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Heparan sulfate proteoglycans and the emergence of neuronal connectivity

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With the identification of the molecular determinants of neuronal connectivity, our understanding of the extracellular information that controls axon guidance and synapse formation has evolved from single factors towards the complexity that neurons face in a living organism. As we move in this direction — ready to see the forest for the trees — attention is returning to one of the most ancient regulators of cell–cell interaction: the extracellular matrix. Among many matrix components that influence neuronal connectivity, recent studies of the heparan sulfate proteoglycans suggest that these ancient molecules function as versatile extracellular scaffolds that both sculpt the landscape of extracellular cues and modulate the way that neurons perceive the world around them.

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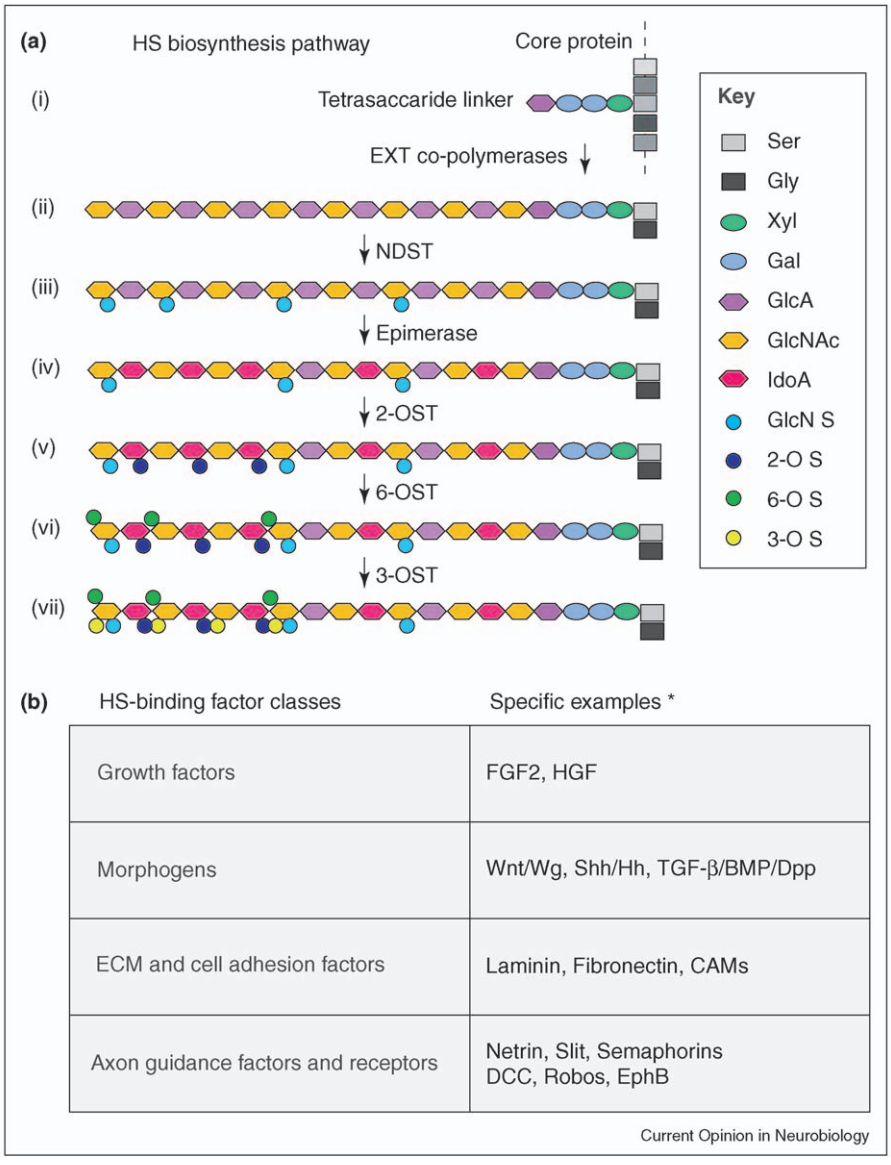
Introduction

To build or rebuild a fully functional nervous system, neurons must often send their axons far across complex terrain. Successful navigation of this challenging extracellular wilderness and productive interactions with target cells require an array of local and long-range axon guidance cues that influence the cell motility machinery within the growing tip of the axon (reviewed in [1,2]). Although a host of secreted factors that instruct axon guidance decisions have been identified, an emerging body of evidence suggests that the logic of their presentation to neuronal receptors requires interactions with the extracellular matrix (ECM), a thick cross-linked forest of secreted molecules characteristic of all metazoan organisms. Although several matrix components are known to regulate axon growth (reviewed in [3]), recent work suggests that one group of ECM proteins is particularly important for shaping and modulating the guidance factor landscape: the heparan sulfate proteoglycans (HSPGs).

HSPGs are cell-surface and secreted or released proteins decorated with a specific type of sulfated carbohydrate polymer: the glycosaminoglycan (GAG) heparan sulfate (HS) (reviewed in [4,5]). HS chains are made up of a repeated disaccharide (glucuronic acid and N-acetyl glucosamine) that is polymerized onto a tetrasaccharide linker covalently attached to specific serine residues in a proteoglycan core protein (see Figure 1a). Each step in the chain of assembly is catalyzed by a separate class of enzymes, from the peptide-O-xylosyltransferase that initiates the linker, to the exostosin (EXT)-family copolymerases that elongate the polymer (Figure 1a). Subsequent modification of the polymer through variable epimerization, de-acetylation and multiple sulfation reactions yields characteristic micro-heterogeneity leading to an astounding diversity of potential isoforms (e.g. up to10^{36} types of HS isoforms; reviewed in [6,7]). Distinct HS isoforms can display tissue-specificity in distribution [8,9] and binding-specificity in functional interactions [6,7], thus expanding the functional repertoire of this polymer. The extracellular functions of HS have been most extensively characterized in facilitating growth factor–receptor interactions (e.g. fibroblast growth factors; reviewed in [10]) and in regulating the distributions of secreted morphogens (e.g. Wingless and Hedgehog; reviewed in [11,12]). However, HS chains bind to a wide variety of secreted and cell surface proteins (see Figure 1b for partial list), suggesting that this carbohydrate is a highly versatile extracellular scaffolding molecule.

