Glycosaminoglycans in extracellular matrix organisation: Are concepts from soft matter physics key to understanding the formation of perineuronal nets?

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Short title: GAGs, soft matter physics and PNN formation

Abstract

Conventional wisdom has it that proteins fold and assemble into definite structures, and that this defines their function. Glycosaminoglycans (GAGs) are different. In most cases the structures they form have a low degree of order, even when interacting with proteins. Here, we discuss how physical features common to all GAGs – hydrophilicity, charge, linearity and semi-flexibility – underpin the overall properties of GAG-rich matrices. By integrating soft matter physics concepts (e.g. polymer brushes and phase separation) with our molecular understanding of GAG-protein interactions, we can better comprehend how GAG-rich matrices assemble, what their properties are, and how they function. Taking perineuronal nets (PNNs) – a GAG-rich matrix enveloping neurons – as a relevant example, we propose that microphase separation determines the holey PNN anatomy that is pivotal to PNN functions.

Highlights (mandatory)

- Glycosaminoglycan physical properties contribute to distinct biochemical functions.
- Hyaluronan is a unique scaffolding material, enabling matrix self-organisation.
- GAG-binding proteins organise GAG matrices
- A balance of repulsion and cross-linking defines GAG matrix organisation.
- Soft matter physics principles underpin the formation of holes in perineuronal nets.

Introduction

Glycosaminoglycans (GAGs) are polymers of unbranched polysaccharides composed of repeating disaccharides units, where a uronic acid [either glucuronic acid (GlcA) or its epimer iduronic acid (IdoA)] or a galactose links to a hexosamine [either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc)]. The GAG family has five members: hyaluronan (HA), heparan sulphate (HS; including the highly sulphated form heparin), chondroitin sulphate (CS), dermatan sulphate (DS) and keratan sulphate (KS). Their presence in all vertebrate tissues implicates their diverse functions and importance.

For example, HA, the largest member of the GAG family, is well known for functions as a shock absorber in synovial, pleural and peritoneal fluid, as an organiser of lecticans (a subfamily of CS proteoglycans, CSPGs) in a wide range of tissues, in the processes of ovulation and fertilisation, and also for its role in inflammation (e.g. as part of the endothelial glycocalyx that controls homing of leukocytes to inflamed tissues). HS is important to the binding and regulation of growth factors, chemokines and complement components for normal physiological and immune functions [1-3]. CS contributes to the load-bearing properties of cartilage and is also important in controlling neurogenesis, neuronal plasticity and regeneration after injury to the central nervous system [4].

GAGs are abundant in extracellular environments, and most of the functions of GAGs rely on their spatially and temporally controlled incorporation into (or release from) extracellular matrices. Questions that remain largely unresolved are: how precisely are GAGs organised in different extracellular matrix contexts, and how do they contribute to the organisation of the extracellular space? In this opinion paper, we propose that two key factors are crucial to answer these questions: (i) the intrinsic physical properties of GAGs and, (ii) the interaction of GAGs, together with their core proteins, with specific GAG-binding proteins that modulate their structure. We shall use the example of perineuronal nets (PNNs) in the central nervous system to illustrate how these two factors may act together.

Molecular characteristics of GAGs

Conventionally, we picture the function of biomolecules and the specificity of biomolecular interactions as being encoded in molecular structure. To this end, most protein and nucleic acid polymers have evolved to fold – spontaneously or upon a stimulus – into secondary and higher order structures. GAGs, however, are distinct: intrinsically, they lack a defined secondary or higher order structure, but rather dynamically sample a wide range of (low energy) conformations. This property has profound implications, not only for the biochemistry of GAG-protein interactions but – importantly – also for the physical properties of GAGs.

Biochemistry of GAGs – specificity and tunability of GAG-protein interactions

Except for HA (and chondroitin), which are not sulphated and thus biochemically homogeneous, all other GAGs show variations in terms of epimerisation, the degree of sulphation and the distribution of the sulphation motifs [3,5]. This endows GAGs with a diverse array of binding epitopes and specificities in the interaction with proteins, including cell surface receptors. This aspect has been studied extensively for HS. It will be focused upon by Lindahl and Kjellen in another contribution to this issue, and shall here only be covered briefly.

