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The hyaluronan and proteoglycan link proteins: organizers of the brain extracellular matrix and key molecules for neuronal function and plasticity

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Title page

Title: The hyaluronan and proteoglycan link proteins: organizers of the brain extracellular matrix

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Abbreviation

Hapln, hyaluronan and proteoglycanbinding link protein; CNS, central nervous system; VVA, *Vicia villosa* agglutinin; WFA, *Wisteria floribunda* agglutinin; CSPG, chondroitin sulfate proteoglycans; GAG, glycosaminoglycan; TN-R, tenascin-R; HAS, hyaluronan synthase; Otx2, orthodenticle homeobox 2; AIS, axon initial segment; NF186, neurofascin186; PNS, peripheral nervous system

Abstract

The hyaluronan and proteoglycanbinding link protein (Hapln) is a key molecule in the formation and control of hyaluronan-based condensed perineuronal matrix in the adult brain. This review summarizes the recent advances in understanding the role of Haplns in formation and control of two distinct types of perineuronal matrices, one for “classical” PNN and the other for the specialized extracellular matrix (ECM) at the node of Ranvier in the central nervous system (CNS). We introduce the structural components of each ECM organization including the basic concept of supramolecular structure named “HLT model”. We furthermore summarize the developmental and physiological role of perineuronal ECMs from the studies of Haplns and related molecules. Finally, we also discuss the potential mechanism modulating PNNs in the adult CNS. This layer of organized matrices may exert a direct effect via core protein or sugar moiety from the structure or by acting as a binding site for biologically active molecules, which are important for neuronal plasticity and saltatory conduction.

Keywords

Hyaluronan, proteoglycan, link protein, perineuronal net, plasticity, node of Ranvier, saltatory conduction

Highlights

- Hapln is a key molecule in the formation and control of perineuronal matrix in the adult brain.
- Two distinct types of hyaluronan-condensed perineuronal matrices exist: perineuronal net and perinodal matrix.
- Essential components of perineuronal nets are the link proteins Hapln1/Crtl1 and Hapln4/Bral2.
- Perineuronal nets play a key role in the control of plasticity in the adult CNS.
- Physiological and developmental roles of perinodal matrices are summarized.

Introduction

The neural extracellular matrix (ECM) is a scaffold that fills the intercellular spaces in the central nervous system (CNS). The intercellular space filled up with ECM is 20% of the adult brain volume (Nicholson and Syková, 1998). The striking feature of the brain ECM is the prominence of proteoglycans and hyaluronan with the exception of basement membrane structures (Ruoslahti, 1996). Perineuronal nets (PNNs) are reticular structures made of ECM molecules that surround the cell body of many neurons, and extend along their dendrites, which were first described by Camillo Golgi in 1882 (Celio et al., 1998). However, the relevance of the brain ECM is dismissed until the beginning of the 1980s because of technical difficulties to visualize and analyse it. Meanwhile, a number of methods has been developed to visualize PNNs using plant lectins such as *Vicia villosa* agglutinin (VVA) and *Wisteria floribunda* agglutinin (WFA) that labels selectively N-acetylgalactosamines (Brückner et al., 1993); monoclonal antibodies to chondroitin sulfate proteoglycans (CSPG) (Couchman et al., 1984; Hockfield and McKay, 1983; Watanabe et al., 1989); the cationic iron colloid staining method detecting sulfate groups of glycosaminoglycan (GAG) chains (Murakami and Ohtsuka 2003) or the combined applications (Murakami et al., 1996). Intriguing co-localization of hyaluronan and CSPG in rat cerebral cortex was reported (Bignami et al., 1992). Molecular cloning has facilitated the identifications of key components of PNNs including a family of CSPG called lecticans, tenascin-R (TN-R) and link protein (Table 1; Bandtlow and Zimmermann, 2000; Yamaguchi, 2000). PNNs are thus consisted of heterogeneous molecules, namely different sets of neurons express distinct complements of cell-surface antigens (Lander et al., 1997).

Link protein-1 (Crtl1) is primarily characterized as an abundant ECM protein in cartilage. Crtl1 can stabilize the noncovalent binding of aggrecan to hyaluronan, giving cartilage its load bearing property. Anti-cartilage aggrecan and anti-Crtl1 antibodies demonstrated the very similar PNN-type labelling in the CNS (Asher et al., 1995). Crtl1 was also co-purified from P7 rat brain by immunoaffinity chromatography with the 1F6 mAb for neurocan (Meyer-Puttlitz et al., 1995). Those evidence indicate that the structure of neural ECM is similar to that of cartilage. The Spicer lab and our lab independently found new members of link protein family in search for novel

hyaluronan-binding molecules (Bekku et al., 2003; Hirakawa et al., 2000; Ogawa et al., 2004; Spicer et al., 2003). The new family is designated as hyaluronan and proteoglycan binding link protein (Hapln) gene family (Spicer et al., 2003). Among four members, three of them including Crt11 are predominantly expressed in the CNS/brain (Table 1). In this issue, we term them Hapln1/Crt11, Hapln2/Bral1 and Hapln4/Bral4.

This review summarizes the recent advances in understanding the role of Haplns in formation and control of perineuronal ECM: one for “classical” PNN and the other for the specialized ECM at the CNS node of Ranvier. We introduce the structural components of each ECM organization including the basic concept of supramolecular structure named “HLT model”. We furthermore discuss the developmental and physiological role of perineuronal ECMs from the studies of Haplns and related molecules. This layer of organized matrix appears as a coat on the neuronal surface, which is important for neuronal plasticity and saltatory conduction.

Haplns and Lecticans

Hyaluronan is a naturally linear, unbranched high molecular mass GAG composed of repeating units of glucuronic acid and N-acetylglucosamine. Hyaluronan forms a highly hydrated pericellular coat around many cells. The ability to form a hyaluronan-linked proteoglycan-rich extracellular environment appears to be one of the hallmarks of vertebrate development, which is not found in protostomes or cnidaria (Hynes 2012; Tammi et al., 2002). The link module superfamily proteins possess a common structural domain, ~100 amino acid residues in length, from which the family name is derived. The superfamily of link module-containing molecules, which has currently 14 members in humans, includes lecticans and Haplns (Blundell et al., 2005). Lecticans are a family of CSPGs, encompassing aggrecan, versican, neurocan and brevican (Table 1). They display a high degree of homology in their N- and C- terminal globular domains (Yamaguchi, 2000).

Haplns consist of an immunoglobulin (Ig) module and two contiguous link modules (Fig. 1). The Ig module and link module are also known as the A-subdomain and B-subdomain or loop, respectively. The structure of Haplns is highly homologous to that of the G1 domain of lecticans (Fig.

1), which share the common property of binding to hyaluronan. The four Hapln proteins share between 45 and 58% amino acid identity, and the homology in the link modules is extremely high (Bekku et al., 2003; Blundell et al., 2005). The structure of link module was primarily determined for the link module of TSG-6 (Blundell et al., 2003). Subsequently, the structure of the link module of CD44 was determined by X-ray crystallography and NMR spectroscopy (Teriete et al., 2004). Those structures of TSG-6 and CD44, determined at high resolution, could reveal that the backbone topology is highly conserved. Further sequence alignment of the link modules of TSG-6 and CD44 with those of Haplns and the G1 domain of lecticans could indicate the conservation of several amino acid residues which have been implicated as important for hyaluronan binding in previous studies (Matsumoto et al., 2003; Ogawa et al., 2004). Using the link module structure of TSG-6 and CD44, molecular modeling of tandem link modules of versican G1 was performed (Matsumoto et al., 2003). There is another molecular model from cartilage link protein (Hapln1/Crtl1) in its hyaluronan-bound form (Blundell et al., 2005). The clusters of predicted hydrophobic and basic amino acid residues on the contact surface with hyaluronan could be localized with both molecular models. Interestingly, aromatic/hydrophobic residues also clustered on the external faces at either end of the Hapln1/Crtl1. These residues are well conserved within the link modules of Haplns and lecticans (Blundell et al., 2003).

