

# **Morphological analysis of the extracellular matrix in the human central nervous system**

**PhD thesis**

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**Budapest  
2014**

## INTRODUCTION

The central nervous system of vertebrates is not solely composed of cells. A substantial part of its volume is filled by the meshwork of macromolecules which are forming the extracellular matrix. These molecules are produced locally; they form an organized network and remain in contact with the surface of the cells that produce them. Matrix molecules are already present in the narrow spaces between the cells of the developing embryo. They play an important role in development whilst they can be found in nearly all areas of the adult CNS. The extracellular matrix in the narrow space between neurons, glial cells and their extensions is a multicomponent structure, being a common product of the neuron and glia. The functional versatility of the different brain regions is reflected in the composition and establishment of the extracellular matrix. Today it is known that the extracellular matrix composes of over 20% of total brain substance. In the central nervous system of vertebrates the molecules of the amorphous ground substance are present in the largest amount, which are thus in focus of this thesis.

Molecules of the extracellular matrix are chemically heterogeneous. Glycosaminoglycans (GAGs), built up by polysaccharide chains, bind to proteins, to form the so-called proteoglycans. Proteoglycans are proteins that are heavily glycosylated. The basic proteoglycan unit consists of a "core protein" with covalently attached GAG chains. Glycosaminoglycans are long non-branching polysaccharides consisting of repeating disaccharide units. According to the combination of these sugars, the GAG chains are subclassified into heparin/heparan-, keratan-, dermatan- or chondroitin sulfates and hyaluronan. Hyaluronan is an anionic, nonsulfated and core protein-free glycosaminoglycan. Most of the mammalian central nervous system studied are chondroitin sulfate proteoglycans, lecticans being the most common ones. As a main matrix component, we labelled the aggrecan molecule of the lectican family which is produced by neurons. Aggrecan is a high molecular weight proteoglycan. It exhibits a bottlebrush structure in which chondroitin sulfate and keratan sulfate chains are attached to an extended protein core. The interaction between hyaluronan and lecticans is reinforced by small link proteins. Lecticans, together with the molecules bound to them – including hyalorunan, tenascins, link proteins are others – make up finally for the composite meshwork of the extracellular matrix.

In addition to the amorphous presence of the matrix, its molecules typically condense around the somatodendritic compartment of certain neurons, called perineuronal nets, to support neuronal function. Alternatively, matrix molecules can aggregate around axon terminals to form so-called axonal coats to ensure a local scaffold for individual synapses.

The existence of perineuronal nets has been implicated by Golgi, Lugaro, Donaggio, Martinotti, Ramón y Cajal and Meyer. However, Ramón y Cajal credits Golgi with the discovery of perineuronal nets because he was the first to draw attention to them and gave the first precise description in 1893. Today perineuronal nets are known to be essential components of the mature central nervous system of mammals, including human.

Perineuronal nets are believed to carry important functions. In addition to connect the extracellular domain with the intracellular cytoskeleton, perineuronal nets were implicated in supporting ion homeostasis around fast-spiking neurons, in the protection of neurons against neurodegeneration or in the reduction of synaptic plasticity at the end of the sensitive period of the brain.

This thesis investigates the phenotypic appearance of the extracellular matrix in the human central nervous system. We examined the matrix structure and morphological diversity of the various levels of human spinal cord. We studied the relationship between the perineuronal nets and fundamental matrix components. Multiple labelling showed relations in the extracellular matrix to various neuronal, neurotransmitter and receptor subtypes. Our results in the human lateral geniculate body brought a surprising turn, since establishment of the extracellular matrix principally differed from previous phenotypic descriptions obtained in mammals. Similarly, the distribution and association of perineuronal nets to diverse neuronal subsets in the human hippocampus showed differences to findings recently reported in mammals.

