low levels of signal observed with in situ hybridization.

Staining of the adult rat nervous system with 343-1 indicated that the glypican protein product is expressed on fibers, and is likely to be associated with axons. High levels of immunoreactivity were seen in the hippocampus (Fig. 10A,C). The associational and commissural fiber layer, containing axons arising from hippocampal pyramidal cells, was heavily stained, and cell bodies within the pyramidal cell layer were also detected. Consistent with the relative levels of glypican expression observed by in situ hybridization, lower levels of glypican were seen in the cell bodies of the dentate gyrus than in the pyramidal cell layer. Less immunoreactivity was observed in the mossy fibers projecting from the dentate gyrus to the pyramidal cell layer. In addition to axons arising from the dentate gyrus, pyramidal cell dendrites are also located in the mossy fiber layer. An evenly distributed membrane protein might
Figure 8. Western blot of adult rat brain soluble fraction. A, Western blot of 20 μg of adult rat brain soluble fraction with 343-1. SDS/PAGE was performed in nonreducing conditions. B, As in A, but SDS/PAGE was performed under reducing conditions. Lane 1, heparitinase- and chondroitinase-treated; lane 2, chondroitinase-treated; lane 3, heparitinase-treated; lane 4, untreated.
Figure 9. Glypican and cerebroglycan protein in the E14 rat telencephalon. A, Sagittal section through E14 rat telencephalon stained with 343-1. B, Sagittal section adjacent to that in A, stained with 521-2. In both section, rostral is down and dorsal is to the left. Abbreviations: p, preplate; v, ventricular zone. Scale bar: 100 μm.
Figure 10. Glypican protein in the adult rat hippocampus. A, Coronal section through adult rat cerebellum stained with 343-1; view of CA3 area of the pyramidal cell layer. B, Coronal section adjacent to that in A, stained with antibodies to KLH (control); view of CA3 area of the pyramidal cell layer. C, Same section as in A; view of dentate gyrus. D, Same section as in B; view of dentate gyrus. Abbreviations: ac, associational and commissural axons; dg, dentate gyrus; m, mossy fibers; p, pyramidal cell layer. Scale bar: 50 μm.
be expected to found in layers containing axons and dendrites, while a membrane polarized to axons would be expected to be localized only to those layers containing axons. These data suggest that glypican might be polarized in pyramidal cells of the hippocampus.

Staining of other regions of the adult brain further demonstrates the localization of glypican to fibers. A high level of staining was observed in the granule cell layer of the cerebellum (Fig. 11). As cerebellar granule cells do not express glypican mRNA (see Fig. 4 in Chapter 2), glypican is likely being supplied by afferents projecting into the granule layer from cells that do express glypican. These afferents, known as cerebellar mossy fibers, consist mainly of axons of the deep cerebellar nuclei (the expression of glypican mRNA in the deep cerebellar nuclei has not been determined). Neurons from the locus coeruleus also project to the cerebellum.

Glypican staining was also observed in the cerebral cortex (Fig. 12A). Scattered staining was seen in the deep layers of the cortex, where neurons express relatively low levels of glypican. High levels of staining were observed, however, in layer IV of the cortex, the cells of which does not express glypican. Afferent projections to layer IV derive largely from the dorsal thalamus, which expresses high levels of glypican mRNA; the immunoreactivity in layer IV is likely to be of thalamic origin.

In the ventrolateral region of forebrain, 343-1 staining was observed in piriform cortex (Fig. 12B), a region that was found by in situ hybridization to express glypican. The fiber layer of the piriform cortex, as well as the cell body layer was stained. The lateral olfactory tract showed no immunoreactivity for 343-1, which might be expected as the olfactory bulb, from which the lateral olfactory tract projects, expresses little glypican (data not shown). However, the glypican epitope may be undetectable in this region with our current staining protocol. It has been found that different procedures, such as different fixations, are required to visualize Thy-1, another GPI-linked protein, in different regions of the nervous system (Morris and Grosveld, 1989).
Figure 11. Glypican protein in the adult rat cerebellum. A, Sagittal section through adult rat cerebellum stained with 343-1. B, Adjacent section to that in C, stained with antibodies to KLH (control). C, Adjacent section to that in A, stained with 343-1 in the presence of a tenfold molar excess of the peptide antigen. Abbreviations: g, granule cell layer; m, molecular layer; p, Purkinje cell layer; w, white matter. Scale bar: 100 μm.
Figure 12. **Glypican protein in adult rat forebrain.** A, Coronal section through adult rat cerebral cortex at the level of the striatum stained with 343-1. The cortical layers II-VI are indicated on the left. B, Coronal section through adult rat piriform cortex at the level of the striatum stained with 343-1. *Open arrow* indicates the cell bodies of the piriform cortex; *closed arrow* indicates the fiber layer of the piriform cortex; *asterisk* indicates the lateral olfactory tract. Abbreviations: s, striatum; w, white matter. Scale bar: 135 μm.
E13.5 mouse embryos were also stained with 343-1. These experiments demonstrate association of glypican with fibers during neural development. In the E13.5 mouse embryonic nervous system, glypican immunostaining is observed in the brainstem (Fig. 13A). This staining has a "tract-like" appearance, and is presumably associated with ascending and descending fibers that project through the brainstem. In addition, glypican appeared to be expressed on spinal commissural axons (data not shown).

Glypican immunostaining was observed in the peripheral and central roots of E13.5 mouse dorsal root ganglia (Fig. 13B), with much less staining in the cell body region. This staining pattern is consistent with polarization of glypican to axons (as opposed to cell bodies and dendrites). This staining pattern may not be representative, however, as these experiments were performed with affinity-purified antibodies from an earlier test bleed than those used on rat tissue. These antibodies, when used to stain adult rat hippocampus, did not stain cell bodies as intensely as did the antibodies from the later test bleed. Furthermore, even if the pattern is an accurate representation of glypican distribution, the evidence for polarization is not conclusive. The concentration of membranes in axons is much greater than in cell bodies; hence, even a uniformly distributed membrane protein would be stained more intensely in axons as opposed to cell bodies (Morris and Grosveld, 1989).

Unfortunately, due to the widespread distribution of glypican in the nervous system, I was unable to address the question of the axonal polarization of glypican in vivo. Hippocampal neurons cultured for several day expressed glypican but did not polarize it to axons; instead, glypican was expressed in an apparently uniform fashion on cell bodies, dendrites, and axons (Litwack et al., 1994b). In addition, the staining experiments shown here do not rule out the possibility that glypican in the roots of dorsal root ganglia is expressed by Schwann cells; Schwann cells in vitro express a GPI-linked HSPG that is reported to be glypican (Carey et al., 1993).
Figure 13. **Glypican protein in the developing mouse nervous system.** A, Sagittal section through E13.5 mouse brainstem stained with 343-1. Rostral is to the right, dorsal is to the top. B, Sagittal section through E13.5 mouse dorsal root ganglia stained with 343-1. Abbreviations: cr, central root; d, dorsal root ganglion; pr, peripheral root. Scale bar: 50 μm.
Control sections in all staining experiments were stained with an antiserum to KLH, which was prepared by affinity purification on a KLH column using the same serum used to purify 343-1. In all cases no specific pattern of staining was observed (Fig. 10B,D,11B; data not shown). Furthermore, addition of a tenfold molar excess of the peptide antigen with respect to antibody concentration abolished almost all specific staining in adult hippocampus and cerebellum (Fig. 11C; data not shown). With both controls a low level of signal was observed in hippocampal cell bodies (e.g., Fig. 10B).

Polarization of cerebroglycan to axons. The axonal polarization of GPI-linked HSPGs could be addressed more directly by immunostaining for cerebroglycan, which is more restricted than glypican in its expression (Stipp et al., 1994). In order to perform these experiments, an anti-cerebroglycan antiserum, designated 521-2, was prepared as described in Materials and Methods. In immunoblots of P0 rat brain membranes treated with heparitinase, this antiserum detected a single band at the size previously observed for the cerebroglycan core protein (Fig. 7B) (Herndon and Lander, 1990; Stipp et al., 1994). As with glypican, the failure of 521-2 to detect a high molecular weight smear (cerebroglycan core protein with attached HS) is likely due to poor retention of PGs by nitrocellulose.

Like 343-1, 521-2 gave staining results that were consistent with the pattern of message expression obtained from in situ hybridization experiments. First, 521-2 specifically stained nervous system (Fig. 14A). Second, in E14 rat high levels of staining were associated with the layer of postmitotic neurons, with little staining observed in the ventricular zone (Fig. 9B). As with in situ hybridization experiments, the patterns of glypican and cerebroglycan staining were complementary, and suggest that at

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2 As with glypican, more signal was detected in membranes treated with heparitinase and chondroitinase than with heparitinase alone. See footnote 1 for discussion.
Figure 14. **Localization of cerebroglycan on nerve fibers.** A, Sagittal section through E19 rat head stained with 521-2. B, Coronal section through P0 rat brain stained with 521-2. C, Transverse section through E13 rat spinal cord stained with 521-2. *Open arrow* indicates spinal commissural axons. Abbreviations: c, hippocampal commissural fibers; d, dorsal root ganglion; h, hippocampus; i, internal capsule; iz, intermediate zone; m, motoneurons; o, optic tract; vr, ventral root. Scale bars: *A-B*, 200 μm; *C*, 50 μm.
this stage cells may turn off glypican expression as they turn on cerebroglycan expression.

Like glypican, cerebroglycan is expressed on fibers. In the developing cerebral cortex, cerebroglycan is associated most heavily with the intermediate zone (Fig. 14A), which contains developing cortical and thalamic axons, as well as migrating cortical neurons. Cerebroglycan is also associated with hippocampal fibers (Fig. 14B) and the internal capsule (Fig. 14B), which consists of cortical and thalamic axons. In the developing spinal cord, cerebroglycan is expressed on commissural axons, axons of motoneurons, and is also found in the dorsal root ganglia (Fig. 14C).