The interaction between Hapln1/Crtl1 and aggrecan is mediated through association of their N-terminal Ig modules (Grover and Roughley, 1994; Matsumoto et al., 2003; Perin et al., 1987). Thereafter, the interaction of Hapln1/Crtl1 with versican G1 and neurocan G1 was demonstrated (Matsumoto et al., 2003; Rauch et al., 2004; Shi et al., 2004). Hapln1/Crtl1 binding with neurocan G1 interaction was demonstrated to be mediated by the Ig module of neurocan G1 on overlay blot analysis, indicating hyaluronan independent interaction (Rauch et al., 2004). In contrast, Hapln1/Crtl1 and versican G1 interaction occurred via their link modules (Matsumoto et al., 2003). Hapln1/Crtl1 and versican G1 did not bind directly to each other in solution yet formed ternary complexes with hyaluronan oligomer (Seyfried et al., 2005). Molecular modeling supported the ability of Hapln1/Crtl1 and aggrecan G1 link modules together to form a tandem array on a single hyaluronan chain (Blundell et al., 2005), though this model did not include the exact position of the Ig

modules. Those studies indicate that there is considerable complexity in the details of Hapln-CSPG interactions, and this may lead to aggregates with hyaluronan of diverse quaternary structure.

The HLT model

The current concept of supramolecular organization and assembly of PNN was first proposed by Ruoslahti (1996) and designated as “HLT (hyaluronan, lecticans and TN-R)” model by Yamaguchi (2000) (Fig. 2). The model is based on the intensive studies of recombinantly expressed N-terminal G1 domains and C-terminal G3 domains with individual ligands (Aspberg et al., 1997; Matsumoto et al., 2003; Shi et al., 2004; Watanabe et al., 1997). The G1 domain is important for its binding to backbone hyaluronan to form a tripartite complex with Hapln. The G3 domain mediates crosslinking to diverse ECM molecules during their assembly. The C-type lectin subdomain of lecticans binds to the fibronectin type III repeats 3-5 of TN-R, which was demonstrated by the crystal structure of the recombinant complex (Aspberg et al., 1997; Lundell et al., 2004). TN-R forms characteristic trimeric structures through association of their N-terminal domains. The trimeric structure was further confirmed by electron microscopy of complexes between native aggrecan and TN-R (Lundell et al., 2004). The HLT model turn out to be a very far-sighted model not only for the PNN molecular organization but also for the perinodal ECM molecular organization (Bekku et al., 2009).

Perineuronal net

ECM in the brain is composed of molecules synthesized by neurons and glial cells (Table 1). During development, ECM plays crucial roles in proliferation, migration and differentiation of neural cells. In the mature brain, ECM undergoes a slow turnover and supports multiple physiological processes (Dityatev and Fellin, 2008). The hyaluronan-based condensed pericellular matrix is formed around some neuronal cell bodies and nodes of Ranvier late in postnatal development at around the time either when synaptic contacts are stabilized or when myelination is finished. The lattice-like PNN that surround cell bodies, proximal dendrites and axon initial segments is formed as synapses stabilize and critical periods for plasticity close (Fig. 3; Celio et al., 1998; Wang and Fawcett, 2012). The PNN and

perinodal matrix (PM) share common features of molecular organization represented by the HLT model (Fig. 2).

The PNN is a unique ECM structure that is most prominently displayed around GABAergic interneurons, with parvalbumin expressing cells having the highest level of colocalization (Brückner et al., 1993; Celio and Chiquet-Ehrismann, 1993; Morris and Henderson, 2000). The involvement of PNNs in neuronal plasticity has been extensively studied over recent years. Thereafter, the PNN is increasingly recognized as a potent site for critical period plasticity.

PNN structure

As mentioned above, the PNN structure is based on the HLT complex. Hyaluronan is synthesized by hyaluronan synthases (HAS), which are located in the plasma membrane. Three isoforms of HAS exist (HAS-1, -2 and -3), encoded by individual genes, which synthesize hyaluronan chains of various length and at different speed, with HAS-1 and -2 generating hyaluronan chains of up to 2000 kD at fast speed, and HAS-3 producing hyaluronan shorter chains at slow speed (Itano et al., 1999; Spicer et al., 1997). Interestingly, PNN-bearing neurons show differential HAS expression depending on CNS regions and developmental time point (see for review Kwok et al., 2011). Neurons in the cerebellar nuclei and visual cortex express HAS-2 and -3, while neurons in the spinal cord express HAS-1 and -3 (Carulli et al., 2006, 2010; Galtrey et al., 2008). However, while HAS-1 and HAS-3 are expressed in developing spinal cord, HAS-3 is the only isoform identified in the mature spinal cord (Galtrey et al., 2008). These changes in HAS expression may have a functional role because the different HASs, synthesizing hyaluronan of different lengths, may confer different structure and mechanical properties to the PNN, and thus affect their properties. Furthermore, because HASs retain the growing hyaluronan polymer while synthesizing and extruding it from the cell surface (Toole, 2004), they might act as anchor molecules for the PNN. Indeed, transfection of HAS-3 gene in human embryonic kidney (HEK) cells is sufficient for the formation of a PNN-like matrix on the cell surface (Kwok et al., 2010).

All four lecticans are found in PNNs (Carulli et al., 2006, 2010; Hagihara et al., 1999; Matsui et al., 1998; Zimmermann and Dours-Zimmermann, 2008). Aggrecan is present on almost all

PNN-positive neurons, while other lecticans are found on subpopulations of PNN neurons, as shown by co-labeling of WFA with antibodies against different CSPG core proteins (Galtrey et al., 2008). Versican seems to be present as a proteolytic remnant of the juvenile-type V0 and V1 isoforms. Neurocan in the PNNs may present as a mixture of full length- and truncated products (Neurocan-N) (Bekku and Oohashi, 2010; Zimmermann and Dours-Zimmermann, 2008). Notably, aggrecan is necessary for PNN formation, as shown in the HEK cell model (Kwok et al., 2010) and in organotypic cultures derived from aggrecan knockout mice (Giamanco et al., 2010). As with hyaluronan, the properties of lecticans (namely the size of the lectican molecules or their glycosylation profile) may affect the compactness of the PNN, and therefore PNN functions. For instance, a lectican with higher affinity for TN-R (such as brevican) might form a more highly cross-linked PNN than those with lower affinity (e.g. neurocan; Aspberg et al., 1997; Yamaguchi, 2000). Interestingly, it has been recently shown that a developmental increase in the 4-sulfation/6-sulfation (4S/6S) ratio of CSPGs has a role in the closure of the critical period for ocular dominance plasticity in the mouse visual cortex, and condensation of CSPGs into PNNs is prevented by cell-autonomous overexpression of chondroitin 6-sulfation, which maintains a low 4S/6S ratio (Miyata et al., 2012).

The interaction between hyaluronan and lecticans is stabilized by Haplns. Two are the link proteins found in PNNs: Hapln1/Crtl1 and Hapln4/Bral2. These Haplns are expressed exclusively in neurons bearing a PNN (Bekku et al., 2003; Carulli et al., 2006; Galtrey et al., 2008; Rauch et al., 2004) and are essential for the proper assembly of the PNN structure. For example, in the HEK model, the overexpression of Hapln1/Crtl1 in HAS-3 expressing cells, which form a diffuse pericellular matrix, results in the formation of a compact pericellular matrix (Kwok et al., 2010). In animals lacking Hapln1/Crtl1 in the CNS, PNNs are strongly attenuated, showing a complete absence of WFA staining around the dendrites and faint and diffuse staining around the soma (Carulli et al., 2010). Similarly, in mutant mice lacking Hapln4/Bral2, which is mainly expressed in the brainstem and cerebellum, PNNs in these areas are reduced, with a complete lack of brevican and a general attenuation of the majority of the other PNN components (Bekku et al., 2012). However, there was no attenuation in the PNN formation of aggrecan even at the same neuronal surface, which indicate the independent binding of aggrecan in the PNN. Dissociated expression of aggrecan and brevican was

reported in the medial nucleus of the trapezoid body by Blosa et al. (2013). We speculate that Haplns are regulating the micro-organization of PNN via specific interaction with lecticans (e.g. Hapln4/Bral2 and brevican, Hapln1/Crtl1 and other lecticans) (Fig. 4; Oohashi, unpublished observations).