## **AIMS**

The extracellular matrix of the mammalian central nervous system has been studied in several species. Similar knowledge of human samples are incomplete and only of limited availability. Focusing on three regions of the central nervous system of different structure and territorial hierarchy, we aimed to unravel the establishment and chemical heterogeneity of the extracellular matrix in humans. We examined the main chondroitin sulfate proteoglycan components of the perineuronal matrix: aggrecan and brevican.

1. Therapeutic efforts have focused on the spinal cord to facilitate the axon regeneration via breaking down the extracellular matrix. These studies ignore the immediate vicinity of neuronal somatodendritic compartments. We wanted to investigate the neurochemical make-up and cranio-caudal segment diagram of the perineuronal and perisynaptic matrix around different neuronal groups and terminals in the spinal cord.
2. In the midbrain, we focused on the lateral geniculate body, since our preliminary observations showed an absence of perineuronal nets in this area. Further, the lateral geniculate body was selected as an aplastic brain region to study the brain extracellular matrix.
3. Hippocampus is a brain region known for its plastic properties. We have investigated the extracellular matrix of this area focusing both on basic description and mapping, phylogenetic uniqueness and perineuronal and perisynaptic recruitment.

## **METHODS**

### *Human samples*

Human tissue samples were provided by the 2<sup>nd</sup> Department of Pathology number of Semmelweis University, under license of Semmelweis University ethical use. Our samples were obtained from otherwise healthy cadavers without any history of neurodegenerative diseases. We took two whole brain samples with a post mortem delay (PMD) of 15-18 hours, and two whole spinal cord samples with a post mortem delay (PMD) of 12-15 hours.

### *Fixation*

Instead of the most often used immersion fixations in human samples we fixed organs of the central nervous system through blood vessels. The brain and spinal cord were first perfused *in situ* through the arterial and venous system (common carotid and vertebral arteries, superior sagittal sinus). Spinal cord fixation was completed via subarachnoid fixative administration.

### *Sectioning*

Samples were processed for light microscopy and immunofluorescence testing. We used 40 µm thick sections. Before the staining process we used cryoprotection and cryostat for making sections.

### ***Reducing the non-specific endogenous peroxidase activity, blocking step***

After washing several times in phosphate buffer (PB), sections dedicated for light microscopy were placed in peroxidase blocking solution in order to reduce endogenous peroxidase activity. Sodium borohydride was used to reduce the amount of generated aldehydes. This was followed by the serum blocking step using bovine serum albumin, casein and normal donkey serum diluted in PB saline with Triton, in order to avoid non-specific secondary antibody binding.

### ***Lectin histochemistry***

The free-floating sections were reacted in PB diluted biotinylated Wisteria floribunda agglutinin (WFA) for overnight at room temperature. After washing with PB, the tissue has been placed in diluted avidin-biotin complex. The linked avidin-biotin complex (ABC) has been visualized by chromogen (3,3'-diaminobenzidine, DAB) to precipitate presence of nickel ammonium sulfate in order to see a higher intense reaction. After multiple washing with PB, sections were mounted onto gelatin-coated slides.

### ***Immunohistochemistry***

Sections were incubated with the primary antibodies diluted in a solution of PB saline with Triton containing normal donkey serum through overnight. In case of multiple labelings the primary antibodies were diluted in the same solution (cocktail).

### ***Detecting aggrecan- and brevican based core protein with primary antibodies***

The core protein of aggrecan molecule was recognized by the HAG7D4, Cat 301 and Cat-315 antibodies, whilst the linker region of aggrecan was detected by the anti-Ctrl-1 antibody. The brevican component of the matrix was traced with anti-brevican antibody, the tenascin molecule with anti-tenascin antibody. The hyaluronan was visualized by protein binding reaction. The free-floating sections were pretreated with hyaluronidase, thereafter reacted with specific biotinylated hyaluronan-binding protein.