Of course, an additional layer of specificity for the localization, expression and function of HS lies in its carrier proteins. Conserved signaling motifs and observed functional specificity among HSPGs also indicate that they function as more than simple vehicles for HS. HSPG core proteins fall into four major families: the transmembrane Syndecans, the glycosyl-phosphoinositide (GPI)-linked Glypicans, and the secreted Perlecans and Agrins (see Figure 2a; reviewed in [4,5]). Additional proteoglycans might also carry HS as a minor carbohydrate, whereas some HSPGs carry small amounts of chondroitin sulfate (CS) — a distinct but similar GAG (reviewed in [13]). Among the major HSPG families, Syndecans and Glypicans are the most diverse and are localized at the cell surface by membrane attachment. However, enzymatic processing can also release these proteoglycans into the extracellular space (Figure 2a and b), making all HSPGs part of the extracellular matrix. Although HSPGs are

Figure 1



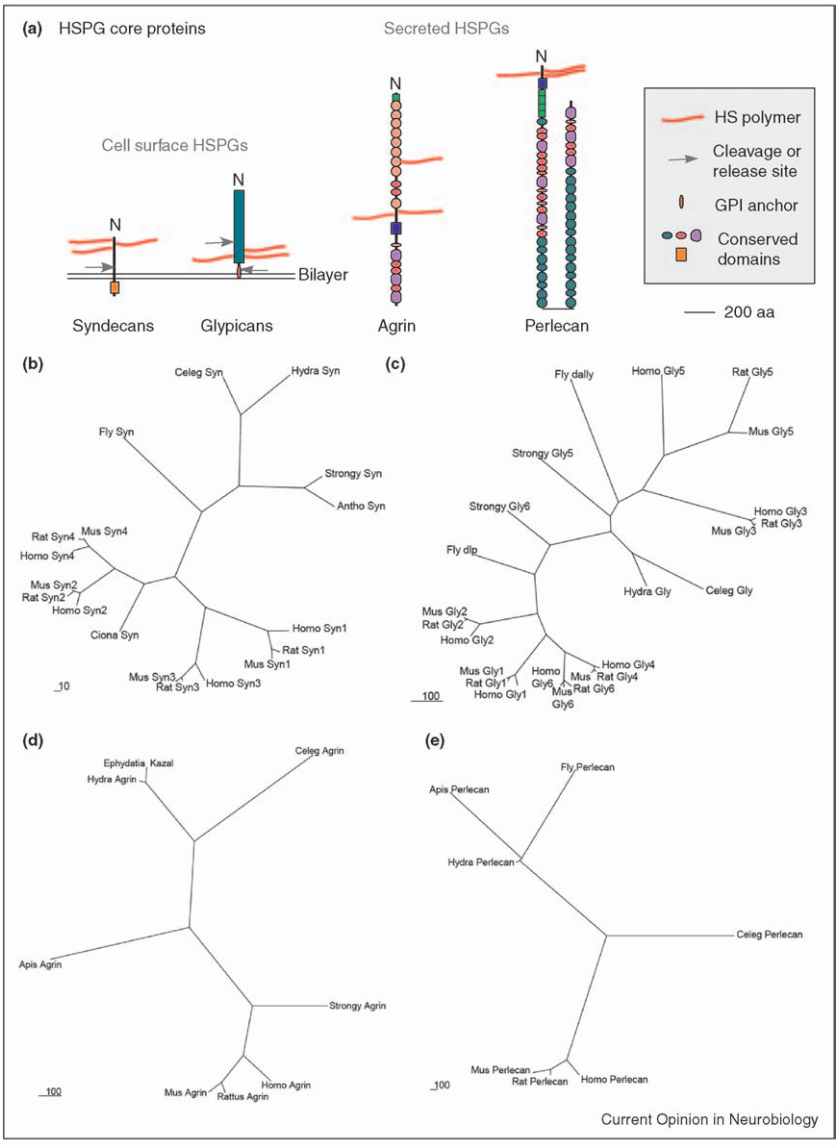
Heparan sulfate biosynthesis and HS-binding proteins. **(a)** A summary of the HS biosynthetic pathway. **(i)** The pathway starts with the addition of the Xyl-Gal-Gal-GlcA linker at specific serine residues, and **(ii)** the elongation of the HS chain by the EXT family of co-polymerases. **(iii)** It proceeds to the subsequent de-acetylation and sulfation (NDST), **(iv)** epimerization and **(v–vii)** sulfation of the polymer by 2-O, 3-O and 6-O-sulfotransferase enzymes (2-OST, 3-OST and 6-OST). Because sulfation and epimerization reactions are variable, micro-heterogeneity is observed within each HS polymer, creating distinct domains of charge density [5]. In addition, tissue-specific diversity in HS modifications exists [8,9]. **(b)** A partial list of factors that bind to HS and/or require HS for activity illustrates the potential complexity of HS function *in vivo* (* this list is not exhaustive). These factors include growth factors and morphogens that pattern early development, in addition to components of the extracellular matrix (ECM) and the factors and receptors that direct formation of axon pathways in the developing nervous system [21].

thought to function in many tissues and stages of development, genetic analysis of their functions *in vivo* point to the nervous system as an important arena in which these molecules regulate cellular morphogenesis.