A widely held view of GAG-protein interactions is that these are dominated by charges. Although this is often correct, recent studies suggest that in some cases there is a high level of specificity of GAG-protein interaction regardless of their charge composition [3,6]. Making use of a library of HS/heparin octasaccharides, Ashikari-Hada et al. [7] reported five main groups of HS binding growth factors, which showed differential affinity to distinct sulphation patterns in the HS. Such differential binding has also been observed in other HS binding proteins, such as sulphatases.

Similar to HS, CS also demonstrates specificity in its interactions with proteins, with the level and distribution of sulphations (and possibly the associated chain flexibility) tuning recognition and affinity. An early example is pleiotrophin that binds preferentially to chondroitin-4-sulphate and chondroitin-2,6-sulphate, and this differentially modulates pleiotrophin binding in the course of brain development [8]. Recent studies also showed that the soluble transcription factor Otx2 [9] and the chemorepulsive

guidance molecule semaphorin 3A bind with high affinity to chondroitin 4,6-sulphate, but not to chondroitin 2,6-sulphate, which bears the same charge/mass ratio, and that this binding regulates the function of PNNs [4]. Moreover, the inflammation-associated and GAG-binding protein TSG-6 was found to bind better to chondroitin-4-sulphate than chondroitin-6-sulphate [10]. Even for the simple structure of HA, there are clear examples where charge is not at all involved in its binding to proteins (Box 1).

These studies exemplify that the sulphation code, as a combination of the type, the level and the distribution of sulphations, is an important regulator of the interaction of proteins with sulphated GAGs [11]. The situation is obviously different for HA given that it is a structurally simple GAG where the lack of biosynthetic modifications has been preserved throughout vertebrate evolution. The biochemical and functional diversity of HA instead results from the range of HA-binding proteins (HABPs) that differentially interact with HA and regulate the formation of supramolecular structures, due in part to the fact that many protein molecules can bind simultaneously to a single HA chain (multivalency) (Box 1).

Physical properties of GAG chains – mechanically soft and space filling

Although chemically distinct, all members of the GAG family share the same physical properties:

Hydrophilicity. At physiological ionic strength and pH, all GAGs are very well solvated by water.

Charge. At physiological pH, all GAGs are negatively charged and repel each other. GAGs carry one carboxyl group and between zero and three sulphate groups per disaccharide, that is, between one and four deprotonation sites per nm of contour length. Sulphated GAGs have the highest charge density of all biomolecules known, and this entails a high osmotic swelling pressure owing to counterions (Donnan effect).

Linearity. All GAG chains are un-branched, linear polymers. For such a simple topology, the exact chemical makeup can be largely neglected to predict the gross morphology of individual chains. Instead, a set of just three effective physical parameters is sufficient: (i) the contour length (one nm for each disaccharide unit; a measure of GAG size; Fig. 1 a), (ii) the effective excluded volume (the average volume of a disaccharide unit – on the order of 1 nm^3, increasing with sulphation; a measure of the GAG's bulkiness plus the osmotically driven repulsion between GAG chains), and (iii) the persistence length (a measure of GAG chain rigidity; Fig. 1a).

Semi-flexibility. Over the length of a few disaccharides, GAGs appear stiff owing to their conformationally constrained pyranose rings and glycosidic bonds, but on larger length scales, GAG chains readily flex [12,13]. The persistence length defines the contour length range over which a stiff appearance is retained. Persistence lengths between 4 and 10 nm have been reported for GAGs at physiological ionic strength from experiments and computer simulations [12,14]. This value is high compared to most other biological molecules (e.g. 0.4 nm for peptide chains, 2 nm for single-stranded nucleic acid chains) and synthetic polymers (e.g. 0.4 nm for polyethylene glycol) (Fig. 1a).

The combination of these properties has profound consequences:

GAGs fill space. Owing to their high persistence length, solvation and excluded volume, GAG chains are remarkably efficient at filling space. An individual HA chain of 2 MDa molecular weight, for example, forms a swollen random coil (Fig. 1a) that pervades and dynamically samples a volume of approximately 150 nm diameter [15]. For comparison, a bacterial ribosome with the same molecular weight only measures 20 nm in diameter and thus occupies approximately 400 times less volume.