### ***Marking the synaptic markers and dendrites of neurons with primary antibodies***

For synaptic markers anti-glutamic acid decarboxylase (65/67) antibody, anti-vesicular glutamate transporter-1 antibody or anti-parvalbumin antibody were used. Neurons were identified by the anti-choline acetyltransferase antibody or anti-substance P antibody. Gamma-aminobutyric acid (GABA) receptors were detected by using the anti-monoclonal GABA<sub>A</sub>  $\beta$ 2 antibody. Against the glycine receptor (Gly-R) the monoclonal GlyR4a antibody was used.

Dendrites of neurons were visualized with the SMI 311 antibody, for glial cells detection anti-glial fibrillary acidic protein (GFAP) antibody was used.

### ***Light microscopic analysis of the epitopes in single labelings***

After repeated washing, slices were reacted with biotinylated donkey anti-mouse, rabbit or goat anti-immunoglobulin serum (secondary antibody) for 60 minutes. Subsequently, sections were reacted with the preformed avidin-biotin complex (ABC) for one hour at room temperature. The immunoprecipitate was visualized by using amplified nickel ammonium sulfate DAB reaction. Sections were mounted on gelatine-coated slides and coverslipped with DePex for light-microscopical examination.

### ***Multiple immunofluorescence labelings***

For double- and triple immunofluorescence labellings synaptic or neuronal markers and aggrecan or brevican antibodies were combined. Then the sections were reacted with different fluorescent secondary antibodies, i.e. in a mixture of different colored carbocyanin (Cy2, Cy3, Cy5)-conjugated donkey anti-mouse, goat or rabbit immunoglobulin. The sections were mounted on gelatine-coated slides, dried and coverslipped with glycerol.

### ***Electron microscopy***

Samples were removed from the lateral geniculate body as well as from the CA1 region of the hippocampus. Fifty-micron thick sections were processed for HAG7D4, CTRL-1 or brevican immunocytochemistry as described above. This was followed by osmium-tetroxid treatment at room temperature followed by embedding in Durcupan. Ultrathin sections were cut and mounted slot grids pre-treated with Formwar.

## ***Imaging***

The sections labelled with immunoperoxidase reaction were captured by light microscope. Immunofluorescent labellings were examined with a Nikon Eclipse E800 microscope attached to a Bio-Rad Radiance 2100 Rainbow confocal laser scanning system. Sections for ultrastructural analysis were studied under JEOL1200 EMX electron microscope.

## **RESULTS**

### **Distribution and presentation of the extracellular matrix in the human spinal cord**

#### ***General histochemical considerations***

The perineuronal and perisynaptic extracellular matrix in the human spinal cord was represented by unique distribution patterns and a great structural and chemical diversity. Description and characterisation of the chondroitin sulfate proteoglycan-based extracellular matrix were based on the analysis of representative spinal cord segments. Chemical heterogeneity was dissected by detecting five fundamental extracellular matrix components: hyaluronan, aggrecan, brevican, stabiliser link protein 1 (CRTL-1 or HAPLN-1) and tenascin-R.

Hyaluronan and tenascin-R are ubiquitous components of the extracellular matrix; this was confirmed in both white and grey matters of the human spinal cord. Grey matter was more intensely labelled with condensed matrix assemblies around perisomatic compartment of neurons likely forming PNs. Notably, the substantia gelatinosa showed largely spared hyaluronan but not tenascin-R immunoreactivity in the dorsal horn, especially in its thoracic, lumbar and sacral segments.

Aggrecan, brevican and HAPLN-1 were detected predominantly in matrix aggregates around neuronal somata, dendrites and terminals. Since these structures were concentrated in the grey matter, distinct extracellular matrix structures were detected in these regions leaving the white matter practically spared from aggrecan, brevican and HAPLN-1 immunoreactivity. We revealed that perineuronal nets show a partial overlap of their basic extracellular matrix components: aggrecan delineates neuronal somata and dendrites with different intensities whereas brevican and HAPLN-1 widely overlap around more distal dendritic compartments as well. Finally, multiple labelling experiments gave basic information on the relation of extracellular matrix to neuronal, transmitter and receptor subtypes in the human spinal cord.