Heparan sulfate and the formation of axon pathways

Early evidence that HS regulates the growth and guidance of axons *in vivo* came from simple experiments in the insect nervous system in which whole embryo culture could be exposed to high levels of exogenous carbohydrate polymers or enzymes to disassemble endogenous GAG sidechains, and identified axon pathways could be analyzed at single cell resolution [14]. In this pioneering study, HS emerged as the only GAG among several tested to have a significant role in the directional specificity of both central and peripheral axon pathfinding. Similar experiments in the amphibian visual system suggested

Figure 2



Heparan sulfate proteoglycan core protein families. **(a)** The four major HSPG families are illustrated for comparison of overall structure. The transmembrane Syndecans carry several HS chains near their extracellular N-terminus, however, the highest degree of sequence conservation is found in the cytoplasmic tail (orange box). The glycosyl-phosphoinositol (GPI)-anchor Glypicans have a large globular domain (blue box) N-terminal to the HS addition sites. Secreted Agrins carry HS chains in a central region, surrounded by several conserved motifs (orange, magenta, purple). The large, secreted Perlecans are composed of many Immunoglobulin domains (green circles), and carry several HS chains near the extreme N-terminus. (b–e) Parsimony-based phylogenetic reconstructions of the core heparan sulfate proteoglycans. **(b)** Syndecan (Syn) phylogeny. The diversification of syndecan into four members occurred after the split between urochordata (Tunicates, here represented by Ciona) and the rest of the chordates. **(c)** Glypican (Gly) phylogeny. Dally and dlp are the two glypicans found in *Drosophila melanogaster*. On the basis of the present phylogenetic reconstruction, dally and dlp, the two fly proteins, are sister to the diverse glypican subfamilies glypicans 3 and 5, and glypicans 1, 2, 4, and 6, respectively. The diversification of glypican into subfamilies appears to have occurred only in the vertebrata. **(d)** Agrin phylogeny. We included here the Kazal-like protein (sALK from the genus Ephydatia) together with Agrin proteins from major metazoan lineages. This was done to test the hypothesis that Agrin and Kazal are close paralogs and that extant Kazal, which is found ubiquitously throughout Metazoans, approximates the ancestral form of Agrin. The polyphyly of the 'true' Agrin shown here supports this hypothesis. **(e)** Perlecan phylogeny. Arthropod and Cnidarian perlecans appear to be highly diverged from perlecans found in chordate lineages. (b–e) Gene family members were identified using the basic local alignment search tool (BLAST) and unpublished motif detection algorithms written by the authors [77,79]. These tools were used in combination to mine whole genomic (when available) and expressed sequence tag sequence data from the following taxa: Anthocidaris crassispina (Antho); Apis mellifera (Apis); *Caenorhabditis elegans* (Celeg); Ciona savignyi (Ciona); *Drosophila melanogaster* (Fly); Ephydatia fluviatilis (Ephydatia); Homo sapiens (Homo); Hydra magnipapillata (Hydra); *Mus musculus* (Mus); Rattus norvegicus (Rat); Strongylocentrotus purpuratus (Strongy). Gene trees do not always contain the same taxa either, because data were too sparse to determine the absence of a protein family member unambiguously (e.g. with Antho, Ciona, Hydra, and Ephydatia) or because the protein family member was found to be truly absent from the lineage. Addition of other vertebrate lineages (e.g. *Xenopus*, Danio) did not affect the results. Trees were built using maximum parsimony (100 heuristic random replicate searches with Tree Bisection Reconnection [TBR] branch swapping). All trees are unrooted and branch lengths are to scale, reflecting numbers of nonsynonymous substitutions.

that HS is required for aspects of axon growth, guidance and target interaction [15]. Subsequent analysis in this system also suggested that specific HS sulfation patterns underlie aspects of retinal axon targeting [16]. Because the manipulation of HS in both vertebrate and invertebrate systems led to a variety of axonal phenotypes, one could anticipate that HS function in axonal development might be mediated by a variety of extracellular partners.

Biochemical studies of protein binding to HS, or the fully sulfated polymer heparin (e.g. [17–20]), implicated HS in the actions of many different factors known to influence the growth and/or guidance of developing axons (see Figure 1b). This list includes neurotrophic factors, ECM and cell adhesion molecules, in addition to chemotropic factors and morphogens (reviewed in [21]). However, the role of HS in the neuronal functions of these candidates has been tested in only a few cases. Numerous experiments in cell culture have suggested that HS facilitates axon outgrowth (e.g. [22–28]). However, evidence that HS is required for specific directional cues is more recent. One study of the growth cone repellent factor Slit showed that enzymatic removal of HS prevents efficient binding of Slit to the cell surface, and attenuates its repellent activity for cell migration and axon outgrowth [29]. More recently, another class of repellent Semaphorin 5A (Sema5A) was shown to be bifunctional, with attractive activity that requires HS on the surface of responding axons, and repellent activity that requires extracellular CS [30••]. The latter results were particularly exciting because they suggest that spatial patterns of particular GAGs and their carrier proteoglycans can confer specific guidance information, as opposed to having a purely permissive role.