TN-R is important for PNN structure, as it strengthens the macromolecular assembly of the PNN. As mentioned above, TN-R knockout mice show abnormal PNN staining, with irregular distribution of WFA around soma and dendrites (Brückner et al., 2000; Morawski et al., 2014; Weber et al., 1999).

PNN functions

PNNs have been observed throughout the CNS of different organisms, including frogs, birds and mammals (Celio et al., 1998; Balmer et al., 2009; Gaál et al., 2014; Morawski et al., 2009). WFA-positive nets are localised around different neuronal types, such as parvalbumin-expressing GABAergic neurons in cortical areas (see above), as well as cortical pyramidal neurons (Alpár et al., 2006; Hartig et al., 2001; Takahashi-Iwanaga et al., 1998; Wegner et al., 2003), glutamatergic neurons in the cerebellar nuclei (Carulli et al., 2006) and glycinergic neurons of the medial nucleus of the trapezoid body (Hartig et al., 2001). As to the cellular origin of PNN components, molecules that are crucial for PNN assembly, namely HAS, link proteins and aggrecan, are exclusively synthesized by neurons, whereas astrocytes, oligodendrocyte precursors and oligodendrocytes produce other ECM molecules, which are then likely incorporated into the PNN (Carulli et al., 2006, 2007; Lander et al., 1998; see table I). In accordance, when primary hippocampal neurons are cultivated in indirect coculture with astrocytes lacking four matrix molecules (neurocan, brevican, TN-R and TN-C), the neuronal culture by itself is able to form PNNs. In this model hippocampal neurons lacking neurocan, brevican, TN-R and TN-C display rudimentary PNNs and reduced synapse number, suggesting that PNNs are important for synapse formation/stabilization (Geissler et al., 2013).

The organization of specific ECM molecules into PNNs *in vivo* requires the presence of appropriate stimuli and occurs during postnatal development, in concomitance with the end of the critical period for experience-dependent plasticity (Carulli et al., 2010; Kalb and Hockfield, 1990; McRae et al., 2007; Pizzorusso et al., 2002; Ye and Miao, 2013). Critical periods are temporal

windows in which experience has profound effects on synapse refinement and wiring of neuronal circuits and, as a consequence, on the development of specific functions and adaptive behaviors. This has been shown by the seminal studies of Hubel and Wiesel on ocular dominance plasticity (Wiesel and Hubel, 1965). Visual deprivation of one eye during the critical period yields important anatomical remodelling of thalamocortical fibers, with an expansion of the axon arborization conveying visual inputs, and a retraction of thalamic axons conveying no input, leading to loss of visual acuity of the deprived eye (Antonini and Stryker, 1993, 1996). Notably, of the various PNN components, the only two whose mRNAs were up-regulated at the time of PNN formation in the visual cortex are the link proteins Hapln1/Crt11 and Hapln4/Bral2 (Carulli et al., 2010), suggesting that Haplns are crucial components for the assembly of ECM molecules into a PNN. Moreover, the expression of Haplns mRNA is activity-dependent (Carulli et al., 2010). The first demonstration of a role of PNNs in restricting experience-dependent plasticity came from Maffei's and Fawcett's groups. They showed that removal of CSPGs by chondroitinase [a bacterial enzyme which degrades GAGs, including chondroitin sulfate and hyaluronan, disrupting the structure of the perineuronal net; see Galtrey and Fawcett (2007) for review] in adult animals restores juvenile levels of plasticity in the rat visual cortex, and allow a complete recovery of visual acuity in rats which experienced monocular deprivation during the critical period (Pizzorusso et al., 2002, 2006). In addition, the decrease in spine density caused by long-term monocular deprivation is recovered by chondroitinase treatment (Pizzorusso et al., 2006). Notably, Carulli et al., (2010) showed that PNNs rather than other modifications in the brain ECM (such as changes in CSPG expression levels) control CNS plasticity, and digestion of PNNs is how chondroitinase reactivates plasticity. Namely, in adult mice lacking Hapln1/Crt11, which exhibit vestigial PNNs but no changes in the overall levels of matrix components, plasticity in the visual and somatosensory systems is strongly enhanced. Interestingly, it has been recently demonstrated that PNNs in the mouse visual cortex, and specifically disulfated (2,6) CS (also named CS-D) and disulfated (4,6) CS (also named CS-E), provide binding sites to orthodenticle homeobox 2 (Otx2) homeoprotein. Namely, PNNs permit Otx2 capture and transfer into GABAergic interneurons expressing parvalbumin. These events are necessary and sufficient to open, then close, the critical period (Beurdeley et al., 2012). In this context, gain- and loss-of-function experiments

(Beurdeley et al., 2012; Spatazza et al., 2013; Sugiyama et al., 2008) indicate that Otx2 internalization enhances the expression of several markers of parvalbumin cell maturation, including PNN formation, and therefore control visual cortex plasticity during development and in adulthood.

PNNs contribute to the closure of critical period also in other systems. For example, PNN maturation in the amygdala is a crucial event for the transition from a juvenile form of fear memories, which can be fully erased by extinction, to an adult form of fear memories, which are erasure-resistant (Gogolla et al., 2009). Xue et al. (2014) demonstrated that PNNs in the amygdala also protect drug-related memories from erasure. In the same context, removal of PNNs from the prelimbic region of the medial prefrontal cortex of adult rats impairs the acquisition and reconsolidation of a cocaine-induced conditioned place preference memory (Slaker et al., 2015). Interestingly, developmental song learning in birds shares key characteristics with sensory critical periods in mammals, suggesting shared underlying mechanisms. In a song area important for sensorimotor integration in the zebra finch, the percentage of neurons with perineuronal nets correlates with song maturity. Shifting the vocal critical period with tutor song deprivation decreases the staining intensity of CS in PNNs (Balmer et al., 2009), suggesting a role of PNNs in the regulation of critical period for song learning.

Other recent studies show a prominent role of PNNs in controlling cognitive abilities. Removal of the PNNs in the auditory cortex of adult Mongolian gerbils promotes a significant increase in re-learning performance, and thus high cognitive flexibility required for reversal learning of previously acquired behavioral habits (Happel et al., 2014). Chondroitinase administration into the perirhinal cortex has been shown to enhance object recognition task, i.e. the forms of plasticity which may underlie explicit learning and memory (Romberg et al., 2013). Accordingly, in Hapln1/Crt11 knockout mice long-term object recognition memory is prolonged similarly to animals which received a chondroitinase injection, and chondroitinase treatment does not affect object recognition in those mice, supporting the evidence that PNNs, rather the general ECM, regulate recognition memory (Romberg et al., 2013). Moreover, chondroitinase application into the perirhinal cortex of mice with tau pathology, which show profound loss of object recognition memory, is effective in restoring such memory to normal levels, indicating that chondroitinase treatment promotes plasticity also in the

presence of the widespread neuronal dysfunction/loss caused by neurodegeneration (Yang et al., 2015).

A bulk of evidence show that PNN digestion promotes anatomical plasticity in the injured nervous system, mainly by inducing sprouting of damaged or intact neurites. For example, Tropea et al. (2003) show that after a partial retinal injury, chondroitinase promotes sprouting of undamaged retinal axons in the denervated superior colliculus. PNN digestion in the partially denervated cuneate nucleus results in collateral sprouting of intact somatosensory fibers, which is accompanied by the formation of functional connections (Massey et al., 2006). Similarly, in adult rats that underwent cervical spared-root lesion [rhizotomy of cervical level (C)5, C6, C8, and thoracic level 1, sparing C7], a focal intraspinal injection of chondroitinase restores sensory function, due to reorganization of intact C7 primary afferent terminals within adjacent segments (Cafferty et al., 2008). Additional evidence of spinal plasticity after PNN digestion come from the experiments by Galtrey et al. (2007). In a model of peripheral nerve lesion and repair, PNN removal in the ventral spinal cord produces increased sprouting in the cord and enhanced functional recovery even following inaccurate reinnervation of the forelimb muscles and sensory structures by peripheral nerve fibers.