We performed a detailed immunohistochemical analysis of sections of the spinal cord as well. We have made comparison between different segments of the spinal cord matrix composition. Key observations were: (1) the lack of aggrecan immunoreactivity in the dorsal horn of the, (2) CRTL-1 and brevican were present in the substantia gelatinosa unlike the Lissauer-zone, (3) the Clarke-Stilling and intermediomedial nuclei were clearly demarcated by the presence of thick perineuronal nets and (4) a most motor neurons in the anterior horn were surrounded

by perineuronal nets. Perineuronal nets were typically found in regions of the spinal with distant connections, including motor, sensory or autonomic functions. This is in contrast to previous descriptions attributing the presence of perineuronal nets to local circuit neurons. In the dorsal horn, a large number of afferent fibers penetrate the gray matter through the Lissauer-zone. We observed that isolated matrix aggregates appear in our specimens CRTL-1 or brevican containing axonal coats, possibly around synapses.

### ***Extracellular matrix relations to special types of neurons transmitters and receptors***

The relation of the extracellular matrix assembly to neuronal, transmitter and receptor subtypes were investigated in the cervical segments of the human spinal cord.

Perineuronal nets were composed of chemically different components. This was recapitulated in the human spinal cord with distinct compartmentalisation around the surrounded neuron. Aggrecan, HAPLN-1 and brevican are fundamental elements of perineuronal nets with a wide, overall overlap between HAPLN-1 and brevican but less between aggrecan in the distal neuronal compartment.

The most conspicuous neuronal populations in the spinal cord are the large cholinergic motoneurons that populate the ventral horn. Most of these neurons (cervical segment: 71%; thoracic segment: 64%; lumbar segment: 64%, sacral segment: 81%) were surrounded by aggrecan-based perineuronal nets ensheathing the somatodendritic compartment of the cells with different intensity. Nevertheless, not all cholinergic, cholin-acetyl transferase-immunoreactive neurons of the ventral horn were surrounded by perineuronal nets and perineuronal nets were not exclusively associated to cholinergic neurons as shown by complementary cholin-acetyl transferase and aggrecan immunopatterns. In contrast, brevican was detected with high intensity around neuronal somata and distal parts of dendrites. The brevican-immunoreactive matrix formed small round or oval-shaped structures representing ACs that contacted the neuronal surface.

Firing patterns of neuronal cells and circuits are powerfully controlled by inhibitory synapses. In the spinal cord, inhibitory terminals operate with both GABA as well as glycine as neurotransmitters. We hypothesised that inhibitory terminals carry a matrix scaffold. Since single perisynaptic matrix assemblies, hence, axonal coats were selectively labelled with the anti-brevican and anti-HAPLN-1 antibodies we investigated the co-localisation of these matrix components with glycine and GABA<sub>A</sub> receptor markers. Glycine receptors were found widely distributed in the grey matter, predominantly without a relation to HAPLN-1 containing structures. In contrast, brevican-immunoreactive axonal coats repeatedly displayed colocalisation to glycine receptors. Similar correlations were found concerning HAPLN-1 and GABA<sub>A</sub> receptors with a clear overlap along dendrites of large motoneuron.

Distribution of excitatory contacts was investigated by stainings for vesicular glutamate transporter type 1 (VGLUT-1). VGLUT-1 is widely distributed throughout the grey matter of the spinal cord. In the ventral horn, somata of large cholinergic motoneurons were virtually spared of VGLUT-1 immunoreactivity. However,

dendrites were contacted frequently. In the dorsal horn, HAPLN-1-positive dendrites were found to be partially contacted by VGLUT-1-immunoreactive profiles. In the transition zone from the dorsal horn into the posterior nerve roots a strong VGLUT-1-immunopositive zone was detected.