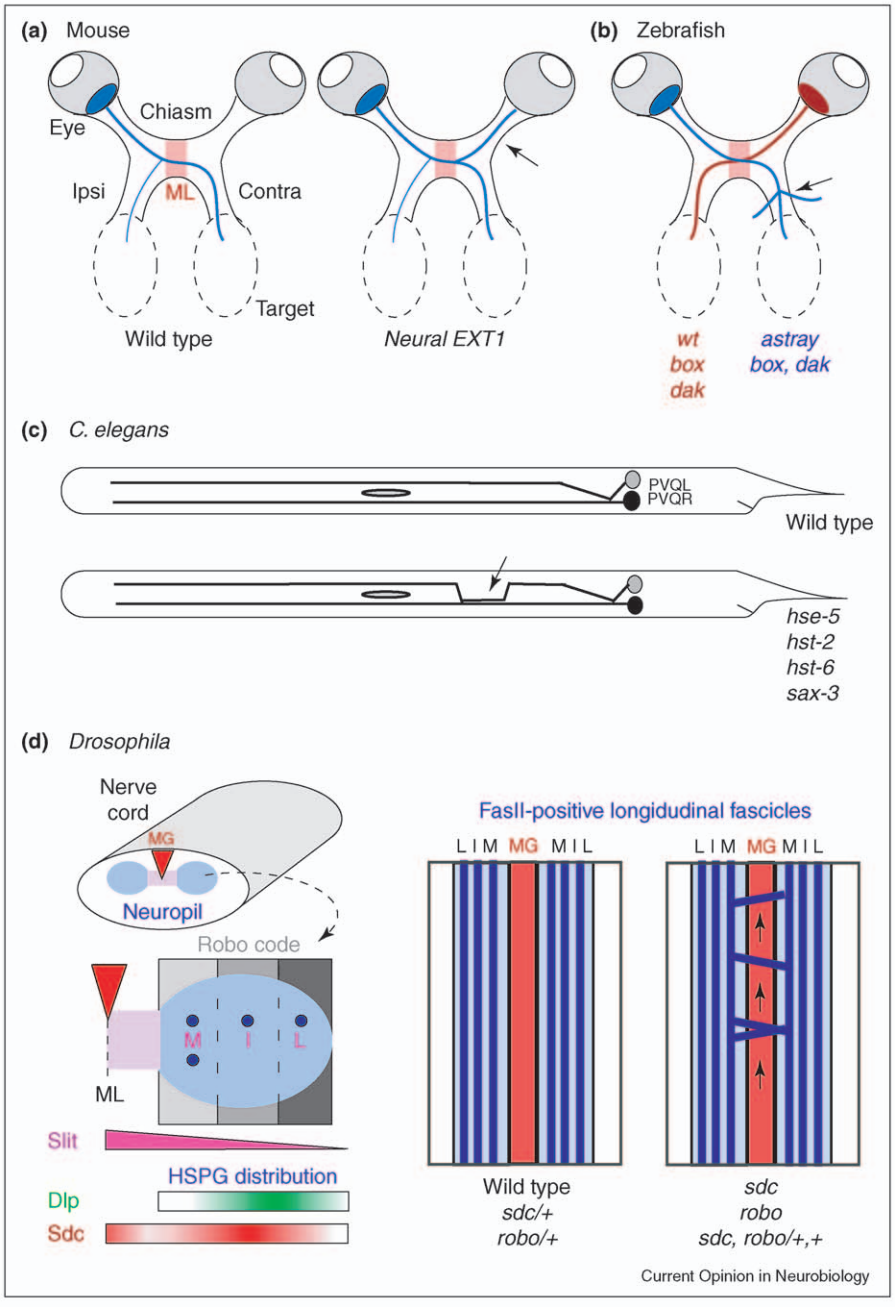
Heparan sulfate and axon guidance at the CNS midline

Although cell culture experiments provided the first data implicating HS as a modulator of growth cone repellent signals, perhaps the most compelling accumulation of evidence has come from genetic analysis of axon guidance at the CNS midline. The midline, a conserved feature of bilaterian organisms, functions as an organizing center and intermediate target that regulates the crossing of axons to integrate neural function on the two sides of the body. In addition to midline attractants (e.g. Netrin/UNC-6) that lure axons to cross over to the contralateral side, midline repellents (e.g. Slit) are presented by midline cells to prevent inappropriate crossing of ipsilateral axons and to define axon trajectories as they leave the midline. Although Netrin, Slit and their receptors bind to heparin, HS or HSPGs [17,19,31–33,34••], to date only Slit and its Roundabout/SAX-3 (Robo)-family receptors are known to require HS for efficient function *in vivo*.

The first *in vivo* evidence that HS is necessary for Slit function at the midline came from conditional loss of a key enzyme that catalyzes an early step in HS biosynthesis. CNS-specific elimination of the HS-polymerase gene EXT1 induces several defects in brain development, including absence of the major commissures that carry axons across the midline in the murine brain and visual system [35]. At the optic chiasm, where in EXT1 mutants retinal axons fail to project normally towards the contralateral side of the brain (see Figure 3a), a strong genetic interaction was observed between EXT1 and Slit2 [35]. Interestingly, independent forward genetics in zebrafish identified two additional members of the EXT family, *dackel/ext2* and *boxer/extl3*, as genes required for accurate guidance of retinal axons in the optic tract [36••]. Although elimination of both *ext2* and *extl3* is required to obtain a strong defect in midline crossing of retinal axons, suggesting some redundancy between the two enzymes, this double mutant phenotype is highly reminiscent of mutations in the Slit receptor astray/robo2 (see Figure 3b; [36••]). More recent analysis of mutants lacking the murine GlcNAc N-deacetylase/N-sulfotransferase Ndst1, which catalyzes an intermediate step in sulfation of the HS polymer, revealed defects in anterior commissure formation [37••]. The severe cerebral and craniofacial phenotypes caused by general loss of Ndst1 are suggestive of defects in Shh and FGF signaling and complicate the interpretation of axonal abnormalities, but are consistent with the EXT1 CNS phenotypes.