Besides a clear involvement of PNNs in the control of neuronal plasticity, PNNs may also serve other functions. A common feature of neurons surrounded by PNNs is their fast-spiking activity. Therefore it has been proposed that PNN, due to the highly negative charge of GAG and hyaluronan chains, binds cations such as sodium, potassium and calcium, in the neuron microenvironment, preventing the diffusion of such cations, and thus providing a suitable milieu in support of the high activity of the neurons (Hartig et al., 1994, 1999, 2001; Morris and Henderson, 2000; Reimers et al., 2007; Wintergest et al., 1996). However, conflicting results come from *in vivo* studies. PNN digestion by chondroitinase has no effect on baseline activity level of neurons in the visual cortex (Pizzorusso et al., 2002), whereas enhances hippocampal activity, presumably due to a decrease in fast-spiking interneuron function (Shah and Lodge, 2013). Degradation of PNNs in slices of the mouse anterior cingulate cortex, in which PNNs are found around parvalbumin interneurons, does not disrupt the fast rhythmic activity but enhances the power of the local high-frequency oscillatory neuronal activity (Steullet et al., 2014). ECM CSPGs have also been shown to be involved in the control of the

direction of GABA signalling (resulting in excitation or inhibition), constraining the local Cl^- in the extracellular compartment (Glykys et al., 2014).

In addition to this physiological mechanism, the polyanionic PNN components might also interact with ions of potential pathophysiological relevance such as iron ions involved in generating oxidative stress. Therefore, PNNs, through scavenging and binding of redoxactive iron, may be able to reduce the potentially deleterious local oxidative potential in the neuronal microenvironment, thereby protecting neurons against oxidative damage (see for review Suttikus et al., 2014). Cabungcal et al. (2013) show that parvalbumin cells enwrapped with mature PNNs are better protected than immature parvalbumin cells surrounded by less-condensed nets, and enzymatic degradation of PNNs renders mature parvalbumin cells more susceptible to oxidative stress. Indeed, both in normal-aged brain and Alzheimer's disease brain, neurons ensheathed by a PNN are less frequently affected by lipofuscin accumulation than neurons without a net. Lipofuscin is an intralysosomal pigment generated by iron-catalysed oxidative processes. These findings suggests a neuroprotective function of PNNs against neurodegeneration induced by oxidative stress (see for review Morawski et al. 2014). Importantly, protection of PNN-ensheathed neurons against iron-induced neurodegeneration is directly mediated by the net structure (Suttikus et al., 2014). Moreover, in human brains with Alzheimer's disease, cortical and subcortical neurons that are enwrapped by PNNs are less frequently affected by neurofibrillary degeneration (Morawski et al., 2010, 2012).

Compelling evidence supports a role of PNNs also in the control of synaptic functions (Dityatev and Schachner, 2006; Frischknecht and Gundelfinger, 2012). PNNs restrict the lateral diffusion of AMPA receptors and thus the exchange of desensitized receptors for naive functional ones from extrasynaptic sites, therefore allowing synaptic desensitization and affecting synaptic depression during high-frequency stimulation (Frichknecht et al., 2009). Consistently, treatment of primary hippocampal neurons with chondroitinase results in increased excitability of interneurons (Dityatev et al., 2007).

How PNNs control plasticity

The mechanisms underlying the plasticity-restricting effects of PNNs are not entirely elucidated. Because PNNs are composed of growth-inhibitory molecules, such as CSPGs, they may exert their inhibitory effect through these components, and the efficiency of chondroitinase treatment in promoting axonal sprouting points in this direction. Specifically, CSPGs, and particularly their GAG moieties, may act by i) binding to a specific receptor; ii) blocking the growth-promoting activity of integrins; iii) presenting growth-inhibitory proteins.

i) Known CS/CSPG receptors are: contactin-1 (Mikami et al., 2009), receptor protein tyrosine phosphatase sigma (RPTP σ ; Shen et al., 2009), leukocyte common antigen-related phosphatase (LAR; Fisher et al., 2011) and Nogo receptor (NgR) 1 and 3 (Dickendesher et al., 2012). In vitro experiments show that contactin-1 interacts with CS-E, but the main effects of this interaction is the stimulation of neurite outgrowth (Mikami et al., 2009). RPTP σ can bind with high affinity to neurocan and aggrecan and strongly inhibits axon outgrowth and regeneration (Sapieha et al., 2005; Shen et al., 2009; Fry et al., 2010; Lang et al., 2015). LAR interacts with high levels of extracellular CSPGs, including versican, in both the normal and injured CNS. LAR deletion in knock-out mice overcomes growth restriction of CSPGs on neurites in neuronal cultures, and treatments with LAR-targeting peptides or LAR knockout significantly improve axonal growth and behavioral recovery after spinal cord injury (Fisher et al., 2011; Xu et al., 2015). NgR1 and NgR3 bind with high specificity to different types of monosulfated and disulfated GAGs. Experiments on cultures of cerebellar granule neurons from *Ngr* single and compound mutants show that NgR1 and NgR3 participate in CSPG-mediated neurite outgrowth inhibition (Dickendesher et al., 2012). However, no evidence so far exist showing a relationship between CSPG receptors and PNNs.

ii) A potential mechanism of CSPG-mediated repulsion may be through control of integrins. Previous studies revealed an interaction between β 1-integrins and CSPGs (Tan et al., 2011; Wu et al., 2002). Integrins possess a growth-promoting activity which depends on the substrate which the neurons contact. Reduced integrin activation is present in neurons cultured on substrate coated with CSPGs, which display growth inhibition and CSPG digestion release the negative effects of CSPG on integrin activation and neurite outgrowth (Afshari et al., 2010). CSPG digestion in organotypic hippocampal slices enhances the motility of dendritic spines of CA1 pyramidal cells and induces the appearance of

spine head protrusions, in parallel with the activation of β 1-integrins and phosphorylation of focal adhesion kinase at synaptic sites (Orlando et al., 2012).

iii) Another potential mechanism of action of PNNs may rely on the binding of GAG chains to ligands harboring growth-inhibitory or repulsive effects. Recently, the repulsive guidance molecule semaphorin3A (Sema3A) has been identified as a PNN component (De Wit et al., 2005; Vo et al., 2013). Chondroitinase injection into the cortex abolishes Sema3A staining around the injection site. In mice lacking the link protein Hapln1/Crtl1 in the CNS there are no Sema3A-positive PNN (Vo et al., 2013). A biochemical analysis shows that Sema3A protein binds with high-affinity to CS-GAGs, aggrecan and versican extracted from PNNs in the adult rat brain (Vo et al., 2013). Particularly, Sema3A has been shown to bind PNNs via CS-E (Dick et al., 2013), which is enriched in PNNs (Deepa et al., 2006). Notably, the combination of Sema3A and PNN-GAGs is a potent inhibitor of axon growth (Dick et al., 2013). Because PNN-bearing neurons and/or synaptic terminals embedded in PNNs may express the Sema3A receptor plexinA4 (Gutekunst et al., 2010; Vo et al., 2013), Sema3A signaling may be responsible for the PNN inhibitory effect on axon growth and synapse dynamics.