GAG chains are compliant. Owing to their high persistence length, it is relatively easy to stretch GAG chains out of their equilibrium conformation.

Self-organisation of GAGs from a soft matter physics perspective

In the previous section, we adopted a soft matter physics perspective to identify common physical properties of GAG polymers. Here, we use the same approach to identify a set of organisational features that are common to virtually all GAG-rich extracellular matrices. They implicate a combined role of the

physical properties of the GAG polymers and the biochemical interaction of GAGs with their protein binding partners.

GAG brushes

GAGs, when densely grafted to a core protein (such as the ~100 CS and ~30 KS chains on aggrecan), form a 'bottle brush' (Fig. 1b); extrusion and retention of HA at the cell surface (by HA synthases) can give rise to a 'planar brush' (Fig. 1b). While isolated in solution, GAG chains flex dynamically and their average extension is determined by their radius of gyration (R_g; Fig. 1a,c; left). When anchored with one end to a substrate (e.g. a cell surface or core protein) and at high density (anchor distance < R_g), the conformation of the GAG chain is altered: the repulsion between chains causes their stretching away from the substrate, and the result is a 'GAG brush' (Fig. 1c). It is notable that the level of repulsion between polymer chains depends on the topology of the substrate. For a given substrate surface area, for example, the same number of polymer chains can more easily be accommodated on small spheres or narrow cylinders than on a plane. Rilla et al. have observed cell-surface anchored HA to promote the formation of membrane protrusions [16,17] and the budding of extracellular vesicles [18]. The energy gains associated with an easier packing of HA chains in cylindrical and spherical brushes as compared to a planar brush (Fig. 1b) may well be a major driving force for these processes.

The GAG grafting is normally determined by intracellular processes. For all sulphated GAGs, the grafting density is defined by the number of GAG-attachment sites on the proteoglycan core protein, which include serine (for CS/DS/HS/KSII) or asparagine (for KSI) residues, from which the intracellular enzyme machinery would start the GAG synthesis. In the case of HA, the grafting density will be determined by the density of HA synthases on the cell surface.

GAGs and GAG-rich proteoglycans promote swelling and ultra-soft matrices

Integration of GAGs and proteoglycans into pericellular and extracellular matrices leads to swelling, and the matrices thus formed can be extremely mechanically soft. GAGs and proteoglycans, owing to their high charge and osmotic swelling pressure, are efficient space fillers. This was illustrated using reconstituted planar HA brushes as a well-defined artificial matrix model [19] and also with HA-rich cell coats [20,21], where the intercalation of exogenous aggrecan entailed substantial swelling and softening (Fig. 1d). A striking in vivo example of a soft GAG-rich matrix is the expansion of the cumulus cell-oocyte complex (COC) prior to ovulation. We have recently reported Young's modulus values below 1 Pa for the COC matrix [22], which to our knowledge makes it the softest elastic biological material known.

GAG crosslinking induces compaction, rigidification and phase separation

The above-described swelling effect is counteracted by proteins that interconnect (crosslink) GAGs and/or the corresponding proteoglycan core proteins. For example, complexes made from TSG-6, pentraxin 3 and the heavy chains of inter-α-inhibitor (Box 1) are vital to stabilize the COC matrix [23,24]. In addition, chemokines and growth factors have been shown to crosslink HS in vitro [25,26], and tenascin R has been proposed as an aggrecan crosslinker in the central nervous system [4,27]. The effects of these interactions are matrix compaction, rigidification and stabilisation.