After polymerization and the initial deacetylation and sulfation of HS chains carried out by Ndst-family enzymes, HS is further modified by epimerization of glucuronic acid to iduronic acid, and additional sulfation at three different sites in the polymer (see Figure 1a). These latter steps in HS biosynthesis are variable, and, thus, introduce a large degree of structural heterogeneity [6,7]. *Caenorhabditis elegans* mutants lacking either the C5-epimerase (HSE-5) and the 6O-sulfotransferase (HST-6) were identified as genetic modifiers of mutations in *kal-1*, the ortholog of the human Kallmann Syndrome gene [38••]. Comparison of these mutants with mutants lacking the 2O-sulfotransferase (HST-2) revealed that the fidelity of midline axon guidance does rely upon HS modification enzymes. The *hse-5*, *hst-2* and *hst-6* mutants shared phenotypes with mutants lacking Slit (*slt-1*) and Robo (*sax-3*), in addition to Ephrin (*vab-1*) and Integrin (*ina-1*) (see Figure 3c). However, when double mutant analysis was applied to test the functional relationships between the HS-modifying enzymes and specific guidance signaling pathways, a pattern of specificity was revealed. The action of the Slit–Robo pathway required HS modifications in some but not all neurons. Moreover, the functions of Ephrin, Integrin and KAL-1 were dependent on different sulfotransferases in different contexts. These findings reinforce the notion that HS is a tool to modulate a

Figure 3



Heparan sulfate modulates midline axon guidance. **(a)** In the murine visual system, retinal ganglion cell axons make major contralateral (Contra) and minor ipsilateral (Ipsi) projections from the optic chiasm to the lateral geniculate nucleus (Target). Loss of EXT1 activity in neurons results in ectopic axon projection toward the contralateral retina (arrow; [35]). **(b)** In zebrafish, wild type retinal axons (red) project solely to the contralateral optic tectum, whereas in the absence of Astray (Robo2) or both of the EXT-family enzymes Boxer and Dackel (blue) axons display ectopic branching (arrow) [36••]. **(c)** In *C. elegans*, the longitudinal axons of PVQL neurons display ectopic midline crossing in the absence of the HS modification enzymes hse-5, hst-2 and hst-6 (arrow), highly reminiscent of mutants lacking the Robo-family receptor SAX-3 [37••]. **(d)** In the *Drosophila* CNS (nerve cord), midline glial cells (MG) express the secreted repellent Slit. An overlapping pattern of Robo receptor expression (Robo code) on the surface of axons in the longitudinal neuropil (light blue) determines lateral position of longitudinal axon tracts relative to the midline. Longitudinal axons are organized into fascicles that can be visualized with cell-surface markers like Fasciclin II (dark blue circles). The HSPGs Dlp and Sdc, localized in an overlapping pattern in the neuropil, function to improve the efficiency of midline repulsion. Mutants in *sdc* show midline crossing reminiscent of a weak *robo* mutant, either homozygous or when combined *in trans* with a *robo* allele (robo/+ and sdc/+ indicating heterozygotes) [34••,39•]. Abbreviations: I, intermediate; L, lateral; M, medial; ML, midline.

variety of signaling pathways. But, in addition, the data also imply that specific sulfation patterns might provide an 'HS code' to endow different cells with distinct profiles of ligand sensitivities depending on the distribution or activity of HS-modifying enzymes [38••]. This information-rich view of HS function would depend on discrete cell-type specificity in HS modifications, and is yet to be rigorously tested. An alternative is that the efficiency of receptor activation varies depending on the context of ligand presentation, and that HS might facilitate interactions by concentrating ligands in challenging circumstances (e.g. distant ligand source), but not be required in others (e.g. proximal source). Quantitative assays for ligand–receptor binding and signal output will be needed to complement genetic manipulation of HS biosynthesis to resolve these issues.

Heparan sulfate proteoglycans modulate axon guidance *in vivo*

Although HS modifications are a likely source of functional specialization, additional specificity could be introduced at the level of the HSPG core proteins. Again, genetic analysis has shown that HSPGs are important for the function of Slit and Robo during midline axon guidance. Studies of *Drosophila* mutants with defects in the single *syndecan* (*sdc*) gene revealed ectopic passage of axons across the CNS midline and strong dose-sensitive genetic interactions with Slit and Robo (see Figure 3d; [34••,39•]). Rescue experiments with cell type-specific expression showed that Sdc must function in neurons for normal midline repulsion, not in the midline cells that secrete Slit [34••,39•], consistent with the localization of Sdc and Slit to axon surfaces [34••,39•,40]. Moreover, development of a gentle immunohistochemical preparation with increased sensitivity for extracellular Slit showed significant decreases in Slit localization to axons in the *Sdc* mutant [34••]. This was consistent with the role proposed for HS in shaping extracellular morphogen distributions during early pattern formation (reviewed in [11,12]). However, the biochemical association observed between endogenous Sdc and both Slit and Robo also suggested that the HSPG might be functioning as co-receptor for the repellent ligand [34••]. Although the presence of conserved signaling motifs in the Sdc cytoplasmic domain might infer an active role for Sdc in the response to Slit, the ability of the glypican Dallylike (Dlp) to rescue partially the *sdc* mutant suggested that cell-surface HS served the main role in Robo activation [34••].