Modulation of PNNs in the adult CNS

In parallel with recent discoveries that highlight that the adult CNS retains a certain degree of plasticity, activity-dependent changes to PNNs have been shown to occur even in the adult CNS. Since the seminal work by Rosenzweig and coworkers (see Rosenzweig and Bennett (1996) for review), it is known that exposure to enriched sensory, motor, cognitive and social stimulation (i.e. enriched environment) strongly promotes neuronal plasticity, enhancing learning and memory and leading to improved functional recovery after different kinds of CNS lesion or disease (Bennet et al., 1969; Diamond et al., 1976, see for reviews Nithianantharajah and Hannan, 2006; van Praag et al., 2000). Motor training is also an effective paradigm for increasing plasticity (Cotman et al., 2007; Ding et al., 2006; Gómez-Pinilla et al., 2002; Neeper et al., 1995; van Praag et al., 1999). Only recently, however, changes in PNN expression following environmental enrichment/training have been highlighted, which can elicit plastic phenomena. When rats which were monocularly deprived

during the critical period are reared in an enriched environment as adults, they show recovered visual acuity and cortical response to the deprived eye and in parallel a decrease in PNN number in the visual cortex (Sale et al., 2007). This decrease is accompanied by decreased GABA levels. The importance of GABA for the integrity of the PNN structure in the adult cortex is elegantly shown by Harauzov et al. (2010). Reducing GABAergic transmission in the adult visual cortex by microperfusion of 3-mercaptopropionic acid (an inhibitor of the activity of GABA synthetic enzyme glutamic acid decarboxylase) or of picrotoxin (which antagonizes GABA action on its GABA_A receptors) leads to a significant decrease of WFA- and aggrecan-positive PNNs and promotes ocular dominance plasticity. Enriched environment also promotes structural plasticity in the cerebellum, which is accompanied by a strong PNN reduction and a putative increase in the excitatory/inhibitory ratio. In this model, decreased synthesis of Hapln1/Crt11 and other PNN components is observed, suggesting that external stimuli may have a plasticity-permissive role by affecting the synthesis of link proteins (Foscarin et al., 2011). Indeed, structural changes found after enriched environment, such as an enlargement of Purkinje axon boutons, are also observed in Hapln1/Crt11 mice (Foscarin et al., 2011).

Intriguingly, activity can differentially regulate PNN depending on the CNS area. For example, PNNs are differentially regulated in select regions of the CNS in response to endurance exercise training, with a decreased expression in many brain regions and increased expression in the lumbar spinal cord (Smith et al., 2015). Peripheral nerve injury, which causes massive stripping of synapses on axotomized spinal motoneurons (Svensson et al., 1993), induces a disintegration of PNNs surrounding the motoneurons. However, activity-dependent therapy (treadmill running) reduces PNN destructuring, whilst blockade of sensory inputs from the homolateral hindlimb reduces PNN immunoreactivity around intact motoneurons, indicating that sensory inputs are key players in the maintenance of PNNs in the spinal cord (Arbat-Plana et al., 2015). Accordingly, unilateral labyrinthectomy, which induces a partial degeneration of the vestibular nerve and thus a loss of excitatory inputs to the vestibular nuclei, is accompanied by a strong reduction of PNNs in those nuclei, which then re-form during compensatory axonal plasticity and behavioural recovery of the mice (Carulli, unpublished observations). Partial deprivation of cerebellar nuclei neurons of their

main GABAergic inputs, the Purkinje cells results in a strong decrease of PNNs and their content of Sema3A in denervated regions (Carulli et al., 2013). Therefore, PNNs in the adult CNS may be differentially modulated by excitatory/inhibitory activity in distinct regions.

Perinodal matrix

Neurons are highly polarized cells with multiple distinct membrane domains. Neurons receive excitatory and inhibitory synaptic inputs on their cell bodies and dendrites. Both inhibitory postsynaptic potentials and excitatory postsynaptic potentials are summed in the axon initial segment (AIS) and once a triggering threshold is exceeded, an action potential propagates through the rest of the axon. To increase the speed by which action potentials are conducted, axons are wrapped in an insulating sheath of myelin. At the node of Ranvier, where the myelin sheath is interrupted, the action potentials become regenerated. As the action potentials reach axon terminal, neurotransmitters are released to propagate the signal across the synaptic cleft to the next cell (Rasband, 2010).

Molecular composition of PM

The nodes of Ranvier are highly enriched in voltage-gated sodium channels and are exposed to extracellular space, thereby enabling action potential regeneration and saltatory conduction. Several cell adhesion molecules have been defined as specifically localizing on the axonal membrane at the nodes (e.g. NF186: see review by Susuki and Rasband, 2008). Compared to such ion channels and cell adhesion molecules at the node, extracellular constituents at the nodes have largely been unknown until recently. Hyaluronectin (also named GHAP) was a protein isolated from acid extracts of human brain by affinity chromatography on immobilized hyaluronan and was a first extracellular molecule implicated to localize at the node of Ranvier (Delpech and Halavent, 1981). Hyaluronectin turned out to be an enzymatically cleaved fragment of versican, which contains the entire hyaluronan-binding G1 domain. Thereafter, several extracellular molecules of proteoglycans (e.g. phosphacan) and glycoproteins (e.g. TN-R) were found to localize at the nodes of Ranvier (Weber et al., 1999; Ratcliffe et al., 2000). *Tnr*-deficient mice were phenotypically normal, although they exhibited a significant decrease in conduction velocity as compared with wild-type mice. Immunostaining of

phosphacan, a high-affinity ligand for TN-R, showed it to be weak and diffuse at the nodes in the knockouts when compared to wild-type mice. However, there was no apparent change in expression and distribution of the Na⁺ channels, which are thought to bind TN-R via their β 2 subunit (Weber et al., 1999; Srinivasan et al., 1998; Xiao et al., 1999). Today we know that this distinctive ECM is mainly composed of lectican family of CSPG and their binding partners (Dours-Zimmermann et al., 2009). Versican V2 and Hapln2/Bral1 are most ubiquitously located at the nodes (Schmalfeldt et al., 2000; Oohashi et al., 2002). Brevican and neurocan are also known to localize at the nodes in an axon diameter-dependent manner (Fig. 5; Bekku et al., 2009; Bekku and Oohashi, 2010). Of particular note is that the HLT model made from PNN fits to this PM except for the differences that versican V2 is the most common lectican and aggrecan is absent at the node (Bekku et al., 2009; Dours-Zimmermann et al., 2009).

Physiological role of PM

The nodes of particularly large diameter have a more elaborate ECM assembly than the nodes of smaller axons, as if the ECM could further speed up axonal velocity by a reduction in the resistance of the extracellular medium (Bekku et al., 2009). We thus proposed that the hyaluronan-associated ECM could serve as an “extracellular ion pool” at the perinodal extracellular space because both hyaluronan and chondroitin sulfate provide a strong negatively charged environment corresponding to the nodal diameter (Oohashi et al., 2002; Bekku et al., 2009).

To assess the physiological role of PM, *Hapln2/Bral1*-deficient mice were generated (Bekku et al., 2010). The importance of Hapln2/Bral1 in stabilizing the lectican CSPGs and other associated matrix components at the node of Ranvier were demonstrated by *Hapln2/Bral1*-deficient mice (Bekku et al., 2010; Bekku and Oohashi 2010). CNS nerve conduction is markedly decreased in *Hapln2/Bral1*-deficient mice even though there were no differences between wild-type and mutant mice in the clustering or transition of ion channels at the nodes or in the tissue morphology around the nodes of Ranvier. However, changes in the extracellular space diffusion parameters, measured by the real-time iontophoretic method and diffusion-weighted magnetic resonance imaging, suggest a reduction in the diffusion hindrances in the white matter of mutant mice (Bekku et al., 2010). While

the clustering of nodal sodium channels and paranodal structures appear unaffected in both *Vcan* *V0/V2*- and *Tnr*-deficient mice, effect on the conduction velocity is different in the three types of mutants (*V0/V2*-, *Tnr*- and *Hapln2/Brall1*-deficient mice), probably due to the differences of degree of disturbed ECM assembly (Bekku et al., 2010; Dours-Zimmermann et al., 2009; Weber et al., 1999).