Like glypican, the pattern of cerebroglycan staining suggests that it is polarized to axons. In dorsal root ganglia, high levels of cerebroglycan are found on the peripheral roots, while the cell bodies express little or no cerebroglycan (Fig. 15A). In order to conclusively address the polarization of cerebroglycan, the expression of cerebroglycan in the P21 rat dentate gyrus was examined. In the dentate gyrus, granule cell axons project through the hilar region to area CA3 of the pyramidal cell layer (Bayer, 1980; Gaarskjaer, 1986). Granule cell dendrites, however, project in the opposite direction. As the membrane density of axons and dendrites are likely to be relatively similar, an antibody that recognizes a uniformly distributed membrane protein should stain with similar intensity in both regions. Cerebroglycan staining, however, was localized to the hilar region; in this region, individual axons are apparent (Fig. 15B). No significant staining is seen, however, in the region containing granule cell dendrites. While occasional fibers in this region expressed cerebroglycan, they did not appear to be dendritic, and are likely to be the axons of hippocampal afferents.

In all experiments involving 521-2, control sections stained with equivalent concentrations of the KLH-specific antibodies (described above) or commercially obtained nonimmune rabbit IgG showed no specific pattern of staining.
Figure 15. Axonal polarization of cerebroglycan. **A**, Sagittal section through E14 rat dorsal root ganglia, stained with 521-2. **B**, Sagittal section through P21 rat hippocampus, stained with 521-2. *Asterisk* indicates hilar region of the dentate gyrus. Abbreviations: d, dorsal root ganglion; pr, peripheral root. Scale bars, A, 50 μm; B, 30 μm.
**Discussion.**

A major fraction of cell surface HS in the developing and adult rat nervous system can be accounted for by GPI-anchored HSPG core proteins (Herndon and Lander, 1990). The two GPI-anchored core proteins identified by Herndon and Lander (1990) have been cloned; one (previously designated M12) is glypican (David et al., 1990; Karthikeyan et al., 1992; Litwack et al., 1994a), while the other (previously designated M13) is a related PG, cerebroglycan (Stipp et al., 1994). The patterns of expression of both cores are restricted, and suggest that they may have important functions in brain development and function. In this Chapter, I have further characterized glypican expression during embryogenesis, and have localized the protein products via immunohistochemistry, as a means of further deducing their roles in nervous system development.

*Glypican and cerebroglycan are expressed by different populations of cells in the early developing nervous system.*

*In situ* hybridization experiments indicate that glypican expression in the early nervous system is restricted. In the telencephalon of E14 rat, glypican is found at high levels in the ventricular zone, a region consisting of proliferating neuroepithelia; much lower levels of expression are seen elsewhere in the embryo. The ventricular zone expression of glypican contrasts with the expression of a related HSPG, cerebroglycan. Little or no expression of glypican is found in the preplate, a layer of differentiated neurons lying pial to the ventricular zone, of the developing cerebral cortex. Cerebroglycan, however, is highly expressed in the preplate as well as in other layers of differentiated neurons, while little is expressed in ventricular zones. These patterns of expression are confirmed by antibody staining experiments, which additionally demonstrate that glypican is not significantly retained by cells that have migrated into the preplate.

These complementary patterns of expression suggest that neurons switch HSPG expression during development. Neuroepithelial cells may express glypican, and upon differentiation,
down-regulate glypican and up-regulate cerebroglycan. Switching between two homologous versions of GPI-anchored HSPGs suggests that there may be some functional differences between these two proteins, despite their structural similarity. Glypican, for instance, may function to act as a receptor for members of the fibroblast growth factor (FGF) family. Members of the FGF family are expressed in the ventricular zone, and have been shown to support survival, proliferation, and differentiation of cultured neuroepithelial cells (Murphy et al., 1990). Interestingly, the binding specificity for particular FGFs of at least one HSPG from cultured neuroepithelial cells of different ages correlates with the expression of those FGFs in the ventricular zone at those ages (Nurcombe et al., 1993). As HS is necessary for FGF action (see Chapter 1 for discussion), HSPGs have been suggested to act as coreceptors for FGFs (Bernfield et al., 1992). Glypican could act as such a coreceptor. Furthermore, glypican is associated with other regions of proliferating cells, such as the hilar region of the dentate gyrus, which contains precursors to hippocampal granule cells, the ganglionic eminence, which contains the precursors of striatal neurons, and the external granule cell layer of the cerebellum, which gives rise to granule cells. It may be that there is a general requirement for glypican in neural precursor cells, and that glypican is so required in order to assist in FGF function in those cells.

While glypican expression suggests a role in FGF function, the expression of cerebroglycan in newly differentiated neurons suggests a role in axon outgrowth (Stipp et al., 1994). Cell surface HS is likely to affect axon outgrowth. Many proteins that promote axon outgrowth, such as laminin and fibronectin, bind HS. Cell surface HS has been suggested to mediate neurite outgrowth on heparin-binding peptides derived from fibronectin (Haugen et al., 1992a). Furthermore, exogenously added heparin can disrupt axon guidance in some systems (Wang and Denburg, 1992). Consistent with the idea that cerebroglycan is important for axon growth is the finding that it is localized to fibers and, in at least some cells, is polarized to axons.
It is unclear if a glypican-cerebroglycan switch is occurring at other times or locations during neural development. In some regions, such as the developing cortex, it is likely that at least some cells are expressing both PGs, as both glypican and cerebroglycan are expressed in the ventricular zone and the cortical plate. However, in the absence of double-labeling it is unclear if the same cells in both regions are expressing both PGs.

*Glypican expression is widespread during later stages of neural development.*

At later stages of neural development (E18-P7), glypican expression is widespread. Glypican is expressed in regions such as the hippocampus, cerebral cortex, and thalamus, which express glypican in the adult. In other regions, such as the cerebellum (granule cell layer) and the corpus striatum, glypican expression is transient, as these areas express glypican during development but not in adulthood. While glypican expression is neuronal in the adult, this study did not distinguish between glial and neuronal expression during development (most glial cells, however, appear after birth). The widespread expression of glypican implies that glypican may be involved in a common process that all neural cells undergo.

One such function may again be the interaction of glypican with FGFs. As in earlier embryos, glypican at this stage is coexpressed with members of the FGF family (Gonzalez et al., 1990; Fu et al., 1991; Wilcox and Unnerstall, 1991) and FGF receptors (Wanaka et al., 1991; Peters et al., 1993). In addition to their effects on neuroepithelial cells, FGFs have been shown to have trophic effects on neuronal survival (Unsicker et al., 1987; Walicke, 1988). Such correlations can be made in the adult nervous system as well; glypican is highly expressed in motoneurons, which are dependent on FGF-5 for survival (Hughes et al., 1993). The loss of glypican expression during the maturation of some regions may reflect changes in the requirements of neurons in those regions for trophic factors.

Glypican may also be important for other functions as well. Taking the cerebral cortex as an example, glypican expression
correlates with both stem cells and with postmitotic cells\textsuperscript{3}. Furthermore, glypican expression remains on in all layers of P7 cortex, when most cell migration is completed and much axon growth has occurred (while it is off in layer IV in the adult). Glypican expression may correlate with other phenomena, however; changes in neuronal soma, synaptogenesis, and dendritogenesis, for instance, occur into the fourth week of postnatal life (Miller, 1988). It may be that glypican, at least in some regions, correlates with plasticity, as it is expressed by both the cerebral cortex and the hippocampus.

**Axonal expression and polarization of GPI-anchored HSPGs.**

The idea that GPI-anchored HSPGs are important for axonal growth is supported by the expression of glypican and cerebroglycan on axons\textsuperscript{4}. In the hippocampus, glypican is found on the axons of pyramidal cells; glypican is also found in the fiber tracts of the piriform cortex. In the developing mouse nervous system, glypican was also observed to be on fibers in the brainstem, spinal cord (data not shown), and dorsal root ganglia. The localization of glypican described by Karthikeyan et al. (1994) is generally consistent with the data presented here.

Other regions gave results which, interpreted in the light of *in situ* hybridization experiments, are consistent with axonal localization of glypican. For instance, glypican was observed in the fiber layer of the piriform cortex. Glypican immunoreactivity was also observed in the granule cell layer of the cerebellum. As granule cells do not express glypican (Litwack et al., 1994a; Chapter 2, this thesis), it is likely that glypican is provided by mossy fibers, which consist of axons arising mainly from the deep cerebellar nuclei, that synapse on granule cells; it is not known, however, if neurons of the deep cerebellar nuclei express glypican. Glypican in

\textsuperscript{3}Karthikeyan et al. (1994) suggest that glypican is not found in the cortical plate during development. It is likely that this interpretation results from the levels of background observed in their *in situ* hybridization experiments.

\textsuperscript{4}Glypican and cerebroglycan can also be detected in membrane preparations enriched in growth cones or synaptosomes (Kumbasar et al., 1994; Litwack et al., 1994b).
the granule cell layer could also be derived from the locus coeruleus, which does express glypican (Litwack et al., 1992a; see Fig. 4 in Chapter 2). A similar interpretation can explain the immunoreactivity observed in the cerebral cortex. While glypican was observed in the deep and the superficial layers of the cortex, both of which express glypican, it was also, however, observed in layer IV, which does not express significant glypican in the adult. Glypican in layer IV is likely to be provided by thalamic neurons; the thalamus expresses glypican in the adult, and thalamic neurons synapse on layer IV cortical neurons. In both the cerebellum and the cerebral cortex, glypican was not observed in white matter, contrary to what might be expected if afferents are providing axonal glypican. This may be due to myelination, which could either exclude glypican or mask the glypican epitope. It should also be noted that the immunoreactivity in cerebellum and cerebral cortex could be the result of glypican remaining from development, when both these regions expressed glypican. Turnover rates measured for GPI-linked PGs \textit{in vitro} suggest that this is probably not the case. Turnover rates of GPI-linked PGs \textit{in vivo} are not known, however.

The patterns of immunoreactivity observed suggested that glypican may be polarized at least to some extent to axons. In the adult hippocampus, for instance, glypican was observed in the associational and commissural fibers, with lower levels in the mossy fiber layer. As pyramidal cells, which are the source of associational and commissural axons, project dendrites into the mossy fiber layer (Bayer, 1980), the lower levels of immunoreactivity there suggests that glypican could be polarized to axons and not dendrites. However, hippocampal cell bodies, both in the pyramidal cell layer and in the dentate gyrus, also contained significant immunoreactivity. This was observed in other regions, such as the piriform cortex, where both the fiber layer and the cell bodies were immunoreactive.