Recent data confirm that Syndecan action in midline repulsion is well-conserved. The single worm *Syndecan* (SDN-1) is expressed in the nervous system [41] and appears to function in the Slit pathway [42•]. However, SDN-1 mediates additional cell migration and guidance events in parallel with other HSPGs [42•]. This suggests that HSPG action is highly context-dependent, a result that echoes the analysis of *hse-5*, *hst-2* and *hst-6*. This context-dependence is well illustrated by comparisons of Sdc and Dlp function in *Drosophila*. Although Sdc and Dlp show overlapping function at the *Drosophila* embryonic midline [34••], they have non-overlapping function during retinal axon pathfinding into the adult optic lobes [43••]. Retinal axon trajectories and electrophysiological phenotypes are distinct in *Sdc* and *dlp* mutants, and expression of an *Sdc* transgene in the *dlp* mutant fails to rescue retinal axon defects [43••]. More evidence of HSPG specificity is seen at the CNS midline, where mutations in Perlecan (*TROL*) and the Glypican *dally* fail to show axon guidance defects [39•]. Taken together, these observations suggest a high degree of specialization in the HSPG core proteins.

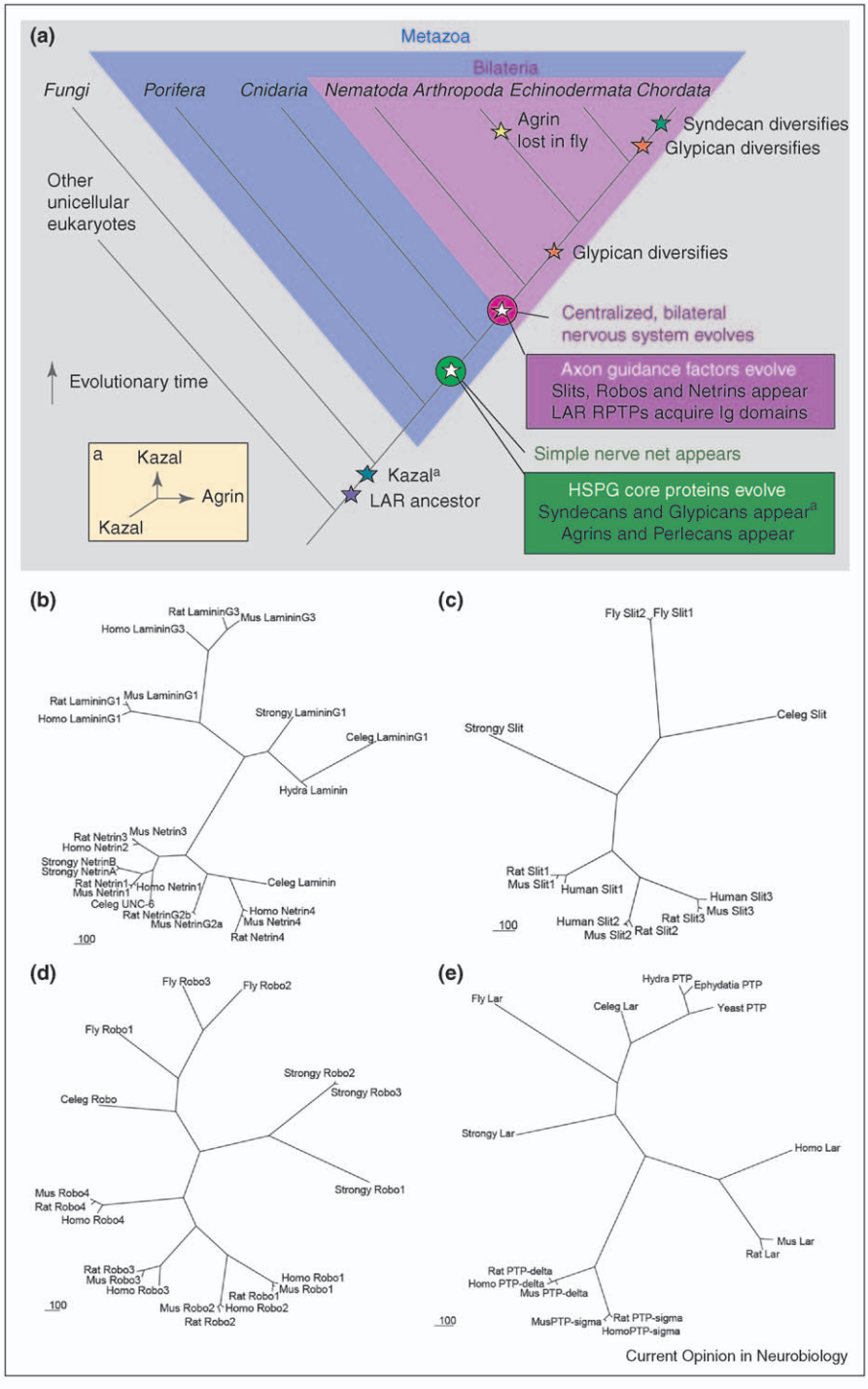
Heparan sulfate proteoglycans and the regeneration of connections

Axons face the challenge of navigating to distant targets not only during embryonic development but also after neural injury. Although the GAG chondroitin sulfate (CS) has received much attention as a possible inhibitor of CNS axon growth after nerve damage (reviewed in [44]), several recent studies suggest that HS might also be important for future control of regeneration. For example, Glypican-1 expression increases after neural damage, in both the central and the peripheral nervous systems [45–47], suggesting that regulation of HSPG expression is part of an injury response and that some HSPGs might be inhibitory for re-growth of axons. Consistent with this idea, enzymatic removal of HS with Heparinase I induced a twofold increase in regenerative peripheral axon sprouts in nerve graft experiments compared with that in untreated controls [48•]. Interestingly, Heparinase III, which cleaves HS closer to the peptide linkage, increased the efficiency of regeneration in a different way, enabling a higher number of axons to extend into the lesion site [48•]. Independent efforts to develop nerve graft material that enhances regeneration after injury suggest that HS cross-linking nearly doubles neurite outgrowth [49]. Although the mechanism(s) by which HS exerts these effects is still unknown, the preliminary results are promising.