The molecular organization of the AIS has many features in common with that of nodes of Ranvier, including ion channels, cell adhesion molecules, cytoskeletal scaffolds and ECM molecules (Rasband, 2010). Presence of three major PNN constituents, lecticans, hyaluronan and tenascin-R, in the axon initial segment (AIS) was first reported by Brückner et al. (2006). However, the ECM seems to be mainly comprised of brevican (John et al., 2006). Of note is hyaluronidase treatment does not abolish brevican labeling in the AIS (Frischknecht et al., 2009). Brevican enrichment in the AIS was mediated via direct interaction with NF186, which was diminished by a knockdown of NF186 (John et al., 2006; Hedstrom et al., 2007). Congenital *BCAN* gene deletion was found in episodic falling syndrome, a neurological disorder with paroxysmal hypertonicity in dogs, which results from a CNS rather than a muscle defect (Gill et al., 2011). The phenotype may be explained by the biological function of the brevican at the nodes, AIS, or PNNs, although a similar phenotype has not been reported in the *Bcan*-deficient mice (Brakebusch et al., 2002).

Developmental role of PM

Clustering of voltage-gated Na⁺ channels is most important functional requirement to generate action potentials at the AIS and regenerate action potentials at the nodes of Ranvier. Actually, disruption of any single nodal ECM gene does not affect the clustering of nodal Na⁺ channels (Bekku et al., 2010; Dours-Zimmermann et al., 2009; Weber et al., 1999). Recent research advances have uncovered overlapping molecular mechanisms on the formation of the peripheral nervous system (PNS) node-formations (Eshed-Eisenbach and Peles, 2013). In the PNS nodes, Schwann cell-derived type II transmembrane protein, glyomedin, is concentrated at the Schwann cell microvilli and induces the clustering of Na⁺ channels at the PNS nodes via interaction with NF186 and NrCAM (Eshed et al., 2005). Subsequently, proteolytically cleaved glyomedin is incorporated into the Schwann cell ECM at the nodal gaps (Eshed et al., 2007). In the CNS, oligodendrocytes

themselves do not contact the nodes but astrocyte foot processes contact nodes to some extent (Bekku et al., 2010). Those glial cells express core CNS nodal ECMs including versican V2 and brevican (Bandtlow and Zimmermann, 2000). Brevican can interact with NF186 in the AIS as described before (Hedstrom et al., 2007). Such growing evidence implies the contribution of glia-derived ECM ligands for clustering Na⁺ channels at the CNS nodes together with other overlapping mechanisms. In a recent paper, Susuki *et al.* addressed the specific contribution of three mechanisms, the paranodal junction as barriers to restrict the position of nodal proteins, axonal cytoskeletal scaffolds and NF186-binding ECM proteins (i.e. perinodal ECMs) by generating mice retaining one mechanism out of three (Susuki et al., 2013). They demonstrate the existence of hierarchy between three mechanisms. CNS nodal ECMs do not play a primary role to recruit Na⁺ channels at the nodes, but play a compensating mechanism for assembling nodes or are important for stabilizing the nodes.

Conclusions

Recent evidence have highlighted that link proteins in the CNS are key molecules for the assembly and function of specialised ECM, characterised by HLT structure. Hapln1/Crt11 and Hapln4/Bral2 are crucial components of PNNs, contributing to PNN formation/maintenance and to the control of plasticity exerted by PNNs. Further knowledge on how to manipulate these molecules may thus have important clinical implications in view of enhancing plasticity in the adult CNS and, as a consequence, promoting functional recovery after CNS damage. Hapln2/Bral1 is an essential element of the PM and plays a key role in the regulation of action potential conduction in myelinated axons. Further studies on Hapln2/Bral1 and the PM may therefore contribute to a better understanding of their function in physiology and pathology.

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Figure legends

Fig. 1. Domain organization of the lecticans and Haplns.

Versican isoforms exist as a result of alternative splicing usage of two large exons encoding GAG- α and GAG- β domains. A GPI-anchored brevican variant arising from alternative transcription termination is omitted.

Abbreviations: Ig, immunoglobulin; GAG, glycosaminoglycan; EGF, epidermal growth factor; CRP, complement regulatory protein

Modified from Day and Prestwich (2002)

Fig. 2. The HLT model of the adult brain ECM.

(A) Lecticans bind hyaluronan and TN-R through their G1 and G3 domains, respectively. The connections are stabilized via a link protein (Hapln). The Lecticans are additionally stabilized by the small glycoprotein TN-R. (B) When an interaction, for example, of Hapln with lecticans, is disrupted, the matrix becomes 'loose'.

Modified from Yamaguchi (2000)

Fig. 3. Representative images of PNNs.

(A) Scheme of the typical reticular structure of a PNN, with representative synaptic boutons included in PNN holes (modified from Lafarga et al. 1984). (B) WFA-positive net enwrapping the soma and dendrites of a neuron in the lateral vestibular nucleus of the adult mouse. (C) WFA-positive PNN (red) in the mouse medial cerebellar nucleus, contacted by Purkinje axon terminals (stained by a-calbindin antibodies, green). In both B and C the holed structure of the PNN is clearly apparent. Scale bars: 10 μ m in B, 5 μ m in C.

Fig. 4. Models of the distribution of lecticans and Haplns at PNN.

Schematic drawing of the composition of the PNN. A hyaluronan backbone is continuously secreted by HAS from the cell membrane. Lecticans are attached to the hyaluronan. (A) The conventional model suggests that HAPLN and lecticans are combined at random represented by yellow background. (B) We suggest that Hapln interacts with specific lecticans (e.g. Hapln4/Bral2 and brevican indicated by green background, Hapln1/Crt11 and other lecticans indicated by red background).

Fig. 5. Molecular organization of PM.

(A) Immunolabeling of mouse optic nerve with neurocan antibody (red) and paranodal marker caspr antibody (green). (B) Structural and molecular composition of the node of Ranvier. Oligodendrocytes (CNS) or Schwann cells (PNS) form the myelin sheath by enwrapping around axons. Nodes are flanked by paranodes, and contacted by perinodal astrocytes/ NG2 glia (CNS) or Schwann cell microvilli (PNS). Adhesion molecules (NF186, NF155, caspr, NrCAM and cntn1) mediate axo-glial attachment, which in turn form multiprotein complexes with ion channels (Na_v , and potassium channels KCNQ2/3 and Kv1.2) that are stabilized by their association with cytoskeletal molecules (ankyrins and spectrins). PM molecules (Vcan V2, Bcan, Ncan, Pcan, HA, TN-R and Hapln2/Bral1 in CNS, Vcan V1, syndecan-3/4 and glm in PNS) fill nodal extracellular space. (C) Axon diameter dependent assembly of hyaluronan binding matrices at the CNS nodes. Minimum unit of the matrix consists of Vcan V2, HA and Hapln2/Bral1 in smaller axon, and more complex matrix surrounds larger diameter axon.

Abbreviations: Bcan, brevican; Bral1, Hapln2/Bral1; cntn1, contactin 1; Gln, gliomedin; HA, hyaluronan; Ncan, neurocan; NF186, neurofascin 186; NF155, neurofascin 155; Pcan, phosphacan; TN-R, tenascin-R; Vcan V1, versican V1; Vcan V2, versican V2. Scale bar, 10 μ m.

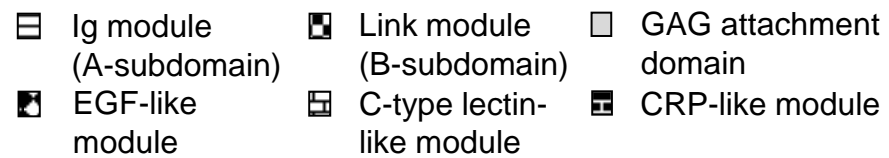
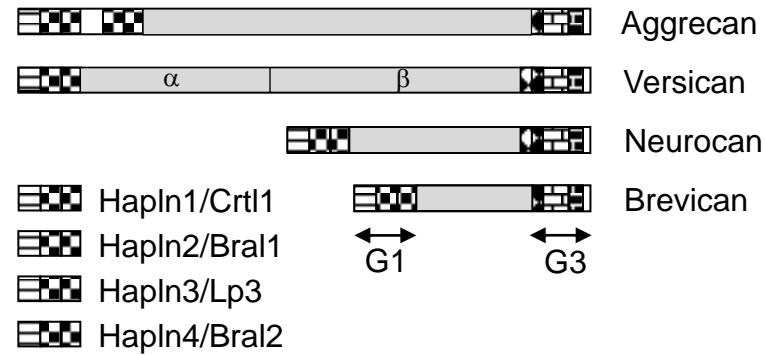
Table 1 The key molecules of the hyaluronan-based condensed perineuronal matrix in the adult brain.