Results in developing mouse were also consistent with axonal polarization of glypican. In dorsal root ganglia, glypican was seen at high levels in central and peripheral roots, but at much lower levels in cell bodies. While consistent with axonal localization, this pattern
could also be explained by Schwann cell expression of glypican. Some work suggests that glypican may be expressed in Schwann cells. It should be noted however, that much of this work was done in cultured Schwann cells. As most, if not all, adherent cells in vitro express glypican (Lories et al., 1992), it may be that glypican expression is induced in cultured cells. In addition, Carey et al. (1993) use an antibody that was raised to a full length glypican fusion protein. Unlike the anti-peptide antibody used here, which was raised to an epitope not conserved among family members, the anti-fusion protein antibody may recognize a glypican homolog.

Unfortunately, the widespread distribution of glypican made it difficult to design an experiment to definitively test the idea that glypican is polarized to axons. Cerebroglycan, however, is much more restricted in development, and is also observed on fibers. Staining of P21 dentate gyrus shows that cerebroglycan is in fact polarized in those cells.

The GPI-anchor may be a signal in some cells for axonal polarization. Many GPI-anchored proteins are expressed on axons. In epithelial cells, the GPI-anchor has been shown to target proteins to the apical surface (Lisanti et al., 1989). While it has been proposed that a similar mechanism occurs in neurons, with the axon being the equivalent of the apical surface, analysis of the expression of several GPI-linked proteins demonstrates that such a generalization is not possible. While as noted above, some proteins (such as F3/F11 and TAG-1 [Dodd et al., 1988; Furley et al., 1990; Faivre-Sarrailh et al., 1992]) are found on axons, some are found on dendrites and cell bodies, such as Thy-1 (Xue et al., 1990; Xue et al., 1991). In fact, the compartmentalization of Thy-1 changes during neuronal differentiation. The present study indicates that some cells can polarize cerebroglycan, and some cells may also polarize glypican. Furthermore, some cells, such as cultured hippocampal neurons, do not polarize cerebroglycan and glypican (Kumbasar et al., 1994; Litwack et al., 1994b). In vitro studies, however, often give results different from those obtained in vivo. Hippocampal neurons in culture polarize Thy-1 to axons (Dotti and Simons, 1990; Dotti et al., 1991), while hippocampal neurons in vivo do not (Xue et
al., 1990; Xue et al., 1991). It has been proposed that, as in epithelial cells (Lisanti et al., 1989), the GPI-linkage is a signal that allows neuronal cells to sort the proteins to axons (Dotti and Simons, 1989; Dotti et al., 1990). It may be that the GPI-linkage acts as a sorting signal in those neurons that do polarize glypican and cerebroglycan. However, it is possible that these two proteins are selectively stabilized on axons, perhaps by binding to other proteins or by being less susceptible to degradation.

The polarization of GPI-anchored HSPGs to axons may serve several functions. As discussed above, cell surface HS is likely to play a role in axon outgrowth. In addition, neuronal response to growth factors may require localized cell surface HSPGs. The trophic factors to which neurons respond, as well as the signals which influence axon growth and guidance, are often target-derived. For instance, motoneuron survival is likely to depend on muscle-derived FGF-5 (Hughes, et al., 1993). The expression of glypican on axons would thus be necessary in order for motoneurons to receive FGF-5 induced signals.

*Nonneural expression of glypican.*

Glypican is widely expressed in tissues outside the nervous system. Particularly high levels of glypican expression are associated with differentiated osteoblasts, although it is unclear at what stage of differentiation glypican is turned on. No glypican expression was observed in chondrocytes at any stage. As in the brain, glypican might bind growth factors. Several heparin binding growth factors have been purified from bone which act as mitogens for osteoblasts (Hauschka et al., 1986).

Clusters of glypican-expressing cells were observed in bone marrow. These could be bone marrow stroma or hematopoietic cells (either stem cells or differentiated cells). The clustered pattern of expression might be suggestive of hematopoietic cells, possibly clones. HS in the stroma compartmentalizes factors such as GM-CSF and IL-3 (Gordon et al., 1987; Roberts et al., 1988). These factors support the proliferation of hematopoietic cells, which must actually contact stroma in order to respond to them. HS in this case is
thought to localize these factors and present them to responsive cells. Glypican could function in the stroma to compartmentalize these factors, or, on the surfaces of hematopoietic cells, it could influence the action of these factors.

Glypican is also expressed in hair follicles. Due to the single time point observed, it is unclear if expression is regulated during the hair follicle growth cycle. Interestingly, glypican in hair follicle is a potential regulator of FGF action. FGF-5 is expressed in hair follicles and is likely to regulate the transition of the hair follicle from anagen (hair growth) to catagen (hair follicle regression), as a targeted mutation in the FGF-5 gene results in delayed progression into catagen (Hebert et al., 1994).
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Chris Stipp and Asli Kumbasar participated in the production of polyclonal antibodies. *In situ* hybridization using cerebroglycan was performed by Chris Stipp. Alexander Yang collaborated in the 521-2 immunostaining. Jon Ivins performed the staining experiment shown in Fig. 15B.
Chapter 4. Decorin in the developing nervous system: floor plate expression and netrin-binding properties.
**Introduction**

Decorin is a chondroitin sulfate/dermatan sulfate proteoglycan (CS/DSPG) with a core protein of about 38 kD (Krusius and Ruoslahti, 1986; Day et al., 1987; Li et al., 1992; Scholzen et al., 1994). It belongs to a family of small interstitial proteoglycans whose other members include another CSPG, biglycan (Fisher et al., 1989; Neame et al., 1989), and two keratan sulfate (KS) PGs, fibromodulin (Oldberg et al., 1989) and lumican (Blochberger et al., 1992a). The core proteins of all these PGs are characterized by a leucine-rich repeat sequence, a motif that is thought to be involved in mediating protein-protein interactions (Kobe and Deisenhofer, 1994). Decorin binds via its core protein to a variety of molecules, including fibrillar collagens (Hedbom and Heinegård, 1989; Bidanset et al., 1992; Hedbom and Heinegård, 1993), fibronectin (Lewandowska et al., 1987; Schmidt et al., 1991; Winnemoller et al., 1991), thrombospondin (Winnemoller et al., 1992), and TGF-β (Yamaguchi et al., 1990).

Decorin is usually found in regions containing collagen, such as skin, tendon, and bone, and decorin is typically associated with collagen fibrils in those regions. The binding of decorin to collagen decreases the rate of collagen fibril formation, and results in smaller fibrils (Vogel et al., 1984; Hedbom and Heinegård, 1989). Recent results suggest, however, that decorin is also expressed in the developing mouse nervous system along the ventral midline of the spinal cord (Scholzen, et al., 1994). This pattern suggests that decorin may be expressed by floor plate. Interestingly, there is little evident pericellular matrix in floor plate, and no obvious collagen fibrils.

The floor plate of developing spinal cord plays an important role in axon growth and guidance (Fig. 1). Commissural neurons, which are located in the dorsal spinal cord, extend axons toward the floor plate. Commissural axons at this stage are thought to be growing in response to diffusible factors released by the floor plate, as axons from dorsal spinal cord explants grow towards floor plate explants in culture (Tessier-Lavigne et al., 1988; Placzek et al., 1990). In addition, commissural exons will grow towards ectopic
Figure 1. **The role of the floor plate in the growth of spinal commissural axons.** A, Commissural axons originate from neurons in the dorsal spinal cord and project along the circumference of the spinal cord. B, At approximately the level of the ventral motoneurons, commissural axons grow directly towards the floor plate, likely in response to diffusible signals released by the floor plate (Tessier-Lavigne et al., 1988; Placzek et al., 1990). C, Once at the midline, axons grow underneath the floor plate; this growth might be influenced by contact-mediated guidance cues from the floor plate or the basement membrane (Chang and Lagenaur, 1990; Klar et al., 1991; Yaginuma et al., 1993). On the contralateral side of the floor plate, axons turn perpendicularly and grow in the direction of the head.
floor plate (Yaginuma and Oppenheim, 1991), and commissural axons in are misguided when floor plate is disrupted (Bovolenta and Dodd, 1991). Once at the floor plate, commissural axons pass just underneath it, and might at this point be guided by contact-mediated interactions with the floor plate (Chang and Lagenaur, 1990; Klar et al., 1991; Yaginuma et al., 1993). After passing underneath the floor plate the axons then turn and extend along the rostrocaudal axis of the spinal cord.

Recently, a protein that can act as a chemotropic factor for spinal commissural axons \textit{in vitro} has been purified from brain (Kennedy et al., 1994; Serafini et al., 1994). This protein, netrin-1, is expressed in the floor plate at a time when commissural axons are believed to be responding to floor plate-derived signals \textit{in vivo}. While it has been argued that netrin-1 acts as a diffusible factor, this protein is found in association with membrane fractions of homogenized brain, and little netrin-1 is found in the conditioned medium of cells expressing exogenous netrin-1 cDNA (although netrin-1 can be removed from cultures of all such cells with high salt washes). These data, along with the homology that has been observed between netrin-1 and laminin, suggest that netrin-1 may be part of the extracellular matrix. Given this possibility, the mechanism by which gradients of netrin-1 are established \textit{in vivo}---if such gradients exist---may not be simple.

Netrin-1 can promote \textit{in vitro} neurite outgrowth from explants of dorsal spinal cord on its own (Kennedy et al., 1994; Serafini et al., 1994). However, its activity is increased in the presence of netrin synergizing activity (NSA), a fraction obtained during the purification of netrin-1. The existence of NSA implies that other factors may play a role in netrin-mediated neurite outgrowth, and that netrin-1 is likely to interact with these factors in the nervous system.