In the dorsal horn of the spinal cord, nociceptive primary afferents typically use substance P as transmitter. We focused our attention on this marker since the clinical relevance of substance P-mediated neurotransmission is exceptional in pain syndromes and therapy. HAPLN-1 immunoreactivity was low in laminae I and II of the dorsal horn but increased abruptly at its ventral border which allowed us to investigate the relationship of substance P immunoreactivity to the surrounding perisynaptic matrix. We found that HAPLN-1 and substance P showed largely non-overlapping distribution.

### **Extracellular matrix distribution and pattern of the lateral geniculate body**

The application of various antibodies against extracellular matrix components resulted in partially overlapping but nonidentical immunostaining patterns around the cellular compartments in the lateral geniculate body. The main observation was that typical perineuronal nets like those described in most brain regions of a variety of vertebrates, including humans, were not found in the human lateral geniculate body. Immunostaining was rather poor around the cell body, whereas small, as axonal coats were present throughout all layers of the nucleus. This is in sharp contrast to previous findings that associated chondroitin sulfate proteoglycan-based extracellular matrix typically with perineuronal nets in analogous nuclei of other mammals or not necessarily in line with the finding that Cat-3xx antibodies are a neuronal surface antigen in the primate lateral geniculate body, suggesting a unique development of extracellular matrix compartmentalization in the lateral geniculate body that is specific to humans. The revealed axonal coats often outlined proximal and intermediate segments of the dendrites, which showed the shape of the neuron and location of soma without further staining.

An overview of immunostaining and lectin staining is provided. For lectin histochemistry using WFA did not label extracellular matrix components or structures in human lateral geniculate body. Neither perineuronal nets nor axonal coats were found in the lateral geniculate body with the application of WFA. The absence of WFA reactivity could not be attributed exclusively to overall tissue or fixation properties, because several typical perineuronal nets were detected within the pulvinar nucleus dorsal to the lateral geniculate body, which were also identified with anti-chondroitin sulfate proteoglycan staining, i.e., with HAG7D4 immunohistochemistry.

### ***Detection of aggrecan core protein with HAG7D4 anti-aggrecan immune reaction***

Although both magno- and parvocellular layers exhibited HAG7D4-immunoreactivity, magnocellular layers stained discernibly more strongly than parvocellular layers. In magnocellular layers 1 and 2, dendrites were clearly outlined in several cases across their 30–40  $\mu\text{m}$  long extent by axonal coats, with sparse



labeling of perisomatic accumulation of extracellular matrix. On the other hand, axonal coats also appeared seemingly sporadically throughout the magnocellular layers of lateral geniculate body. In a few cases, perineuronal nets with very strong perisomatic labeling were also identified in the intercalated layer between magnocellular layers 1. and 2. In parvocellular layers, labeling was far weaker than in magnocellular layers, which allowed the identification of few perineuronal nets.

Perisomatic parts were labeled relatively incompletely, but dendrites were clearly outlined. The peridendritic matrix was not coherently stained; instead, chains of axonal coats established the peridendritic sheath for 40–50  $\mu\text{m}$  or even longer. The number of apparently evenly distributed, independent, nongrouped axonal coats was much lower than in magnocellular layers.

### ***CRTL-1-immunoreactivity in the lateral geniculate body***

In magnocellular layers, strongly stained axonal coats appeared in the relatively lightly stained neuropil. In most cases, they were seemingly unarranged, whereas chains of axonal coats resembling rows of AC-associated synapses formed on dendrites were seen in only a few cases for a maximum of 10  $\mu\text{m}$  in length. In parvocellular layers, only very few and lightly stained items were seen in the immunoreactive neuropil. These items were vaguely identifiable axonal coats that lined up in short chains (maximum 10–20  $\mu\text{m}$  long) resembling dendritic sections as stained with the HAG7D4 antibody.