An important consequence of compaction (if sufficiently strong) is the transition from one homogeneous GAG-rich phase to segregated GAG-rich microphases, i.e. phase separation. To understand the concept, it is instructive to consider theoretical predictions from polymer physics for films of flexible, regular, end-grafted polymers [28,29]. Fig. 1e shows how (i) the polymer grafting density and (ii) the attractive interaction between polymers are expected to affect the morphology of such polymer films. One can see, for example, how a homogeneous brush (I) transforms into a holey film (II) when the attraction between polymers increases (at constant grafting density), or how a film of separate globules (III) transforms into a holey film (II) when the grafting density increases (at constant attraction). The distinct morphologies of the discontinuous films (Fig. 1e) can be understood as a result of two physical parameters, namely, the mean surface density of polymers and the surface tension at the phase boundaries: at higher polymer surface densities, phase boundaries are minimized with cylindrical holes

in a continuous polymer-rich phase (II); at lower polymer surface densities, polymer globules in a continuous polymer-depleted phase (I) are the energetically favourable morphology.

Evidently, brushes of flexible, regular polymers are much simpler than GAG-rich matrices. Nonetheless, the two systems share essential similarities in terms of their physical properties, and the analogy is helpful as a simple conceptual framework to understand how molecular interactions and physical properties jointly define the morphology of GAG-rich matrices.

Hyaluronan – unique as a scaffold

The particular molecular features of HA make this GAG a unique scaffolding material for matrix. Among all GAGs, HA stands out by its size. Its typical molecular weight ranges from 100s of kDa to ~10 MDa (i.e. with corresponding contour lengths of 100s of nm to 10s of μm), whereas the size of all other GAGs is usually below 50 kDa (100 nm). Because of its sheer size, a single HA chain can simultaneously accommodate hundreds of HA-binding proteins (Box 1), and when stretched readily spans distances comparable to the size of a cell.

Fig. 1 demonstrates how HA, arranged as a brush, can indeed serve as scaffold that can be dynamically reorganised. On the one hand, a bulky proteoglycan (such as aggrecan) induces brush swelling and softening (Fig. 1d) [19]; on the other hand, a crosslinking protein (such as TSG-6) causes compaction and rigidification [30], and phase separation (Fig. 1f). Reassuringly, we could obtain two distinct microphase morphologies by modulating the HA grafting density in the presence of TSG-6, as predicted by the theory (Fig. 1e): a holey film ('reticular phase') forms at high HA surface density (Fig. 1f, right) and pinned globules ('granular phase') form at low HA surface density (Fig. 1f, middle). From these extreme examples, one can imagine, how the interplay of swelling and cross-linking, and changes in HA grafting density, would allow a diverse array of structures to be formed and the potential for their dynamic reorganisation in the presence of HABPs; i.e. with profound effects on the morphology and mechanical properties of HA matrices.

How do perineuronal nets form?

Perineuronal nets are a good example of an extracellular matrix structure rich in HA and CSPGs. They are present in the central nervous system (CNS), and crucial in the control of neuronal plasticity both in development and after CNS injury (Box 2).

Composition of PNNs

It is now well established that at least one member of each of four families of molecules is required for PNN formation [31,32]: 1) HA (synthesised by an HA synthase) acting as a scaffold, 2) CSPGs, 3) link proteins (HAPLNs) stabilising the interaction between HA and the CSPG N-terminal G1 domain, and 4) tenascin-R (Tn-R), a trimeric protein that can interlink three CSPG C-terminal G3 domains (Fig. 2a).

PNN morphologies - granular versus reticular

PNNs are traditionally considered as lattice-like or reticular in nature [33,34] (Fig. 2a). Recent studies, however, have shown that immature PNNs appear granular (Fig. 2b, e, g), and that the transition from granular to reticular PNNs is important for PNN function [34]. Indeed, we have observed that PNNs are granular in short-term neuronal culture (< 14 days in vitro) and that the structure gradually changes to reticular upon maturation both in vitro (Fig. 2b-d) and in vivo (Fig. 2e-h).