The expression of decorin along the ventral spinal cord suggests that netrin-1 could be one of several proteins, including other proteins of the extracellular matrix, whose expression overlaps with that of decorin. Further characterization of decorin expression shows that the decorin message and protein product are
expressed in the floor plate at a time when netrin-1 mediated chemotropic growth of commissural axons is occurring. Binding studies indicate that decorin binds to netrin-1 and that this binding is likely to involve the core protein. These data suggest that decorin interacts with netrin-1 in vivo, and raise the possibility that decorin plays a role in netrin-1 mediated neurite outgrowth.
**Materials and Methods.**

**Materials.** Bovine skin decorin was the gift of Dr. L. Rosenberg (Montefiore Medical School). The mouse decorin cDNA and rabbit anti-mouse decorin antiserum were provided by Dr. R. Iozzo (Thomas Jefferson University). The mouse bone morphogenetic protein (BMP)-6 cDNA was provided by Dr. B. Hogan (Vanderbilt University). Extracts of COS cells expressing the netrin-1 transgene and 9E10 supernatant were provided Dr. M. Tessier-Lavigne (UCSF). 125I-labelled urokinase was provided by M. Herndon (MIT). Heparin, chondroitin sulfate A, and chondroitin sulfate B (dermatan sulfate) were obtained from Sigma.

**RNA probes.** A full length mouse decorin cDNA (Scholzen et al., 1994) in pT7/T3a19 (Gibco) was digested with AccI, and digoxigenin-labelled antisense RNA (correspond to bases 1105-1372 of mouse decorin) was synthesized by transcription with T7 RNA polymerase (Genius 4 Kit; Boehringer Mannheim). A digoxigenin-labelled RNA probe corresponding to BMP-6 was produced by EcoRI digestion of mouse BMP-6 in pBluescript (Stratagene) followed by transcription with T7 RNA polymerase (this probe corresponds to bases 395-0 of mouse BMP-6). A digoxigenin-labelled sense RNA (control) was produced by BssHII digestion of mouse BMP-6 in pGEM-3 (Promega), and transcription with T7 RNA polymerase (this probe corresponds to bases 0-395).

**In situ hybridization.** CD-1 mouse embryos were dissected and fixed overnight with 4% paraformaldehyde/PBS. Embryos were then cryoprotected by incubating overnight at 4°C in 5% sucrose/PBS, and then 15% sucrose/PBS. 20 μm cryostat sections were collected onto Probe-On Plus slides (Fisher).

As a pretreatment for hybridization, sections were fixed in 4% paraformaldehyde/PBS for one hour on ice. Sections were washed three times 5 minutes each in PBT (PBS/0.1% Tween-20). After bleaching twice for 45 minutes each in 5:1 (v/v) methanol:30% H2O2, sections were rehydrated stepwise into PBT. Sections were treated with 10 mg/ml proteinase K in PBT for 10 minutes at room
temperature, and fixed for 5 minutes at room temperature with 4% paraformaldehyde/0.2% glutaraldehyde/PBS. Sections were washed twice in PBT, treated with 0.1% sodium borohydride, and washed twice again in PBT.

Sections were incubated in hybridization buffer (50% deionized formamide, 5X SSC [pH 4.5], 50 μg/ml yeast RNA, 50 μg/ml heparin, 1% SDS) for one hour at 58°C. Sections were then hybridized overnight at 58°C in hybridization buffer containing 1 μg/ml digoxigenin-labelled RNA probes.

After hybridization, sections were washed twice 15 minutes each at 60°C in W1 (50% formamide, 5X SSC [pH 4.5], 1% SDS) and twice for 5 minutes at room temperature in W2 (0.5 M NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% Tween-20). RNase treatment was for 30 minutes at 37°C with 20 mg/ml RNase A in W2. Sections then were washed once for five minutes at room temperature in W2, once for five minutes at room temperature in W3 (50% formamide, 2X SSC [pH 4.5]), and twice for 30 minutes at 60°C in W3.

For antibody binding, sections were washed three times five minutes each at room temperature in TBST (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris-HCl [pH 7.5], 0.1 % Tween-20, 5 mM levamisole), and then blocked for one hour at room temperature with 10% heat-inactivated sheep serum (Sigma) in TBST. Sections were incubated overnight at 4°C in a 1:2500 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody (Genius 3 Kit; Boehringer Mannheim) (preabsorbed to mouse brain acetone powder [Sigma]) in 1% sheep serum. The next day, sections were washed extensively in TBST, and then several times in NTMT (0.1 M NaCl, 0.1 M Tris-HCl [pH 9.5], 50 mM MgCl2, 0.1% Tween-20, 5 mM levamisole). Sections were developed with 4.5 ml NBT and 3.5 ml X-phosphate each (Genius 3 Kit; Boehringer Mannheim) per ml of NTMT. Reactions were stopped by washing in PBT, and slides were coverslipped using Gelmount (Biomed).

**Immunohistochemistry.** Immunohistochemistry for decorin was performed as described in Chapter Three, using a 1:100 dilution of a rabbit anti-decorin polyclonal antibody. Staining was developed
using horseradish peroxidase. Control sections were stained only with secondary antibody, and failed to give any specific pattern of immunoreactivity (data not shown).

**Affinity chromatography.** 1 mg of decorin was dialyzed into 0.1 M Hepes (pH 8.0) and coupled to Affigel-15 (Biorad) as per manufacturer's instructions. 1 mg crystalline BSA (Calbiochem) was resuspended in the same buffer and coupled to a separate sample of Affigel-15 as above. Columns were washed extensively in 20 mM Tris-HCl (pH 7.4 @ 4°C) containing a) 0.14 M NaCl, b) 0.14 M NaCl, 1 mg/ml BSA (Calbiochem), c) 1 M NaCl, and then in 0.1 M sodium acetate (pH 4). Columns were reequilibrated in 20 mM Tris-HCl (pH 7.4 @ 4°C), 0.14 M NaCl.

1 M NaCl extracts of COS cells which express a netrin-1 transgene fused to a myc epitope (Serafini et al., 1994) were diluted into 0.14 M NaCl and passed over the columns. After washing with 20 mM Tris-HCl (pH 7.4 @ 4°C), 0.14 M NaCl, columns were eluted with 20 mM Tris-HCl (pH 7.4 @ 4°C), 1 M NaCl. NaCl was added to samples to 1M, and samples, including a series of dilutions of the original cell extract, were applied to Immobilon-P (Millipore) using a dot blot apparatus (Biorad).

Filters were blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.2% Tween-20) for one hour, and then incubated with 9E10 supernatant (an anti-myc monoclonal antibody) including 1% milk for about 1-2 hours at room temperature. Filters were then incubated in a 1:5000 dilution of a horseradish-peroxidase conjugated anti-mouse Ig antibody (Amersham), developed by enhanced chemiluminescence (Amersham), and exposed to XAR-5 film (Kodak). Film images were scanned and pixel intensity calculated over defined areas (specified to include individual samples), with background calculated for the same areas subtracted.

**Western blotting.** COS cell extracts were diluted into 0.2 M NaCl, and separated by 7.5% SDS/PAGE. Samples were then
electrophoretically transferred to Immobilon-P (Millipore). Filters were probed with 9E10 as described above.

**Affinity coelectrophoresis (ACE).** ACE was performed as previously described (Lee and Lander, 1991) with the following modifications. Lanes and well were placed as shown in Fig. 2. All experiments were done in the absence of CHAPS. 1% low melting point agarose (Gibco BRL) was cast onto GelBond (FMC). Descending concentrations of decorin or glycosaminoglycans were cast with agarose into the lanes, and 1 M NaCl extracts containing netrin-1 were diluted to NaCl concentrations below 0.2 M, and cast in 1% agarose into the well. After electrophoresis, the gel was removed from the gelbond and equilibrated in 20 mM Tris-HCl (pH 7.4 @ 4°C), 1M NaCl for 15 minutes. The gel was blotted to Immobilon-P by capillary transfer in the same buffer and probed with 9E10 as described above.

For ACE experiments using commercial GAG preparations, those GAGs were resuspended in ACE running buffer, diluted, and cast into lanes. Decorin-derived DS was prepared by treating decorin with 0.1 N NaOH/0.1M NaBH₄ for 90 minutes at 44°C to cleave the dermatan sulfate chain from the decorin core protein. The reaction was stopped with 0.5 M ammonium formate/50 mM HCl, and the entire mixture was then diluted and cast into lanes. For ACE of decorin-derived DS versus ¹²⁵I-labelled urokinase, the urokinase was embedded in the long well and the DS in the lanes; after electrophoresis, the gel was dried and exposed on a phosphorimager (Molecular Dynamics).
Figure 2. **Affinity coelectrophoresis.** A, A horizontal 1% agarose gel is cast with 9 lanes and 1 well perpendicular to the lanes. Descending concentrations of a test sample, in these experiments decorin of GAGs, are cast into the lanes with agarose. COS cell extracts containing netrin-1 are cast into the well with agarose. B, Netrin-1 migrates towards the anode during electrophoresis. However, the test samples in these experiments migrate towards the anode with much greater rates than does netrin-1. Hence, during electrophoresis the test samples encounter netrin-1. At concentrations of the test sample above its $K_d$ for netrin-1, binding occurs, and the mobility of netrin-1 is increased. At concentrations below $K_d$, no binding occurs, and thus there is no change in the mobility of netrin-1. The mobilities of netrin-1 at the various concentrations of test sample can be used to calculate the $K_d$ of the test sample for netrin-1 (Lee and Lander, 1991; Lim et al., 1991).
**Results.**

*Expression of decorin in the floor plate.*

In *situ* hybridization experiments were performed in order to determine the pattern of decorin expression in the developing spinal cord. In the embryonic day 11 (E11) mouse spinal cord, high levels of expression were observed in the floor plate (Fig. 3A), consistent with an earlier report of decorin expression (Scholzen et al., 1994). Lower levels of decorin expression could be observed in the developing connective tissue surrounding the spinal cord. Immunostaining with anti-decorin antibodies demonstrated that the decorin protein product is also present in the floor plate (Fig. 3B). No signal above background was observed in any other tissue (with the exception of the surrounding connective tissue), suggesting that there is little diffusion of decorin away from the floor plate.

In the E13 spinal cord, high levels of decorin expression persisted (Fig. 3C). Expression was limited, however, to the lateral margins of the floor plate; the medial floor plate was negative for decorin expression. As younger floor plate cells are found more laterally and older cells more medially (M. Tessier-Lavigne, personal communication), these results suggest that decorin expression might be transient in cells of the floor plate. In addition, at E13 higher levels of expression could be observed in the surrounding connective tissue, and in the skin, than at E11. This pattern of expression persisted in the E15 spinal cord (Fig. 3D). The decorin-expressing cells appear at E15 to have moved dorsally with respect to cells in earlier spinal cord; this is the result of continual accumulation of axons beneath the floor plate.