HSPGs and the evolution of guidance information

The picture emerging from recent work on HSPGs in axon guidance, in addition to work on HSPGs in morphogen signaling, presents HS as a multifunctional extracellular scaffold capable of modulating the distribution of interacting ligands and/or their ability to engage receptors to elicit different downstream effects. This view might suggest that HS has evolved solely as a means to fine-tune the interactions of *bone fide* receptor–ligand pairs. However, biochemical studies of HS across a wide range of organisms suggest that HS is one of the oldest forms of GAG chain, dating back to and beyond the very first

Figure 4



The evolution of HSPGs and their nervous system partners. **(a)** A phylogenetic tree of extant species shows the relationship between metazoan (light blue) and bilaterian (light purple) organisms and a series of predicted events in the evolution of HSPGs and some of their associated signaling partners. The first nervous system has been postulated to arise in an ancestor of the Cnidaria, correlating with the appearance of the major HPSG core protein families (blue box). Midline axon guidance factors such as Slit, Netrin and their receptors are absent from Cnidaria, suggesting that they arose in the bilaterian ancestral organism in which midline structures first appeared (purple box). Interestingly, although LAR-family receptors have very early ancestors, this family acquired Ig domains after Cnidaria appear, suggesting that HSPG–LAR interactions are relatively modern. Several events in HSPG diversification are also noted for the Agrin, Syndecan and Glypican families (stars). (b–e) Parsimony-based phylogenetic reconstructions of several axon guidance ligands and receptors found in lineages throughout Metazoa [80]. **(b)** Netrin phylogeny. Using motif detection, we identified all netrin family members and several close paralogs from the Laminin family of axon

metazoan (multicellular) creatures [50–52]. Likewise, a phylogenetic analysis of HSPG sequences indicates that all the major HSPG families predate the emergence of bilaterian organisms (e.g. nematodes, insects and vertebrates), with clear orthologs in Cnidaria (see Figure 4), in which the first nervous system is thought to appear. This temporal correlation between the appearance of HSPGs and the first nervous system is intriguing. However, this means that HSPGs existed long before many of the ligand–receptor systems that they now regulate. Certain morphogens such as Wnt-family proteins are ancient enough to be early HSPG partners (e.g. [53]), and regulate axon guidance and synapse growth in higher organisms (e.g. [54–56]); however, the Slit and Robo families only appear in bilaterian species, a pattern that is also seen for Netrins (see Figure 4). Although this might suggest that many axon guidance factors were opportunistic in their relationship with HS, it also suggests that HSPGs might have functions as receptors and/or ligands in their own right.

Although the absence of many long-range axon guidance factors in the nervous systems of Cnidarian organisms is not surprising, given the distributed 'nerve net' organization characteristic of these species [57], it raises the question of what the first function of HSPGs was in the nervous system. One possibility is that HSPGs first functioned to mediate the formation of functional connections between neurons and their targets, because synapses seem to appear well before long-range axon guidance. It is possible that local neuron–substrate and neuron–target interactions provided enough information to form a functional network of synaptic connections, with ECM used to define a permissive extracellular compartment through which neuronal processes could grow to find their partners. This would argue that ECM is the most ancient determinant of neural connectivity.

Secreted HSPGs have been appreciated as molecules that regulate neuron–target interactions for some time. Agrin was the first HSPG to be appreciated as a mediator of neuron–target interaction and synapse assembly at the vertebrate neuromuscular junction (NMJ; reviewed in [58]). Interestingly, Agrins seem to have evolved from the Kazal gene family through a duplication early in metazoan history (Figure 4a). In addition to Agrin, Perlecan is important for NMJ functional properties as an extracellular anchor for acetylcholinesterase (reviewed in [59]). Recent studies also suggest that the cell surface HSPG Syndecan is involved in synaptic biology in the mammalian hippocampus. Interestingly, HSPG expression in the hippocampus might reflect pathology in this region of the brain [60]. Syndecans localize to hippocampal synapses [61,62], and have been implicated in the morphogenesis of dendritic spines by gain-of-function experiments [63]; however, the developmental role of mammalian cell-surface HSPGs has not been tested by loss-of-function genetics at the synapse.

Heparan sulfate proteoglycan and leukocyte antigen-related receptors in neuron–target interactions

In addition to controlling synaptic assembly, Agrin and other HSPGs may also regulate the initial interactions between motor neurons and their targets. For example, *in vitro* studies suggest that Agrin might function as a target-derived 'stop' signal for motor axons [64,65]. Interestingly, in addition to binding the MuSK receptors that direct clustering of acetylcholine receptors [58], Agrin binds to a protein tyrosine phosphatase (PTP-σ) in the leukocyte antigen-related (LAR) family of receptors [66]. Because PTP-σ is expressed on vertebrate motor axons, and its *Drosophila* ortholog is required for motor axon targeting (reviewed in [67]), it has been tempting to speculate that Agrin functions to regulate LAR-family function. Interestingly, Agrin is not present in *Drosophila* where neuromuscular junctions are glutamatergic and perhaps more analogous to vertebrate central excitatory synapses (Figure 4a). However, recent studies suggest