Integrative hypothesis of PNN formation

One of the key questions is how PNNs, with similar molecular components, form nets of such different morphologies? On the basis of the soft matter physics concepts described above, we propose that CSPGs (stabilised by HAPLNs) act as swelling agents whereas Tn-R acts as a crosslinker. The relative abundance of HA, CSPGs, HAPLNs and Tn-R, and their interactions (dictated by the particular CSPGs and HAPLNs present), would then determine the degree of matrix compaction and the arrangement into microphases. Here, we hypothesise that (1) the size, sulphation and/or abundance of CSPGs, (2) the size and grafting density of HA, and/or (3) the abundance of crosslinker gradually change during the

maturation of neurons, and that the fine balance of all these parameters defines how granular PNNs form first and then evolve into reticular PNNs. The morphological similarities of PNNs (Fig. 2d) and crosslinked HA brushes (Fig. 1f), and the consistency with the simple theoretical predictions (Fig. 1e), are remarkable. It has been reported that CSPG sulphation in the CNS and in PNNs varies with maturation [35], but a more detailed knowledge of the above-mentioned GAG parameters will be crucial for understanding how PNN morphology is defined. Currently, little is known about the functional difference between the globular or holey form of PNNs. Any changes in the above parameters will lead to significant differences in PNNs, influencing PNN structure and ultimately PNN function. There is circumstantial evidence supporting this hypothesis, since deletions of the crosslinker Tn-R, or the stabilising HAPLNs, were found to result in the formation of thicker PNNs with the associated neurons showing altered electrophysiological responses [36].

Conclusions

In this opinion paper, we have described how an integrative approach is required to understand the way in which GAG-rich matrices self-organise. Next to the conventional aspects – the biochemistry of GAGs and their binding partners – the physical properties of GAGs also need to be considered. Starting from simple concepts, soft matter physics can provide inspiration to rationalise how GAG-rich matrices assemble, what their properties are, and ultimately, how they function. Following this approach, we hypothesise that the complex morphology of PNNs arises and matures through a simple phase separation process. Quantitative testing of the theoretical predictions will require a detailed knowledge of the composition, spatial organisation and abundance of molecules in GAG-rich matrices. This remains an important challenge for the future, but if answered can help define the self-organisation of many GAG-rich matrices important for various physiological processes in the context of cartilage and synovial fluid, the COC matrix, the endothelial glycocalyx and PNNs.

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Boxes

Box 1. Hyaluronan-protein interactions – from molecules to diverse supramolecular complexes

HA-binding proteins (hyaladherins), the majority of which belong to the Link module superfamily, show considerable diversity in their interactions with HA. This includes the size of the HA-binding domain (HABD) that can either contain a single Link module (Type A) as found in TSG-6, an extended Link module (Type B) as in CD44 and LYVE-1, or two contiguous Link modules (Type C) present in CSPGs and link proteins, and typified by aggrecan and HAPLN1, respectively [37]. In LYVE-1, the extracellular portion of this lymph vessel receptor can be associated into a covalent dimer, which is required for its binding to HA [38]; see below. While evidence from high-resolution structures [39-41] and modelling studies [42,43] suggests that a common region of the Link module surface is involved in forming a shallow HA-binding groove, different types of molecular interactions (ionic, hydrogen bonds, aromatic ring stacking and van der Waals) are used in different combinations in different hyaladherins. For example, while salt bridges between basic amino acids and the carboxylates of HA have been implicated for LYVE-1 and TSG-6 [43,44], the interaction of CD44 with HA does not involve such bonds [40]. Moreover, in TSG-6 only ~25% of the binding energy can be attributed to ionic interactions; this highlights the importance of aromatic residues such as tyrosine, which can support ring stacking and/or hydrogen bonded interactions with HA [40,42,43].

Although, to date, a co-crystal structure has only been determined for CD44's HABD in complex with HA [40], experiment-based modelling of the TSG-6 Link module [42,43] indicates that these two proteins both introduce a pronounced kink into the HA chain, but where the conformations of the HA captured/stabilised by CD44 and TSG-6 are probably distinct. Even more dramatic 'bending' of HA is likely in the case of Type C hyaladherins, because of the positioning of two Link modules with aligned binding grooves, especially when CSPGs are stabilised by HAPLNs, perhaps giving rise to helical organisations of HA/protein complexes [42]. Here the size of HA recognised is likely of relevance, such that only 5 sugar residues make contact within the binding site on CD44, whereas it is 7 in TSG-6 and ~10 in CSPGs or HAPLNs [40,42,43,45]; as well as impacting on the overall perturbation to the HA chain, the differential length recognition correlates well with relative affinities, i.e. CSPGs/HAPLNs > TSG-6 > CD44.