In *situ* hybridization experiments were also done using a RNA probe for bone morphogenetic protein (BMP)-6 (also known as Vgr-1), a member of the TGF-β family of proteins. BMP-6 expression in the floor plate has previously been reported (Jones et al., 1991); in that study, BMP-6 expression was localized to the cells adjacent to the floor plate. In this study, BMP-6 expression was observed directly in the floor plate, and, like decorin, seemed to be transient (Fig. 4A-C). While BMP-6 expression was observed in the medial floor plate, the lateral margins had higher levels of signal. It may
Figure 3. Floor plate expression of decorin. A, Transverse section through E11 mouse spinal cord hybridized with an antisense decorin RNA probe. Arrow indicates floor plate. B, Transverse section through E11 mouse spinal cord stained with an anti-decorin polyclonal antibody. C, Transverse section through E13 mouse spinal cord hybridized as in A. D, Transverse section through E15 mouse spinal cord hybridized as in A. Scale bar: 100 μm.
Figure 4. **BMP-6 expression in the developing spinal cord.** A, Transverse section through E11 mouse spinal cord hybridized with a BMP-6 antisense RNA probe. B, Transverse section through E13 mouse spinal cord hybridized with a BMP-6 antisense RNA probe. C, Transverse section through E15 mouse spinal cord hybridized with a BMP-6 antisense RNA probe. Abbreviations: f, floor plate; r, roof plate. Scale bar: 100 μm.
be that the previously reported pattern of BMP-6 expression was
due to the use of younger mice than those used in the experiments
presented here. As in the previous report, BMP-6 expression was
observed in the roof plate at all developmental stages checked (Fig.
4A-C).

Comparison of BMP-6 and decorin expression in the floor plate
suggests that BMP-6 is expressed slightly earlier. At E11, when
decorin is expressed in the middle of the floor plate, BMP-6
expression appears to be more lateral (Fig. 4A). At E13 and E15,
ventral BMP-6 expression has significantly declined, and is confined
to the medial floor plate (Fig. 4B,C). In all cases, the sense (control)
strand of BMP-6 gave no signal anywhere in the spinal cord. As
BMP-6 is similar to TGF-β, a protein which binds decorin (Yamaguchi
and Ruoslahti, 1988), it may be that BMP-6 in the floor plate binds
decorin. This hypothesis is currently being tested in collaboration
with A. Yang (UROP).

*Binding of decorin to netrin-1.*

As decorin expression is coincident with that of netrin-1, a
floor plate-derived chemoattractant for spinal commissural axons
(Serafini et al., 1994; Kennedy et al., 1994), we tested decorin for its
ability to bind to netrin-1. High salt extracts of COS cells expressing
recombinant netrin-1 fused to a myc epitope (r-netrin) were used
for these experiments. One band of the molecular weight previously
observed for netrin-1 (Serafini et al., 1994) was observed in
Western blots of the extract probed with the monoclonal
supernatant 9E10 (which recognizes the myc epitope) (Fig. 5). A
small amount of high molecular weight material was observed at the
interface between the stacking and separating gel; this is likely to be
aggregated r-netrin. When these extracts were passed over decorin
conjugated to Affigel, approximately 10-20% of the recoverable r-
netrin was eluted with 1 M NaCl (as measured by immunoreactivity
to the myc epitope) (Fig. 6). In contrast, no r-netrin could be eluted
with 1 M NaCl from a similar column prepared with BSA. These
data show that r-netrin can bind to decorin.
Figure 5. **Western blot of r-netrin.** A Western blot of a 1 M NaCl extract of COS cells which express a netrin-1 transgene. **N**=r-netrin.
Figure 6. **Affinity chromatography of r-netrin.** COS cell extracts containing r-netrin were passed over decorin-Affigel or BSA-Affigel columns; the columns were then eluted with 1 M NaCl and collected into five fractions. R-netrin eluted with 1 M NaCl is expressed as the percentage of total r-netrin recovered. Roughly 50% of the r-netrin applied to each column was recovered (likely due to nonspecific and irreversible absorption of netrin-1 to both columns). The dot blot of the 1 M NaCl fractions is shown for each column; no signal was detected in the eluates from BSA-Affigel columns, even after long exposures. ND, not detectable.
<table>
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<tr>
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<th>Eluted Fractions</th>
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<tr>
<td>Decorin-Affigel</td>
<td>1 2 3 4 5</td>
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1 M NaCl elution (% recovered netrin)

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<tr>
<td>Decorin-Affigel</td>
<td>10-20</td>
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<td>BSA-Affigel</td>
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In order to further characterize this binding, the affinity of r-netrin for decorin was measured by ACE. Horizontal agarose gels were cast, and samples embedded, as indicated in Fig. 2. While r-netrin migrates towards the anode, the mobility of decorin towards the anode is greater. Thus, decorin will encounter r-netrin during electrophoresis. At concentrations of decorin near or above the $K_d$ of r-netrin for decorin, a shift in the mobility of r-netrin occurs (Fig. 7A). The $K_d$ calculated from a plot of $R$ versus decorin concentration is 91.5 nM (Fig. 9A).

**Binding of netrin-1 to glycosaminoglycans (GAGs).**

Netrin-1 is known to bind heparin, as heparin affinity chromatography was one of the methods used to purify netrin-1 (Serafini et al., 1994). It thus seemed likely that $K_d$ measured for r-netrin and decorin reflected an interaction between r-netrin and the DS chain present on decorin. The affinities of r-netrin for various commercial GAGs were measured. R-netrin had a $K_d$ of 85 nM for heparin (Fig. 7B,9B), 1.4 $\mu$M for CS B (porcine skin DS) (Fig. 9B; data not shown), and 2.8 $\mu$M for CS A (Fig. 9B; data not shown). While heparin bound well to r-netrin, the affinities of CS, and particularly of DS, were lower than expected.

As the decorin-derived DS in our particular sample could have had a different fine structure than the DS obtained commercially, we measured the affinity of the DS from decorin for r-netrin. The DS chain was detached from decorin by $\beta$-elimination, and the mixture of DS and decorin core protein was loaded into the ACE gel. This procedure allowed for the measurement of the affinity of r-netrin for decorin-derived DS, and not for the decorin core protein, as the core protein has a low mobility and thus would not encounter r-netrin during electrophoresis (data not shown). No shift in r-netrin was observed below DS concentrations below 1 $\mu$M DS (Fig. 8A). Above this concentration, the r-netrin mobility was retarded in the gel; this is in contrast with results obtained both with intact decorin and with commercially available DS (see below), which caused an
Figure 7. **ACE of r-netrin.** Decorin (A) and heparin (B) vs. r-netrin.
A

B

Decorin (nM)

Heparin (nM)
Figure 8. **ACE of β-eliminated decorin.** A, β-eliminated decorin vs. r-netrin. B, β-eliminated decorin vs. urokinase.
B-eliminated decorin (nM)

A

B
Figure 9. Measurement of $K_d$ of r-netrin for decorin and GAGs. 

A, Plot of R vs. decorin concentration. —■— decorin; —▲— β-eliminated decorin. In this case, R is displayed as the absolute value of R, as R has negative values due to the retardation of netrin-1 with β-eliminated decorin. 

B, Plot of R vs. GAG concentration. —■— heparin; —▲— chondroitin sulfate; —■— dermatan sulfate. $K_d$'s for GAGs were calculated using the following Mr's: heparin, 10,000; chondroitin sulfate, 17,500; dermatan sulfate, 25,000.
A

Decorin
$K_d = 91.5 \text{ nM}$

$\beta$-eliminated decorin
$K_d = 1.3 \text{ } \mu\text{M}$

Heparin
$K_d = 0.85 \mu\text{g/ml}
(85 \text{ nM})$

CS
$K_d = 48.6 \mu\text{g/ml}
(2.8 \mu\text{M})$

DS
$K_d = 35.6 \mu\text{g/ml}
(1.4 \mu\text{M})$

B

R

GAG (μg/ml)
increase in the mobility of netrin-1. Using this shift as a measurement of binding, the $K_d$ of $\beta$-eliminated DS was about 1.4 $\mu$M (Fig. 8A,9B). The same sample had a $K_d$ of about 1.2 $\mu$M for urokinase (Fig. 8B; data not shown); as this is similar to previously measured $K_d$'s for CS and urokinase (M. E. Herndon and A. D. Lander, unpublished results), it is unlikely that the DS was damaged during $\beta$-elimination. The affinity of r-netrin for commercial DS is consistent with that measured for decorin-derived DS; in fact, it is likely that commercial DS, being derived from skin, was originally a component of decorin. These data imply that the affinity of the DS chain of decorin for r-netrin does not account for the affinity of intact decorin for r-netrin, and suggests that the decorin core protein is involved in the interaction of decorin with r-netrin.

\footnote{It is unclear why r-netrin in this experiment was retarded. It may be that there is something inherently different about the DS chain derived from this sample of decorin and the other GAGs tested. For instance, it may be that decorin-derived DS is longer, and thus can bind multiple r-netrin molecules, decreasing the mobility. It may also be, however, that the decrease in mobility was an artifact of the experiment. Since the reagents used to perform the $\beta$-elimination were not removed before ACE, it may be that those reagents were able to encounter r-netrin and affect its mobility. In a previous experiment where the concentration of decorin-derived DS was below 1 $\mu$M, no shift of r-netrin was observed (data not shown), consistent with the interpretation that decorin-derived DS has a significantly lower affinity for r-netrin than intact decorin.}
Discussion.

Decorin expression is transient in the floor plate. It has recently been shown that decorin, a small matrix PG usually associated with collagen fibrils, is expressed along the ventral developing nervous system; this data suggested that decorin might be expressed by the floor plate (Scholzen et al., 1994). In situ hybridization experiments on transverse sections through developing spinal cord confirm these results. Furthermore, these experiments suggest that decorin expression in the floor plate may be transient. As the spinal cord develops (E13 and E15), decorin expression is confined to the lateral margins of the floor plate. The medial floor plate expresses high levels of decorin at E11, but is negative at E13 and 15. As newer floor plate cells are added to the lateral margins, it may be that the older floor plate cells which, after a period, have down-regulated decorin expression.