### ***Immunostainings with the Cat-301 and Cat-315 antibodies***

Among the Cat antibodies, Cat-315 offered the most unique immunostaining. In magnocellular layers, small, often ambiguously identifiable axonal coats were seen in the immunoreactive neuropil that only sporadically formed thin, shorter (up to 15  $\mu\text{m}$  long) and more vaguely traceable chains. In parvocellular layers, neuropil was very weakly labeled which allowed the identification of gracile fiber-like structures that remained undetected with other immunostainings. In some cases, they seemed to outline the shape of dendritic sections but were barely found around somata. Whereas the phenotypical appearance of labeled structures in magnocellular layers was similar to that found with HAG7D4 or CRTL-1 immunostaining, they failed to outline the dendritic shaft and did not allow recognition of neuronal shapes. With the Cat-301 antibody, faint perisomatic and stronger short dendritic labeling was found in the magnocellular layers, and a similar but also rather ambiguous immunostaining was visible in the parvocellular layers. Nevertheless, axonal coats were recognizable in both magno- and parvocellular layers.

### ***Colocalization of the aggrecan core protein with other extracellular matrix markers***

Confocal laser scanning microscopy was utilized to demonstrate that HAG7D4-immunoreactive axonal coats largely colocalized with the CRTL-1-immunopositive structures. In contrast, HAG7D4 immunoreactivity colocalized poorly with Cat-315 immunostaining in the magnocellular layers of the lateral geniculate body, perhaps indicating that Cat-315 was not detecting aggrecan in the human lateral geniculate

body. This assumption is well in line with previous studies suggesting similar hypotheses.

### ***Synaptic markers colocalize with the aggrecan core protein in several cases***

Double-immunofluorescence labeling showed that, although several HAG7D4-ir structures showed staining patterns related to immunostaining with an antibody raised against VGLUT1, many of the staining profiles with these antibodies were non-overlapping. At the same time, compared with their lower number, GAD-immunoreactive profiles overlapped with HAG7D4 reactivity proportionally more frequently. Relatively few PV-immunoreactive structures appeared occasionally close to HAG7D4-immunoreactive structures. Quantitative assessments showed that a low proportion (4–6%) of VGLUT1- immunoreactive or PV- immunoreactive profiles was in intimate contact with HAG7D4- immunoreactive axonal coats, whereas this proportion grew to 38% at GAD- immunoreactive profiles. We were unable to detect VGLUT2 immunoreactivity in our human samples.

### ***Different extracellular matrix components are differentially related to neuronal or glial markers***

Although HAG7D4 and Cat-315 immunostainings revealed structures of similar phenotypes, they were associated with different structures. Dendrites emanating from cell body or more distally were visualized using the SMI-311 antibody, which was irregularly ensheathed by HAG7D4-immunoreactive structures in several cases. In contrast, Cat-315-immunoreactive profiles sometimes colocalized with structures immunoreactive for GFAP. In contrast to previous findings, this finding supports the hypothesis that the Cat-315 antibody possibly detects a carbohydrate epitope on a protein other than aggrecan.

### ***Electron microscopy***

At the ultrastructural level, HAG7D4-immunoreactivity was seen around proximal dendrites but less frequently around soma. The labeled matrix accumulated in the extracellular space, in several cases around axodendritic junctions, and could sometimes be found in close proximity to glial structures.

## **Distribution and presentation of the extracellular matrix in the human hippocampus**

### ***Aggrecan in the human hippocampal formation***

By using antibodies directed against aggrecan's chondroitin sulphate chain-enriched (AB1031 and Cat-301) or N-terminal (HAG7D4) domains, we have studied the distribution and compartmentalization of this chondroitin sulfate proteoglycan component in the human hippocampal formation, including the entorhinal cortex. We detected perineuronal nets in all hippocampal subfields in a region- and layer-specific manner. The distribution of AB1031-immunoreactive, Cat-301-immunoreactive or HAG7D4-immunoreactive perineuronal nets was overlapping, failed to label pyramidal cells, yet differed in their intensity to label the hippocampal neuropil.