Key components	ECM type	Cellular Origin in the Nervous System	CNS specific	Proposed interaction within PNN or NOR or AIS
Aggrecan	PNN	Neurons	No	HapIn1, TN-R
Versican V2	NOR	Oligodendrocytes	Yes	HapIn2, TN-R
Neurocan	PNN, NOR	Neurons/astrocytes	Yes	HapIn1, HapIn2, TN-R
Brevican	PNN, NOR, AIS	Glial cells/neurons	Yes	HapIn2 (in PM), HapIn4 (in PNN) TN-R, NF-186
HapIn1/CrtI1	PNN	Neurons	No	Lecticans except for versican V2
HapIn2/Bral1	NOR	Oligodendrocytes/neurons?	Yes	Versican V2, neurocan, brevican
HapIn4/Bral2	PNN	Neurons	Yes	Brevican
TN-R	PNN, NOR	Neurons/glial cells	Yes	Lecticans, phosphacan
Phosphacan	PNN, NOR	Glial cells/neurons	Yes	TN-R
hyaluronan	PNN, NOR	Neurons	No	Lecticans, HapIns

Abbreviations: PNN, perineuronal net; NOR, node of Ranvier; AIS, axon initial segment

Figure1

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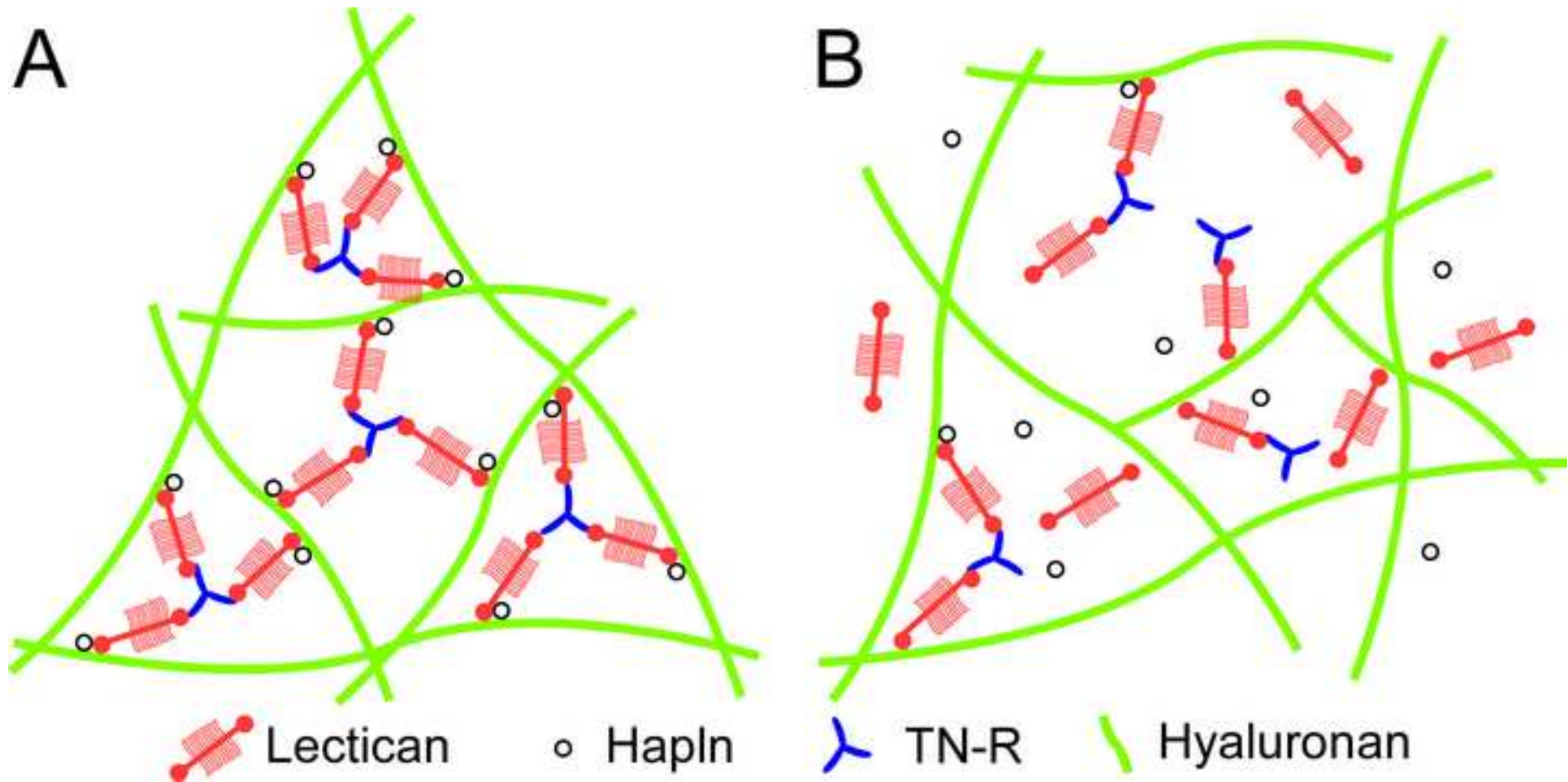