Interestingly, BMP-6, a member of the TGF-β family, is expressed in the floor plate at the same time as decorin (Jones et al., 1991). This expression is also apparently transient, as it is has virtually disappeared by E15. BMP-6 colocalizes well with decorin at E11, when both messages overlap in floor plate expression. Compared to decorin, BMP-6 at this time seems to be more highly expressed along the lateral margins of the floor plate; earlier studies in younger mice found that BMP-6 expression was confined to cells on either side of the floor plate (Jones et al., 1991).

By analogy to TGF-β, this pattern of expression suggests a possible mode of regulation of decorin expression in the floor plate. TGF-β has been shown to induce decorin expression in some cells (Bassols and Massagué, 1990). It is possible that BMP-6, a member of the TGF-β family, may perform such a function in the floor plate. In addition, decorin can bind TGF-β and inhibit the effects of TGF-β on growth (Yamaguchi and Ruoslahti, 1988; Yamaguchi et al., 1990). Hence, it is also possible, by analogy, that decorin in turn regulates BMP-6 action in the floor plate. As a first step in testing these hypotheses, I am currently attempting in collaboration with A. Yang (UROP) to measure the binding of decorin to BMP-6.
Decorin and the extracellular matrix of floor plate.

As predicted by in situ hybridization, immunohistochemistry demonstrates the presence of the decorin protein product in the floor plate. These experiments demonstrate that decorin is largely confined to the floor plate, as no signal above background was detected in other regions of the spinal cord. It has been suggested that other proteins expressed in the floor plate may accumulate in the underlying basal lamina or on passing commissural axons (Klar et al., 1991).

The retention of decorin protein in the floor plate may be related to its ability to bind other proteins known to be in the floor plate. Type II collagen and fibronectin, both of which bind decorin with $K_d$'s of approximately 20 nM in solid phase assays (Hedbom and Heinegård, 1989; Schmidt et al., 1991; Winnemöller et al., 1991), have been reported to be expressed in the floor plate (Cheah et al., 1991; Shiga and Oppenheim, 1991; Su et al., 1991). We have shown in this report that decorin binds netrin-1, which is highly expressed in the floor plate. Other ligands for decorin in the floor plate are possible. F-spondin, a distant homolog of thrombospondin, is expressed in the floor plate (Klar et al., 1991); thrombospondin has been shown to interact with decorin with a $K_d$ of about 5 nM in solid phase assays (Winnemöller et al., 1992). Likewise, as discussed above, BMP-6 is expressed in the floor plate (Jones et al., 1991).

The floor plate expression of decorin is unusual in that decorin is usually expressed in association with collagen fibrils. While both type II collagen message and protein, which can bind decorin, is present in the floor plate (Cheah et al., 1991; Su et al., 1991), no fibrils have been reported; there is not even much evident pericellular matrix (Yaginuma et al., 1991). Nevertheless, the expression of multiple proteins, with known or potential affinities for decorin, suggests that there may be an organized matrix in the floor plate, and that decorin may be important for that organization (Fig. 10). One function of such a matrix could be to influence, by contact-mediated guidance mechanisms, the growth of commissural axons as they pass underneath the floor plate and then turn towards the head (Chang and Lagenaur, 1990; Klar et al., 1991; Yaginuma et
Figure 10. Interactions of decorin with proteins expressed in the floor plate. Some of these interactions have been demonstrated; others are hypothesized by analogy with known decorin-binding proteins (see text), and are indicated by question marks.
Netrin-1

Fibronectin

Netrin-1

F-Spondin

Decorin

Collagen Type II

BMP-6
al., 1993). Such a matrix could play a role analogous to that of basement membranes, which can support the growth of peripheral axons.

**Decorin may play a role in netrin-mediated neurite outgrowth.**

Decorin, either alone or as a component of an organized extracellular matrix, could also play a role in controlling the diffusion of factors out of the floor plate. Diffusible factors have been proposed to guide the growth of commissural axons towards the floor plate (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Recently, a floor plate-expressed protein that acts as a chemoattractant for commissural axons *in vitro*, netrin-1, has been identified (Serafini et al., 1994; Kennedy et al., 1994). Decorin can bind to netrin-1, and both proteins are expressed in the floor plate at a time when commissural axons are extending. This suggests that decorin could regulate netrin-1 activity. Such regulation could involve enhancement or inhibition of axon growth or guidance, controlling the diffusion of netrin-1, or localization of netrin-1 to the floor plate.

The existence of netrin synergizing activity (NSA), which can increase the effect of netrin-1 on axon outgrowth (Serafini et al., 1994), suggests that netrin-1 interacts with other factors *in vivo*. Decorin is one one factor that is likely to bind netrin-1 *in vivo*, and there are likely to be others. The high affinity of netrin-1 for heparin suggests that netrin-1 may also bind to heparan sulfate PGs in the spinal cord. Interestingly, netrin-1 is found in membrane fractions of brain homogenates and netrin-1-expressing COS cells. Netrin-1 can, however, be extracted from the surface of cells with high salt. Such interactions might occur through PGs on the cell surface. The binding of netrin-1 to HSPGs, either on cell surfaces or in the basement membranes surrounding floor plate, may also help organize the matrix of floor plate.

These interactions are unexpected, given the proposed function of netrin-1 as a diffusible factor. Characterization of the effects of decorin on netrin-1 activity will likely be important in understanding the manner in which netrin-1 affects axon growth.
and guidance, and how gradients of netrin-1 are established in the spinal cord.
Chapter 5: The functions of proteoglycan core proteins in the development of the nervous system.
1. Introduction.

PGs, as proteins which bear GAG chains, have the potential to affect a number of functions involving GAG binding proteins. GAGs can accelerate reactions, stabilize complexes of proteins and receptors, act as reservoirs of proteins, induce conformational changes, and participate in extracellular matrix structure. Binding of GAGs to proteins in some cases can directly mediate processes such as cell adhesion.

It is clear as well that PG core proteins are in many cases vital for PG function. The existence of a large number of proteins, with diverse structures, that can bear GAGs, and the in vivo regulation of the expression of these proteins, suggests that these proteins play important functional roles. If the only function of the PG core protein is to bear and present GAG chains, one might expect to find only one or a few cores. The diversity of core proteins may reflect different functions that core proteins may have; the expression of different core proteins may thus allow different responses upon interacting with GAG-binding proteins. The regulated in vivo expression of core proteins may reflect specific requirements of cells or tissues for particular PGs, and thus further suggests that PG core proteins have important functions.

2. Multiple PG core proteins as the basis for diverse PG functions.

The existence of multiple families of PG core proteins, each with different structures and properties, and each possessing multiple homologs, suggests that the PG core proteins play important roles in PG function. Cell surface HS, for example, can be provided by two families of cell surface PGs, to each of which belong four members. One class is the syndecan family of transmembrane HSPGs; the other is the glypican family of GPI-linked HSPGs. The existence of these two families raise the question of why eight distinct gene products falling into two classes are required to provide cell surface HS.

It is likely, based on an examination of the structures of the two families, that syndecans and glypicans are involved in different
functions. Syndecan is a transmembrane protein with a cytoplasmic domain that, being conserved between family members and between species, has been predicted to interact with the cytoskeleton (Bernfield et al., 1992). This prediction has been supported by colocalization studies of syndecan-1 and syndecan-4 with F-actin, and the localization of syndecan-4 to focal adhesions (Woods and Couchman, 1992; Carey et al., 1994; Woods and Couchman, 1994). These properties, likely mediated by the cytoplasmic domain, may make syndecan a particularly good core protein for providing cell surface HS for functions involving cell adhesion.

Glypican, on the other hand, is GPI-linked, and thus is unlikely to interact directly with the cytoskeleton. The GPI-linkage, however, is predicted to impart other properties on glypican. These might include high lateral mobilities in cell membranes (Chan et al., 1991) localization to specialized membrane domains (Lisanti et al., 1994), and the ability to directly signal through intracellular protein tyrosine kinases (Stefanova et al., 1991; Thomas and Samelson, 1992; Rudd et al., 1993). Thus, glypican may induce different cellular responses than syndecan in response to interactions with heparin-binding proteins.

The extracellular domains of these two classes also suggest different functions. The extracellular domain of syndecan is poorly conserved among other family members and between species (with the exception of the GAG attachment sites) (Bernfield et al., 1992), suggesting that the syndecan extracellular domain may have little function beyond the anchoring of GAGs. Glypican and its family members, however, are more conserved, both between members and between species (Stipp et al., 1994). In particular, the conservation of fourteen extracellular cysteines suggests that glypican and its family members may have a conserved three dimensional structure. This conserved structure may in turn reflect a common function. Such a function could include the binding of the glypican core protein to other proteins in the membrane; however, no such interaction has yet been described.
3. Functional implications of the restricted expression of PGs.

That core proteins are important is supported by the fact that PG core proteins are often expressed distinctly in spatially and temporally restricted patterns. Such restrictions in expression can suggest possible functional roles for a protein.

For instance, expression studies suggest that glypican may act as a coreceptor for one or a number of heparin-binding proteins. In the early developing nervous system glypican expression is associated with neuroepithelia (Chapter 3). At this time, neural stem cells are proliferating, and beginning to give rise to differentiated neurons. These processes are likely to be regulated by growth factors, and both expression data and in vitro assays suggest that these growth factors include members of the FGF family (Walicke, 1988; Murphy et al., 1990). The coexpression of glypican with members of the FGF family at this time in development, along with the requirement of HS for the physiological actions of FGF, suggest that glypican may be acting as a coreceptor at this time. In fact, glypican could be acting as a coreceptor for FGFs throughout the lifetime of the nervous system. In adulthood, glypican is highly expressed in neurons that either express members of the FGF family, or that require FGF for survival (Elde et al., 1991; Gómez-Pinilla et al., 1992; Hughes et al., 1993). There is also the possibility that glypican regulates the function of other heparin-binding proteins instead or as well.