An individual HA molecule, because of its immense size, is able to bind simultaneously to a large number of proteins (100s). In the case of the CSPGs, when stabilised by a cooperative interaction with HAPLNs, they have the potential to saturate all binding sites on HA, and lead to the formation of massive and long-lived aggregates that are of very high affinity and do not spontaneously dissociate [46]. A single HA chain is also able to bind simultaneously to multiple (10s) cell surface receptor molecules, e.g. CD44 and LYVE-1 [47,48]. However, in this case the interactions are non-cooperative and weak, meaning that relatively small changes in the density of functional receptors have a dramatic effect on whether HA binding occurs or not; the term 'superselectivity' (based on a concept from soft matter physics) has been coined for such interactions [49], which is apt given that they can be exquisitely regulated. For instance, the crosslinking of HA chains by TSG-6 can enhance or induce the binding of HA to CD44 [50]. In the case of LYVE-1, it will only bind HA if the polysaccharide is correctly organised, for example crosslinked by TSG-6 [48] or as presented on the surface of a dendritic cell [51]; this might be a result of the anti-parallel arrangement of HABDs in the context of the covalent dimer that perhaps preferentially allows binding to an anti-parallel arrangement of sugar chains, e.g. within the context of crosslinked HA [38].

Another type of crosslinking involves the covalent modification of HA with heavy chains (HC) from members of the inter-α-inhibitor family, where this transferase reaction is mediated by TSG-6 (see [23]). HC•HA complexes, which form at sites of inflammation, and in the cumulus matrix just prior to ovulation, are linked together non-covalently via pentraxin-3, an octameric protein that is believed to bind multiple HCs, thereby crosslinking HA chains together [24]; here transient HC-HC interactions may also be involved [52]. Given that relatively few HC molecules are likely to be attached to an individual HA chain [52], the hydrodynamic properties of the polysaccharide are likely to be less affected than for other kinds of HA/protein complexes. However, there are at least 5 different HCs that have the potential to become covalently attached to HA [53], where the diversity of complexes formed

could lead to a vast array of biological outcomes depending on composition and context, including beneficial physiological and protective immune functions or driving pathology [23,53]; for example, some HC•HA complexes are pro-adhesive for leukocytes whereas others are not [50].

Overall, there is huge diversity in how HA is recognised and organised by hyaladherins. A consequence of this is the formation of a dazzling array of supramolecular complexes with dramatically different architectures, physical properties and biological activities.

Box 2. Perineuronal nets in the central nervous system – key to controlling neuronal plasticity

A mature perineuronal net is a layer of aggregated matrix structure wrapping around the surface of neurons, with holes for synapse formation and stabilisation, and for signal transmission (Fig. 2) [31]. PNN removal enhances neuronal plasticity, which is beneficial to the recovery in various neuropathological conditions. The role of PNNs in plasticity was first demonstrated in the visual cortex, where PNN removal enhances ocular dominance (i.e. eye preference) plasticity in the adult [54]. Subsequent studies showed that PNNs also control developmental plasticity in the barrel cortex and the vestibular cortex, and that juvenile plasticity can be extended into adulthood by preventing PNN formation [36]. PNN removal after spinal cord injury also enhances plasticity, e.g. by allowing the de novo sprouting of axons and the formation of new synapses for the recovery of functions [27]. The benefits of PNN removal in regeneration have also been demonstrated in stroke models [55]. Moreover, there is increasing evidence that the PNN is important for memory formation and retention. Gogolla et al. first reported the role of PNNs in controlling memory formation, such that fear memory can be 'erased' by the removal of PNNs in the amygdala [56]. Transgenic animals with attenuated PNNs showed enhanced memory formation in novel object recognition tests [57] and a recent study in an Alzheimer's mouse model found that removal of PNNs using chondroitinase enhances memory retention [58]. Over the last ten years PNNs have been implicated in a wide range of CNS pathologies, in addition to the ones mentioned above, including epilepsy, schizophrenia and addiction [59]. The wealth of examples highlights the importance of neuronal plasticity, and it can be expected that a better understanding of how PNNs control plasticity will advance potential treatments for these diseases.