guidance factors. Only those Laminin proteins producing BLAST E-values of ~1e-50 or lower (lower blast E-values indicate higher sequence similarity) and that had sufficiently long motifs shared with classic netrins (netrin 1–3) were added to the phylogenetic analysis. Laminins gamma 1 (LamininG1) and gamma 3 (Laminin G3) from several lineages and two additional proteins annotated as 'Laminin-like' — one found in *Hydra magnipapillata* (GenBank id: 50350744) and one in *Caenorhabditis elegans* (GenBank id: 17541466) — met these criteria. Netrin G1 is a glycosyl phosphatidylinositol-linked mammalian netrin that is known to be functionally divergent from true netrins [79]. **(c)** Slit phylogeny. Only a single slit protein could be identified in the Echinodermata (represented by *Strongylocentrotus*; other proteins showing marked sequence homology to Slit 1 in this lineage were members of the Notch family). This suggests the possibility that Slit underwent a duplication event in the ancestor of the coelomates and that Slit2 was subsequently lost in Echinoderms. However, the fact that slits in *Drosophila melanogaster* are not members of the monophyletic clades containing Slit1 and Slit2 suggests an alternative explanation for the present phylogenetic reconstruction: Slit1 duplicated independently at the base of the arthropods and chordates, with the arthropod duplication event resulting in two copies, the chordate duplication resulting in three. **(d)** Robo phylogeny. Interestingly, in the arthropod and sea urchin lineages, Robos1, 2, and 3 were found to be more closely related to each other than any are to their orthologs in related species. This phylogenetic pattern suggests that robo split into three members once, at the base of the coelomates, but did not diversify appreciably in function (and in sequence) until later, in the immediate ancestor to modern chordates. An alternative although less parsimonious hypothesis is that robo duplicated more than once, perhaps in the ancestors of all three major Eukaryotic lineages (Arthropods, Hemichordates, Chordates). **(e)** LAR phylogeny. LAR is a conserved protein that has diverged, largely via terminal addition of Ig domains, from its ancestral form, protein tyrosine phosphotase (PTP) that is found in all major metazoan lineages. Lar is a relatively large family of proteins, with ~17 distinct members in vertebrates, many of which were omitted from this analysis for visual clarity. See legend of Figure 2 (b–e) for a description of the methods used for building phylogenies and for explanation of taxa abbreviations. Abbreviations: Yeast: Saccharomyces cerevisiae.

that other HSPG families regulate LAR receptors in *Drosophila*.

Using an *in situ* receptor-affinity approach coupled with a screen of deletion mutations, the HSPG Syndecan (Sdc) was recently identified as a binding partner of LAR in the *Drosophila* embryo [68••]. At this early stage of development, LAR is required for the entry of specific motor neurons into their appropriate target muscle domain (reviewed in [67]). As ventral motor axons reach their targets, Sdc immunoreactivity can be seen on the surface of the ventral muscles, as if to define a permissive runway through the target domain [68••]. Consistent with the high affinity binding seen between Sdc and LAR on *Drosophila* cells, various double mutant experiments reveal that loss of Sdc enhances LAR axon guidance defects up to twofold in specific allelic combinations [64]. However, *Sdc* mutants alone display little or no embryonic motor axon phenotype [68••,69••]. This important detail indicates that additional LAR ligands must exist for motor pathfinding, consistent with the fact that *in situ* LAR-AP (alkaline phosphate) binds to sites (e.g. neuropil) that do not disappear in *Sdc* mutants [68••].

Cell-surface HSPGs regulate distinct aspects of synapse development

A major goal of neuron–target interaction is the formation of functional synaptic connections. Syndecans have been implicated in controlling behavior and synaptic plasticity in mouse [70,71], yet the requirement for cell-surface HSPGs in synaptic development has been a mystery. However, recent analysis in *Drosophila* reveals that Sdc is essential for the normal growth of synapses during larval stages [69••]. Although embryonic NMJs appear normal in *Sdc* mutants, larval NMJs have fewer than normal number of presynaptic varicosities compared with those in wild type [69••]. Elevated expression of *Drosophila* Sdc is sufficient to increase NMJ growth [69••], reminiscent of the accelerated maturation of dendritic spines in hippocampal neurons over-expressing murine Syndecan-2 [63]. Loss of *Drosophila Sdc* is highly reminiscent of the *LAR* mutant phenotype in fly [72], worm [73] and mouse [74], consistent with the co-localization of Sdc and LAR at the *Drosophila* NMJ and a double mutant analysis placing Sdc in the LAR pathway [69••]. Although *LAR* mutants also display defects in both the active zone area and the neurotransmission at the NMJ [72], these functional parameters are normal in *Sdc* mutants [69••], raising the question of what might regulate the additional aspects of LAR function. The answer seems to lie with the glypican Dallylike (Dlp).

In contrast to Sdc, loss of Dlp has no effect on NMJ growth; however, *Dlp* mutants show defects in active zone ultrastructure and electrophysiology that are precisely the opposite of those seen in *LAR* mutants [69••], suggesting that Dlp might inhibit synaptic LAR. Consistent with this model, Dlp localizes to the *Drosophila* NMJ and binds to LAR [69••]. More importantly, double mutant analysis and signaling assays of the LAR substrate protein Enabled indicate that Dlp inhibits LAR phosphatase activity, demonstrating that Dlp functions upstream of the receptor [69••]. These data confirm that HSPGs are functional partners of the LAR receptor *in vivo*, and reveal a clear functional specialization between Syndecan and Glypican at the synapse.

Conclusions

A growing body of evidence on HSPG function in the developing nervous system shows that this class of ECM molecule is vital to the emergence of effective connectivity between neurons and their target cells. This work demonstrates that HSPGs are highly specialized, both at the level of core protein action and at the level of functional impact of HS modifications. In addition, HSPGs are candidates for future therapeutic approaches in the nervous system and beyond (e.g. in the vasculature [75•,76•,77], and in cancer metastasis [78]). Although the genetic analysis of vertebrate HSPGs is just getting started, the diversity of core proteins in the vertebrate genome promises that even greater complexity will be revealed in the extracellular landscape. With HSPG functional specificity recently defined in model organisms, the near future is likely to bring new insights as to the underlying determinants, whether they lie in core protein structure, HS microheterogeneity or a combination of both.

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