The pattern of glypican contrasts to that of the homolog cerebroglycan. While glypican is associated with neural precursors in the early nervous system, cerebroglycan is associated with differentiated neurons. It is unclear if this complementary pattern of expression persists throughout development. While glypican is expressed in the internal and external granule cell layers of the developing cerebellum, it is not known whether glypican is associated with newly differentiated neurons, as is cerebroglycan. Expression of both PGs seems to overlap in the ventricular zone and cortical plate of developing cerebral cortex, but it is unclear if the two PGs are expressed by the same cells--the pattern could be
explained by a switch in expression or by overlapping expression. It may in general be that 1) cells express glypican, switch to cerebroglycan upon differentiation, and then switch back to glypican, or 2) cells express glypican, leave glypican on after turning on cerebroglycan following differentiation, and then turn cerebroglycan off, leaving glypican on or shutting it off depending upon other factors. In order to address these questions, it will be necessary to perform careful double-labeling analysis of both PGs.

Despite the differences in expression, localization of the protein products for these two PGs suggests some commonality. Both glypican and cerebroglycan are localized to nerve fibers and axons, and in some cases they may be selectively expressed on axons. In combination with expression data, these data suggest that cerebroglycan plays a role in axon outgrowth. Glypican may be expressed on axons for other reasons; if its role is to act as a growth factor receptor it may be required on axons in order to allow neuronal response to target-derived signals.

Protein localization data, in combination with preliminary results showing that glypican is found in biochemical preparations of synaptosomes (Kumbasar et al., 1994; Litwack et al., 1994b), further suggest a possible synaptic role. Other PGs are found at synapses such as agrin or Cat-301; it may be that PGs are important for synaptic function or specificity. Further characterization of glypican's localization, including ultrastructural studies, may shed more light on glypican's function.

4. Interactions of PG cores with other proteins.

Their complementary patterns of expression in the early nervous system suggest that glypican and cerebroglycan perform different functions. The structural similarities between the two proteins, however, makes it unclear what those functions are. That there are functional differences, however, is further suggested by preliminary evidence that glypican and cerebroglycan isolated from neonatal rat brain have different affinities for fibronectin (which is also expressed in the neonatal rat brain), presumably as a result of different GAG structures (Herndon and Lander, 1992). Further
characterization of the binding affinities of these PGs for various heparin-binding proteins may elucidate function.

In addition to examining the characteristics of glypican-associated GAGs, it will also be important to search for proteins that bind to the glypican core protein. No such proteins are yet known. Nevertheless, recent evidence suggests that both glypican and cerebroglycan can associate with protein tyrosine kinases (Kumbasar et al., 1994; Litwack et al., 1994b). Establishing the identity of these kinases, and whether they interact directly or indirectly through other proteins with cerebroglycan and glypican, may also aid in identifying functions for these cores.

Other PGs are known to be interact with proteins through their cores. The decorin core proteins binds, likely via its leucine-rich repeats, to collagen type I. This binding is functionally important, as it affects both the rate of fibrillogenesis and the final morphology of collagen fibrils. Other decorin homologs, however, exhibit different binding properties. Biglycan does not bind to collagen; fibromodulin does bind to collagen, but at a different site on the fibril than does decorin (Scott and Haigh, 1988; Hedbom and Heinegård, 1993). Hence, different cores within the same family can exhibit different binding abilities, and such differences might be one reason why multiple family members have evolved.

Testable theories as to the functions of individual PGs can be formed by investigations of both expression and binding of individual core proteins. For instance, I have shown in Chapter 4 that decorin binds to netrin-1, a chemoattractant for spinal commissural axons, and that this interaction is likely to involve the core protein. The specific expression of decorin in the floor plate at a time when commissural axons are presumably responding to floor plate-derived netrin-1 suggests that decorin could affect the activity of netrin-1. Experiments are currently in progress to test the effect of decorin on netrin-1's axon outgrowth-promoting activity (in collaboration with M. Galko and M. Tessier-Lavigne, UCSF).
5. Conclusion.

It may be that particular functions can be assigned to individual PG core proteins. The studies presented in this thesis demonstrate that certain PGs are regulated in their expression in the nervous system, and suggests that this regulation is likely to reflect the functional roles of these molecules. Further characterization of nervous system expression, as well as investigation into the interactions of these PGs with other proteins, is necessary in order to further elucidate the functions of the individual PG core.
Appendix 1: Expression of glypican in myeloma cells leads to loss of heparan sulfate synthesis on PG core proteins.
**Introduction.**

Many of the functions of proteoglycans (PGs) can be attributed to the function of the attached glycosaminoglycan (GAG). One example of this is the attachment of myeloma cells to collagen type I (Sanderson et al., 1992; Sanderson et al., 1994). Myeloma cell adhesion to collagen type I is directly related to the affinity of cell surface HS on each cell type for collagen type I; this cell surface HS is attached to syndecan-1, which is the major, if not the only, HSPG in these cells. The higher affinity HS of MPC-11 myeloma cells can support cell adhesion on collagen type I, while the lower affinity HS of P3 myeloma cells cannot. These differences in affinity are related to differences in sulfation position; while HS from both cells has the same overall levels of O-sulfation, the higher affinity MPC-11 HS has more 4-O-sulfate, a modification apparently involved in high affinity interactions with collagen.

Nevertheless, it is possible that the core protein of syndecan-1 play a role in the adhesion of MPC-11 cells. The conservation of the cytoplasmic domain between members of the syndecan family has led to the suggestion that syndecan can interact with the cytoskeleton. Indeed, members of the syndecan family have been found to colocalize with F-actin, and to participate in focal adhesions (Woods and Couchman, 1992; Carey et al., 1994; Woods and Couchman, 1994). It may be that this property supports cell adhesion via attached HS chains.

Glypican, a GPI-linked HSPG, is likely to have different properties on the cell surface. GPI-linked proteins are predicted to have high lateral mobilities in cell membranes (Chan et al., 1991). They also have been reported to be localized to specialized membrane domains (Lisanti et al., 1994), and to interact with protein tyrosine kinases (Stefanova et al., 1991; Thomas and Samelson, 1992; Rudd et al., 1993). Glypican on cell surfaces, then, might lead to different effects than syndecan.

It may also be that a role of PG core protein is to specify the structure of its attached HS. No evidence exists that this occurs, however. Little difference is seen among different cores in the affinity of their HS for antithrombin III (Mertens et al., 1992).
fact, even the same PG can have attached to it both high and low affinity chains for antithrombin III (Kojima et al., 1992). While one report suggests that different cores from the same source have different affinities for FGF-2, these differences have not been related to HS structure (Aviezer et al., 1994).

In order to investigate the different functional properties of glypican and syndecan, both in terms of HS structure and effects on cell adhesion, cells were infected with a retrovirus possessing a glypican transgene. Some initial differences were observed in terms of the type of GAG attached to the protein. Surprisingly, however, high levels of glypican expression lead to a complete loss of glycanation with HS, but not CS, in these cells. Analysis of such cells may give insight into the mechanisms of HS synthesis.
Materials and Methods.

Infection of P3 cells with glypican. A full length rat glypican cDNA (Litwack et al., 1994a; Chapter 2, this thesis) was inserted into the EcoRI site of the retroviral vector pMV7 (Kirschmeier et al., 1988). This vector uses an LTR promoter for transgene expression, and contains a selectable neo marker. ψ2 cells were transfected with the glypican containing construct using Lipofectamine (Gibco BRL) and selected in the presence of 1 mg/ml G418 (Gibco BRL). Viral conditioned medium from these ψ2 trans cell lines was used to reinfect ψ2 cells, thus creating ψ2 inf cell lines. Viral conditioned medium from the ψ2 inf lines were used to infect P3 myeloma cells (gift of R. Sanderson, U. of Arkansas). Infected P3 cells were grown in the presence of 0.5 mg/ml G418 and individual lines were cloned by the method of limiting dilutions on mouse macrophage feeder layers. The relative levels of glypican expression were determined by fluorescence-activated cell sorting (FACS) (Cancer Center, M. I. T.) using the affinity purified anti-glypican polyclonal antiserum 343-1 at 2.5 mg/ml and a 1:500 dilution of fluorescein-conjugated goat anti-rabbit secondary antibody (Vector).

PG purification. PG purifications and iodinations were performed as previously described (Herndon and Lander, 1990). Immunoprecipitations were performed as described in Chapter 2, except that GAG lyase digestion were performed before immunoprecipitation, and protein A-antibody complexes were boiled directly in sample buffer before analysis by SDS/PAGE. Labeled PGs and immunoprecipitates were analyzed by 9% SDS/PAGE (Laemmli, 1970).

SDS extracts. 5 x 10^6 cells were washed two times in cold HBSS. Cells were resuspended in 50 mM Tris [pH 8 @ 4°C], 1% SDS, and boiled for 10 minutes. After centrifuging for 5 minutes, supernatants were removed and saved at -80°C until further use. In some cases, cells were treated for 30 minutes at 37°C with 0.5 U/ml phosphoinositide-specific phospholipase C (PIPLC; Boehringer Mannheim) and centrifuged before extraction of cell supernatant.
and cell pellets with SDS. Some samples were reduced by boiling with 10 mM dithiothreitol and then alkylating with 50 mM iodoacetamide before Western blotting.

Enzymatic treatments. SDS extracts were diluted 1:10 into 1% Triton X-100. Heparitinase and chondroitinase digestions were performed as described previously (Herndon and Lander, 1990). For cleavage of N-linked sugars, samples in 1% Triton X-100 and 10% β-mercaptoethanol were treated with 5 U/ml N-glycosidase F (Boehringer Mannheim) at 37°C overnight.

Western blots. Western blots were performed as described in Chapter 3, using 5 mg/ml 343-1.

Cell staining. Cells were washed in 5% goat serum/5% fetal calf serum in HBSS. Cells were then incubated for 30 minutes in 2.5 mg/ml 343-1, washed again, and incubated in a 1:50 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Vector) for 30 minutes. After washing extensively, cells were fixed in 10% formalin for 5 minutes, washed in PBS, and then mounted with 50% glycerol on glass slides. In some cases, cells were treated with 0.5 U/ml PIPLC, 10 mM phytic acid, or 1 mg/ml heparin for thirty minutes on ice, or in the case of PIPLC, at 37°C. Mock samples were treated identically, but these reagents were omitted.
Results.