Figure legends

Figure 1. GAG self organisation – the soft matter physics perspective. (**a**) A schematic diagram illustrating the radius of gyration and the contour length of polymer chains, and the sizes of the building blocks and persistence lengths for GAGs, nucleic acids and (poly)peptides. (**b**) Polymer brushes: a bottle brush forms upon anchorage of CS and KS at high density to a core protein (e.g. in aggrecan); HA, when retained via attachment to hyaluronan synthases (HASs), can form a planar brush on the cell surface, spherical brushes around extracellular vesicles and cylindrical brushes around cell membrane protrusions. (**c**) Brushes form when the flexible GAG chains are anchored at high density to a substrate. (**d**) Assemblies of GAGs and GAG-rich proteoglycans are intrinsically mechanically soft and swollen owing to the mutual repulsion of GAGs and the high negative charge providing an osmotic swelling pressure due to associated counter ions. The schematic shows how aggrecan swells and extends HA brushes [19]. (**e**) Crosslinking proteins (such as TSG-6 for HA) cause compaction, phase separation and rigidification. The schematic phase diagram summarises simple predictions from soft matter physics theory for films of flexible, regular, end-grafted polymers as a function of polymer grafting density and level of attraction between polymers [28,29]. As the attraction between polymers increases (in the case of GAGs by crosslinking), the film becomes more compact and rigid, and a homogeneous brush (I) can phase separate into heterogeneous films of distinct morphology: a continuous film with holes (II) or separate globules (III). Boundaries between phases are drawn qualitatively and three-dimensional views (red) illustrate the gross morphology of phases I, II and III. **(f)** Phase separation in crosslinked HA brushes, which provides a simple in vitro model of HA rich extracellular matrix. Hyaluronan brushes were formed on supported lipid bilayers on which the HA anchors are laterally mobile (schematic, **left**), as previously described [30]; root-mean-square anchor spacings were $d \approx 50$ nm (HA $M_W = 1080 \pm 56$ kDa; **middle**) and $d \approx 10$ nm (HA $M_W = 58 \pm 3$ kDa; **right**); both brushes were incubated with 40 µg/mL Link_TSG6 (the recombinant Link module of TSG-6 [39]) at 5 mM NaCl, pH 7.4; under these low salt conditions, Link_TSG6 binds with high affinity, crosslinks and collapses the HA film [30]. Atomic force microscopy of the surface topography (z range encoded from dark on the bottom to bright on the top, see colour bars) reveals the predicted microscopic phase separations: HA/Link_TSG6 globules (‘granular phase’; **middle**) and a holey HA/Link_TSG6 film (‘reticular phase’; **right**) form at low and high HA surface density, respectively.

Figure 2. PNN composition and organisation. (**a**) (**Left**) Schematic diagram illustrating the PNN components and their molecular interactions. A hierarchical assembly of PNN molecules is built upon an HA backbone that is attached by the HA synthase to the neuronal surface. (**Top right**) A schematic diagram shows the formation of synapses in the holes of the PNN. (**Bottom right**) A confocal image showing PNNs stained with Wisteria floribunda agglutinin (WFA; red), a lectin that binds GalNAc in CS, on a cortical neuron in an adult rat brain. Holes are interspersed on the continuous layer of PNN matrix on the neuronal surface. (**b-d**) Histochemical images of PNNs present on cultured cortical neurons at 14 days in vitro (DIV) (**b**) and 28 DIV (**c** and **d**). All PNNs appear granular at 14 DIV, and at 28 DIV a mixture of granular (**c**) and reticular (**d**) morphologies emerge. (**e-h**) Histochemical images of PNNs on cortical neurons from postnatal day 15 (P15; **e** and **g**) and adult (**f** and **h**) rat brain sections. At P15, the cortical neurons in the somatosensory cortex are mostly surrounded by granular PNNs, while the adult neurons are surrounded by reticular PNNs. Histochemistry was performed as described in [60]; brain sections were 30 µm thick. Scale bars are 5 µm (**a-f**) and 2.5 µm (**g-h**).