Perineuronal nets were most frequently seen in the CA1 region, particularly in stratum oriens surrounding multipolar non-pyramidal neurons. Whilst the pyramidal layer was largely devoid of aggrecan-immunoreactive somata, many perineuronal nets of multi- or bipolar phenotypes were identified in strata radiatum and lacunosum-moleculare. The entorhinal cortex could typically be demarcated via the intense labelling of its principal strata. Perineuronal nets were of different phenotypes, including sharply contoured “classical” or “diffuse” subtypes. This diversity was best reflected by the appearance of AB1031-immunoreactive perineuronal nets throughout the human hippocampus. AB1031-immunoreactive, Cat-301-immunoreactive or HAG7D4-immunoreactive matrix assemblies formed peridendritic sheaths that extended for 50  $\mu\text{m}$  over second to third order dendrites. Putative axon initial segments were identified as thin structures emanating from the soma or the most proximal part of a first order dendrite. At the ultrastructural level, aggrecan typically enwrapped postsynaptic compartments on dendrites and dendritic spines. This aggrecan-containing matrix assembly was often seen over axon terminals and presynaptic axons, encapsulating the entire synapse.

### ***CRTL-1 is recruited to axonal coats at excitatory and inhibitory synapses***

The core assembly of brain extracellular matrix is established by “link” proteins, which connect chondroitin sulfate proteoglycans to hyaluronan. The CRTL-1 protein is an indispensable component of perineuronal nets, its production being the major event to limit synaptic plasticity via organizing chondroitin sulfate proteoglycans into perineuronal nets. Animals lacking CRTL-1 have disrupted perineuronal nets and persistent synaptic plasticity.

CRTL-1-immunoreactive perineuronal nets and axonal coats exhibited layer- and region-specific distribution in the profusely stained neuropil. Compared to our aggrecan “maps”, CRTL-1-immunoreactive perineuronal nets in the CA1 subfield were less densely packed, and restricted to strata oriens and radiatum. These were of the sharply contoured “conventional” type of perineuronal nets with extensive peridendritic sheaths. In the CA2 subfield, perineuronal nets were seen in the strata oriens and radiatum. Notably, distinct neuropil labelling in the CA2 subfield formed a “barrier” separating the CA1 and CA3 regions. This finding is supported by earlier data from rodents, reporting dense extracellular matrix proteoglycan and lectin reactivity of the neuropil in the corresponding subfield. In contrast to our anti-aggrecan immunostainings, perineuronal nets were frequently encountered in all layers of the CA3 region with the putative axon initial segment occasionally visible. Alternatively, the perineuronal matrix assembly was restricted to the somata as faint CRTL-1-immunoreactive perisomatic rims. In subicular regions and the entorhinal cortex, perineuronal nets surrounded multipolar neurons. The entorhinal cortex was decorated by prominently labelled islands in the external principal stratum.

The dentate hilus contained many perineuronal nets. However, we have encountered a large number of densely packed structures of 1–3  $\mu\text{m}$  in diameter, axonal coats, at the hilar border of the granular layer. Axonal coats were identified in a similarly high density in the entorhinal cortex; significantly exceeding those in the CA regions. Ultrastructural analysis showed that, in addition to the presence of

CRTL-1 in myelinated axons, extracellular CRTL-1-immunoreactive matrix is restricted to surround terminal axon segments including the synapse.

***Brevican (50 kDa fragment) accumulates in axonal coats***

Brevican is a brain-specific proteoglycan primarily produced by glia. Brevican's roles in synapse development and signalling were shown during the formation of cerebellar glomeruli and by disrupted hippocampal long-term potentiation in brevicin-deficient mice. Brevican is proteolytically cleaved into N-terminal (50 kDa) and intermediate/C-terminal (90 kDa) fragments. Here, we used an antibody raised against brevicin's 50 kDa fragment to show that the brevicin<sup>+</sup> extracellular matrix pattern of the human hippocampal formation is remarkably different from other matrix maps hitherto identified.