In order to create cell lines that coexpress glypican and syndecan-1, P3 myeloma cells were infected with a retrovirus containing a glypican transgene. After selecting for successful infectants, PGs were purified from the initial pool of neo resistant cells (designated P3inf), as well as from uninfected control cells (P3). Purified PGs were treated with GAG lyases and subjected to electrophoresis. In PGs purified from P3inf cells, both the syndecan and glypican core proteins were present at the molecular weights expected; only syndecan was detected in PGs purified from control P3 cells (Fig. 1). Syndecan core protein appeared in response to chondroitinase alone, while glypican core protein appeared in response to heparitinase alone. This suggests that there is a fraction of glypican that possesses only HS, and there is fraction of syndecan that possesses only CS. Immunoprecipitation from the purified PGs of infected cells using 343-1 yielded a band the size of glypican in lanes where sample had been treated with heparitinase; otherwise, a higher molecular weight smear was seen, typical of intact PG (Fig. 1). It is unclear from these experiments whether glypican in P3 cells possesses any CS.

Clonal lines were isolated from P3inf by the method of limiting dilutions. 7 independent clones were grown up for analysis. FACS analysis indicated that all lines had significantly higher glypican expression than P3inf (Fig. 2).

The line expressing the highest levels of glypican as measured by FACS, F6, was selected for further analysis. PGs were purified from F6 and analyzed by immunoprecipitation with 343-1 and SDS/PAGE. Surprisingly, no glypican was detected, either in lanes containing total PGs, or in lanes containing 343-1 immunoprecipitates (Fig. 3). Syndecan-1 could still easily be detected. However, syndecan-1 derived from F6 behaved differently than syndecan-1 from P3 cells or P3inf cells. In these last two lines, the amount of syndecan-1 seemed greater when treated with heparitinase and chondroitinase compare to when treated with chondroitinase alone (Fig. 1); the amount of syndecan-1 from F6 cells seemed equivalent in the two lanes (Fig. 3). Syndecan-
Figure 1. **Analysis of PGs from P3inf cells.** 1, $^{125}$I-labeled PGs purified from uninfected P3 cells. 2, $^{125}$I-labeled PGs purified from P3inf cells. 3, $^{125}$I-labeled PGs purified from uninfected P3 cells immunoprecipitated with 343-1. 4, $^{125}$I-labeled PGs purified from P3inf cells immunoprecipitated with 343-1. Samples were analyzed on a 9% SDS/polyacrylamide gel. Abbreviations: U, untreated; H, heparitinase-treated; C, chondroitinase-treated; HC, heparitinase- and chondroitinase-treated.
Figure 2. FACS analysis of clones derived from P3inf cells. Cells were sorted based on fluorescence intensity after staining with 343-1. Individual clones are labeled C3, G5, F6, etc. "no ab" signifies that no primary or secondary antibody was added to cells before FACS analysis. "no pri. ab" signifies that no primary antibody was added to cells before FACS. "Mean fluorescence" indicates the channel of average fluorescence for that sample.
Figure 3. Analysis of PGs from F6 cells. 1, $^{125}$I-labeled PGs purified from F6 cells. 2, $^{125}$I-labeled PGs purified from F6 cells immunoprecipitated with 343-1. Analysis and abbreviations as in Fig. 1.
1 is normally a hybrid HS/CSPG, but in F6 cells it behaved as a pure CSPG. If this change was due to a loss of HS synthesis, it followed that glypican, an HSPG, might be completely unglycanated in these cells.

To test this hypothesis, F6 cells, as well as C3 cells (the lowest expressing line obtained by FACS), were extracted with SDS, and untreated or heparitinase- and chondroitinase-treated samples were analyzed by Western blotting with 343-1. Significant levels of immunoreactivity were detected both in the presence and absence of GAG lyases (Fig. 4A,B). Two bands were detected at roughly the size of glypican, one of about 66 kD and the other of about 57 kD. The apparent molecular weights of these bands increased upon reduction and alkylation, as would be expected for glypican—the upper band now migrated at about 71 kD, and the lower band migrated at about 64.5 kD. These two bands represent glypican species that have been differentially N-glycosylated, as the two bands are lost and one band of approximately 65 kD appears upon treatment with N-glycosidase F (as compared to 68 kD for the lower band of the doublet in this gel) (Fig. 5). Glypican in fact has two potential N-linked glycosylation sites; this may lead to differential glycosylation in P3 cells and the appearance of two bands.

Additionally, material from F6 contained a significant amount of high molecular weight material that disappeared upon reduction. The C3 line, which expresses less glypican, only had a small amount of high molecular weight material. It is likely that this high molecular weight material represents disulfide-bonded aggregates of glypican. No glypican-like signal is seen in uninfected P3 cells, although in Western blots of P3 cells with 343-1 there is usually a faint band at about 150 kD. The presence of this band does not affect any of the interpretations of data offered in this Appendix.

Thus, a large fraction of glypican from both C3 and F6 is apparently completely unglycanated. In fact, no glypican was ever detected in standard PG preparations from F6 cells\(^1\) suggesting that

\(^1\)As PG purification usually include ion-exchange chromatography, which isolates PGs based on the high negative charge of their attached PGs, unglycanated glypican would be expected to be lost at this step.
Figure 4. **Western blots of C3 and F6 extracts with 343-1.** 15 mg of SDS extracts of all cell lines treated as described were analyzed by Western blotting with 343-1. **A,** Lanes 1, 3, 5, nonreduced samples; lanes 2, 4, 6, reduced and alkylated samples. Lanes 1, 2, uninfected P3 cells; lanes 3, 4 C3 cells; lanes 5, 6, F6 cells. **B,** Lanes 1, 3, 5, untreated samples; lanes 2, 4, heparitinase- and chondroitinase-treated samples. Lane 1, uninfected P3 cells; lanes 2, 3, F6 cells; lanes 4, 5, P3inf cells.
Figure 5. **Two glypican glycoforms are produced in P3 cells.** 1, 15 mg of SDS extract of C3 cells treated with N-glycosidase F. 2, 15 mg of SDS extract of C3 cells, untreated.
all the glypican in these cells is unglycanated. The lack of
 glycanation, however, is not an inherent defect in P3 cells, as P3inf
 contained glycanated glypican (Fig. 1). In addition, Western analysis
 of SDS extracts of P3inf, as performed above, shows that, while there
 is a little unglycanated glypican, GAG lyase treatment results in a
 large increase in signal due to glypican core. As P3inf is a
 heterogeneous cell population, the glycanated and unglycanated
 glypican species are likely arising from different subpopulations of
cells.

The lack of glycanation does not seem to be the result of
improper processing of glypican by P3 cells. A large majority of the
C3 glypican can be collected from medium after treatment of C3
cells with PIPLC. No detectable glypican remains in the SDS extracts
of the treated cells (Fig. 6). Similarly, 343-1 stains the surface of F6
cells, and this staining can be abolished by pretreatment of cells
with PIPLC (Fig. 7) but not heparin or phytic acid (data not shown),
which are reported to remove peripheral membrane HSPGs (Carey
and Evans, 1989). Hence, glypican seems to be properly processed
and sorted to the cell surface in these cells.

It was unclear, however, if the failure to be glycanated was a
glypican-specific defect or if it was a global defect in these cells.
$^{35}$SO$_4$-labeling experiments indicated that syndecan-1 from F6 cells
is predominantly substituted with CS (R. Sanderson, personal
communication). Thus, it is likely that overexpression of glypican in
P3 cells has resulted in a global defect in HS synthesis.
Figure 6. **Effects of PIPLC on glypican in C3 cells.** Western blot of 15 mg C3 fractions with 343-1. *Lanes 1,3,5,7,9,* untreated; *lanes 2,4,6,8,* heparitinase- and chondroitinase-treated. *Lanes 1,2,* SDS lysate of C3 cells; *lanes 3,4,* supernatant of C3 cells treated with PIPLC; *lanes 5,6,* supernatant of cells mock-treated with PIPLC; *lanes 7,8,* SDS extract of C3 cells after PIPLC treatment; *lane 9,* SDS extract of F6 cells.
Discussion.

I have reported here the surprising finding that the overexpression of glypican in P3 myeloma cells leads to a loss of total HS synthesis in the cell. While overexpression of PG cores in cells can lead to changes in GAG synthesis (Shworak et al., 1994b), no effect of this magnitude has yet been reported.

It is unclear, however, if the phenotypes observed in these two lines are the result of some type of clonal aberration, the general result of overexpression of proteins in P3 cells, or the effect of threshold levels of PG core protein expression. In order to demonstrate that this effect is due to the overexpression of a PG core, and that the levels of core are related to the phenotype, I am serially infecting P3 cells with glypican in order to create a set of P3 lines with sequentially increasing levels of glypican core protein expression. An inverse monotonic relationship between the levels of glycanated glypican and number of rounds of infection the cells have undergone will serve to establish the role of core protein expression in this phenotype.

Establishing such a relationship may give insight into the mechanisms controlling HS synthesis. One interpretation of the phenotype shown here is that there are multiple factors controlling HS initiation or elongation, possibly existing as a complex, and that excess core protein competes these factors away from the complex by binding them individually. However, overexpression experiments indicate that, at least in the case of syndecan-4/ryudocan, GAG synthetic enzymes appear to be in excess (Shworak et al., 1994b). Nevertheless, some of the HS synthetic activities, in particular those activities involving HS modifications, are likely to be coupled (Bame et al., 1991). Overexpression of ryudocan can lead to the uncoupling of this reactions, even though HS synthesis in general proceeds and overall levels of modifications are not greatly affected (Shworak et al., 1994b). If such an effect is occurring with this phenotype, it will be necessary to know at what point HS synthesis is failing. I thus plan to analyze any HS or HS precursor that may be remaining on glypican.

These cells, particularly the C3 cells, are likely to be useful in
the study of interactions of the glypican core protein with other molecules. Such studies can be confounded by the presence of GAG, and even GAG lyase treatment leaves some fragment of GAG attached to the core. C3 cells could be used as a source of glypican core protein that has insignificant quantities of GAG attached, and as a source of GAG-free glypican for experimental use.
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