Figure3
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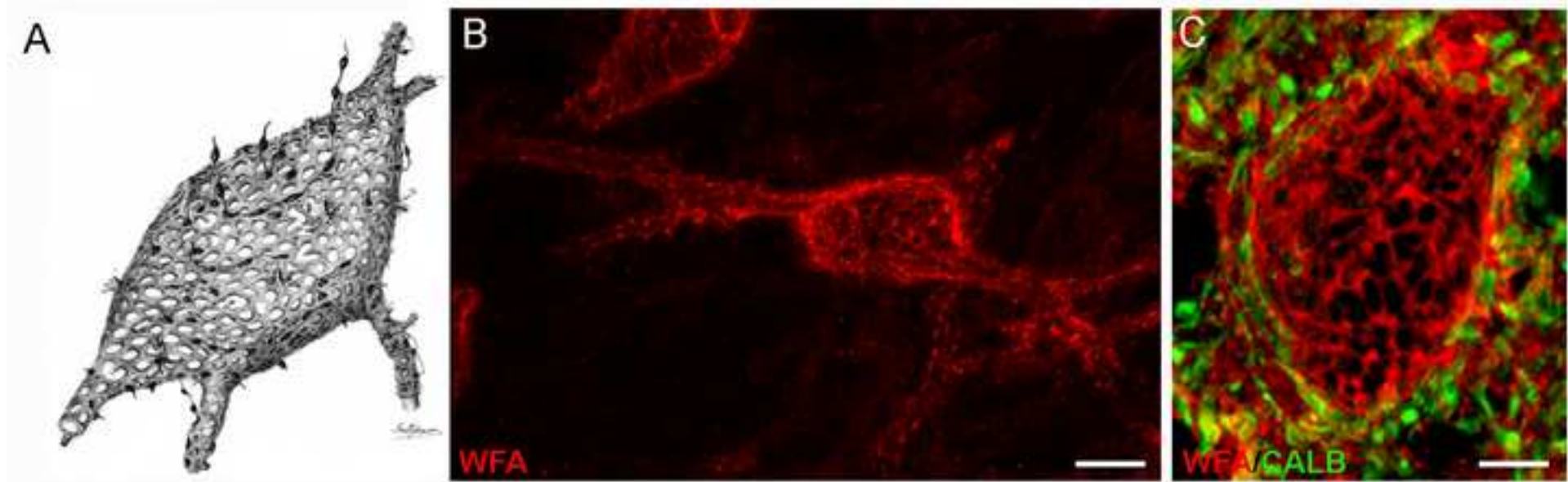
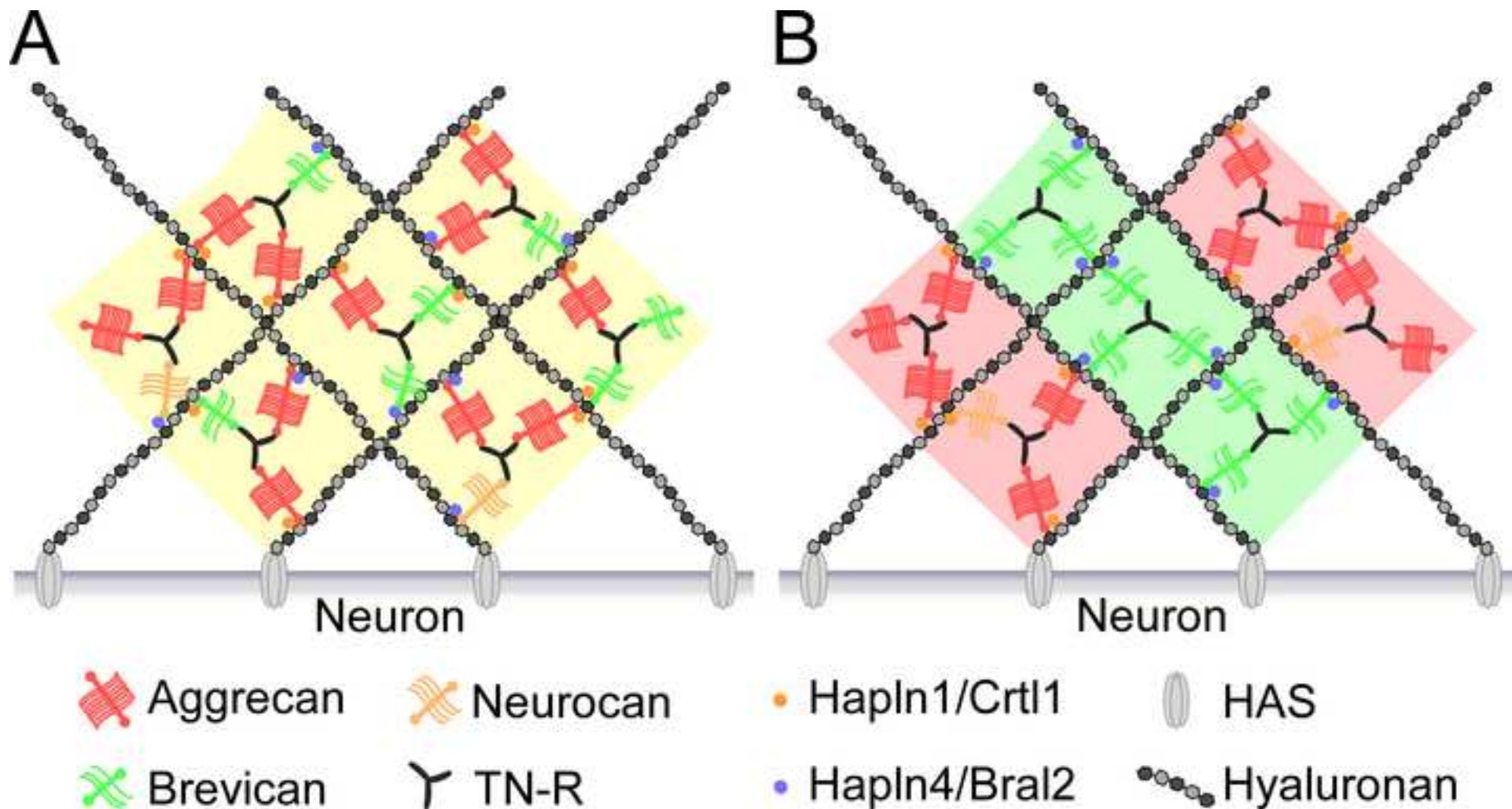


Figure4
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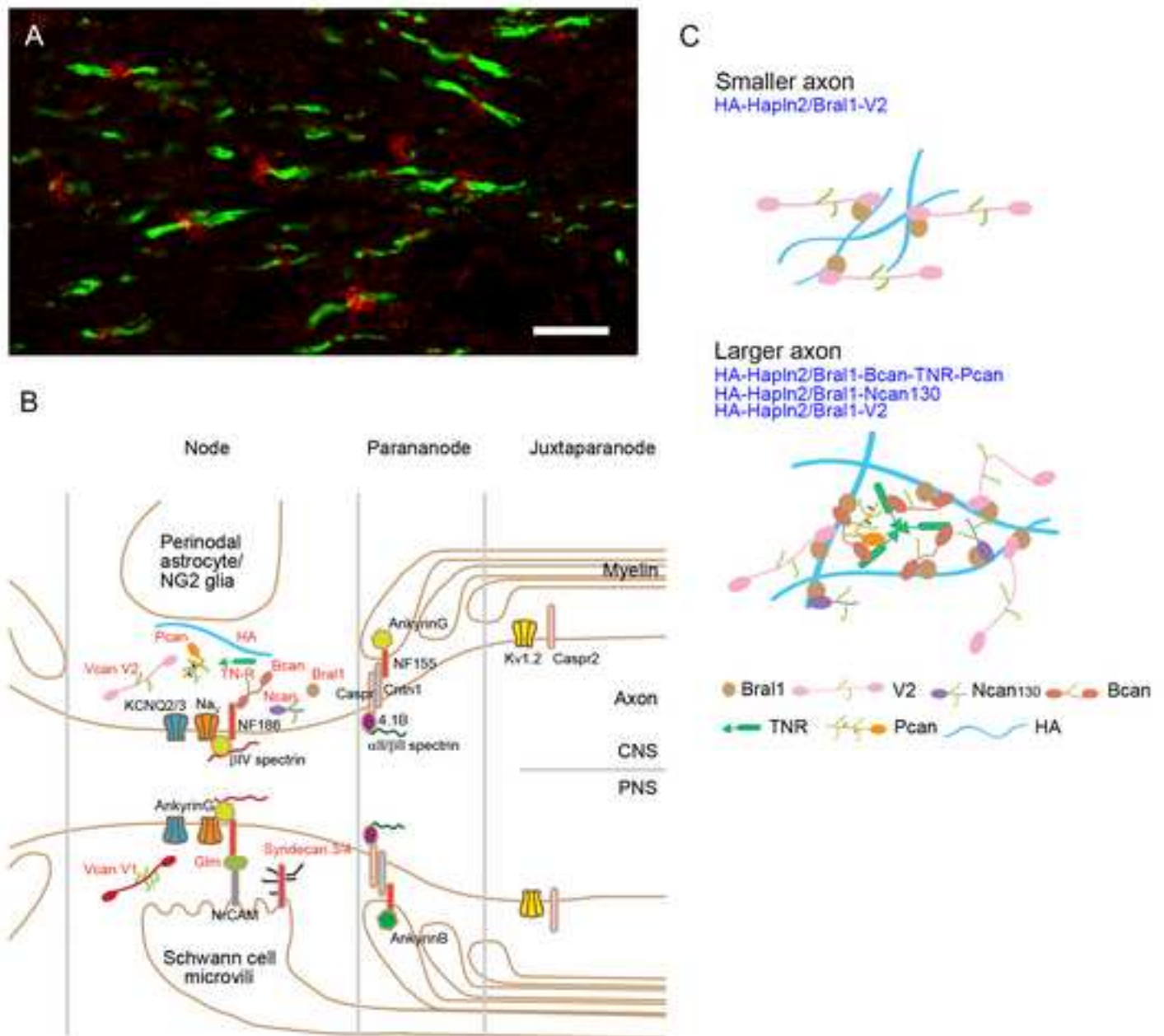


Figure 5. Oohashi et al.