Perineuronal nets were sporadically found in the strata oriens and radiatum of the CA1–CA4 subfields. Instead, brevicin predominantly identified axonal coats, arranged as pearl lace-like strings, at the hilar surface of the dentate granule layer. The orientation of axonal ribbons resembled the aggrecan-immunoreactive peridendritic sheaths at the hilar margin of the granule layer. Although at markedly lower densities, axonal coats were also seen in the CA1–CA3 subfields (except the stratum lacunosum-moleculare) and subiculum. In the entorhinal cortex, brevicin-immunoreactive axonal coats re-appeared at a high density.

We used ultrastructural analysis to confirm that brevicin-immunoreactive axonal coats surrounded synapses. We show that brevicin-immunoreactive matrix assemblies were restricted to individual synapses without forming extensive intercellular scaffolds along the postsynaptic dendrites or somata. Electron microscopy demonstrated the presence of brevicin immunoreactivity in myelinated axons. This finding, and the lack of brevicin immunoreactivity in endo- or lysosomes, suggests that presynaptic neurons actively shape axonal coats.

***Perineuronal nets surround parvalbumin-immunoreactive, calretinin-immunoreactive and calbindin-immunoreactive hippocampal neurons***

Confirming earlier observations in other mammalian species or areas of the human nervous system, the majority of parvalbumin-immunoreactive cells were surrounded by aggrecan-containing perineuronal nets. The association of CRTL-1-immunoreactive perineuronal nets and parvalbumin-immunoreactive cells was analogously high; with CRTL-1-immunoreactive extracellular matrix frequently surrounding neurons in stratum oriens but not stratum radiatum.

Calretinin-immunoreactive and calbindin D28k-immunoreactive neurons of lower mammals lack perineuronal nets. In contrast, a subpopulation of calretinin-immunoreactive neurons was surrounded by aggrecan-immunoreactive or CRTL-1<sup>+</sup> perineuronal nets in the human hippocampus. Similarly, calbindin D28k-immunoreactive neurons were - though less frequently - surrounded by aggrecan-immunoreactive perineuronal nets. Overall, while the presence of perineuronal nets is a general hallmark of inhibitory neurotransmission, our data suggest that the human hippocampus contains a unique variety of perineuronal net-bearing interneurons.

## CONCLUSIONS

The human extracellular matrix of the central nervous system shows regional differences in the distribution and phenotype.

1. We found that the human aggrecan and brevican based extracellular matrix is markedly present in perineuronal nets and axonal coats of the spinal cord. Analyzing the full cranio-caudal extent of the spinal cord, we showed that perineuronal nets are bearing typically neurons with long axons, regardless of what system or pathway are they belong to. The dorsal horn received a large number of afferent fibres which access the grey matter. Whilst a large number of isolated axonal coats was identified, they remained uncoupled to nociceptive afferents.
2. Extracellular matrix of the human lateral geniculate body showed a very different phenotype like previous studies. The matrix of the human lateral geniculate body was not organized into perineuronal nets. Dendrites were contacted by axonal coats appearing as small, oval structures. We demonstrated that these typical structures were associated to synaptic loci on dendrites. The magnocellular layers – opposite to parvocellular layers – showed intense matrix immunoreactivity.
3. In the human hippocampal formation principal cells were devoid of perineuronal nets. Within the group of interneurons not only parvalbumin-containing fast-spiking basket and chandelier cells, but calretinin- and calbindin-containing interneurons were surrounded by perineuronal nets, likely being a human-specific phenomenon. We have shown that the distribution of axonal coats was extremely different; axonal coats occurred in the dentate gyrus and in the entorhinal cortex at outstanding density. Ultrastructural analysis suggested that the perisynaptic matrix components can be produced by presynaptic neurons as well.

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