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ANALYSIS OF PERISYNAPTIC EXTRACELLULAR MATRIX COMPONENTS IN THE BRAIN

PhD thesis

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List of Abbreviations

TIMP tissue inhibitors of metalloproteinases

ADAMTS A Disintegrin and Metalloproteinase with Thrombospondin motifs

CA Cornu Ammonis

CALEB Chicken acidic leucine-rich EGF-like domain containing brain protein

CSPG-5 chondroitin-sulphate proteoglycan-5

DAB-Ni diamino-benzidine-nickel

DIV days in vitro

DMEM Dulbecco's Modified Eagle Medium

DNA deoxyribonucleic acid

E 'x' embrional day x

EGF epidermal growth factor

GABA gamma-aminobutiryc acid

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GFAP glial fibrillary acidic protein

GFP green fluorescent protein

HRP horseradish peroxydase

IHC immunohistochemistry

MAP microtubule-associated protein

NGC neuroglycan C

P 'x' postnatal day x

PAS periodic acid Schiff

PB phosphate buffer

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

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TBS Tris-buffered saline

TH tyrosine-hydroxylase

tPA tissue plasminogen activator

VAChT vesicular acetyl-choline transporter

VGAT vesicular GABA transporter

VGLUT vesicular glutamate transporter

VMAT vesicular monoamine transporter

WB Western-blot

WFA Wisteria floridbunda agglutinin

1 Introduction

1.1 Extracellular matrix in the central nervous system

The term „extracellular matrix" is used to describe the network of proteins and complex carbohydrate molecules present in all tissues between the cells. The extracellular matrix does not passively fill the intercellular space: numerous studies have demonstrated in recent decades that it fulfils versatile tissue functions. These include, but are not limited to, the formation of a microenvironment essential for cellular function, its impact on signal transduction, which thereby shapes cellular functions under physiological (1) (such as cell migration, growth, formation of new connections) and pathological circumstances (such as inflammation (2), fibrosis (3), tumorigenesis (4)).

Whilst it had been widely accepted that most tissue harbour extracellular matrix to varying degrees, its presence in the central nervous system remained enigmatic and unrealistic for a long time. This view was completely overwritten during the last thirty years. At certain loci, the extracellular matrix accounts for even 20% of brain mass (5). This knowledge is also conceptually and functionally relevant: our previous presynapse-postsynapse-glia tripartite synapse model (6) was completed by the active participation of the perisynaptic matrix, which led researchers to introduce the concept of the tetrapartite synapse (7–10). The extracellular matrix of the central nervous system appears in different phenotypes: it may contribute to the diffuse neuropil, but also perineuronal nets or axonal coats surrounding the somatodendritic and synaptic domains of neurons, respectively (11–13).

In addition to its synaptic role, extracellular matrix appears at a second critical locus where molecules from the blood circulation reach the brain tissue. In the blood-brain barrier, highly specialized extracellular matrix elements accumulate to form a special neurovascular unit to safeguard tissue integrity. This unique structure shapes metabolism and signal transduction (14,15). Microglia, typical representatives of the brain immune system, also modulate synapse function by controlling extracellular matrix metabolism (16).

Structural integrity provides the backdrop for physiological processes in which the extracellular matrix of the central nervous system plays a pivotal role. Neurons notoriously need a stable microenvironment, as their signal transduction is fundamentally influenced by changes in ionic composition or presence of various metabolites. Because of their highly specialized function, they are particularly vulnerable to stress and pathological processes such as neurodegeneration or excitotoxicity, against which a well-organized matrix can provide protection (17–19). The

development of the nervous system is partly based on a predetermined genetic program, the execution of which, the migration paths of cells according to their fate, is also determined by matrix molecules (20). In higher organisms, individual variation, hence, the plasticity of the nervous system, is a key factor in adapting to the environment. The degradation or strengthening of individual synapses are also accompanied by parallel processes of matrix degradation and assembly. The regenerative capacities of the central nervous system are extremely limited, partly due to 'scars' containing matrix components, but growth factors released during matrix degradation do in some cases promote growth and cellular reassembly (21,22). Further, alteration of the extracellular matrix is a prerequisite for tumor proliferation (4). Actually, central nervous system tumors typically do not metastasize outside the nervous system because they have adapted to the special matrix of the nervous tissue and are unable to infiltrate tissues elsewhere in the body (23–25).

The composition of the extracellular matrix of the central nervous system is fundamentally different from the collagen-based matrix of other tissues. Both its typical biochemical composition and its phenotype are unique. Based on previous extensive descriptions (26–28) the following sections detail both aspects to demonstrate causality between structure and function.

In general, the extracellular matrix is composed of fibrous proteins (e.g. collagens, elastin) that scaffold the amorphous gel formed by non-fibrous components (29,30) and bind to extracellular matrix receptors of the cells. The extracellular matrix of the central nervous system robustly differs from any other tissue, since non-fibrous components (31,32), mostly glycoproteins, dominate its mass. Glycoproteins are classified according to their carbohydrate content and other biochemical properties (33,34). A characteristic group are proteoglycans, which are characterized by a high carbohydrate:protein ratio and the presence of glucose-aminoglycan side chains (35–37). Glucose-aminoglycans – like keratan sulphate, chondroitin sulphate, heparan sulphate or dermatan sulphate - are long, unbranched molecules composed of disaccharide units, the latter formed by an uronic acid and an amino sugar subunit. Another key component of the central nervous system matrix is hyaluronic acid, the "simplest" representative of the glucose aminoglycan family. Due to its length and large molecular weight (100 to 10 000 kDa) it forms a link between other elements (38–40).

The most characteristic representatives of the proteoglycans in the brain and spinal cord are called lecticans which include aggrecan, versican, neurocan and brevican (27). The latter two molecules are found only in the nervous system. In addition to lecticans, further proteoglycans,

like agrin, glypicans, syndecans, perlecan, decorin, biglycan, contribute to establish brain and spinal cord extracellular matrix. Non-proteoglycane glycoproteins include laminin, fibronectin, tenascins and thrombospondins.

The most important extracellular matrix components of the central nervous system are summarized in *Figure 1*.

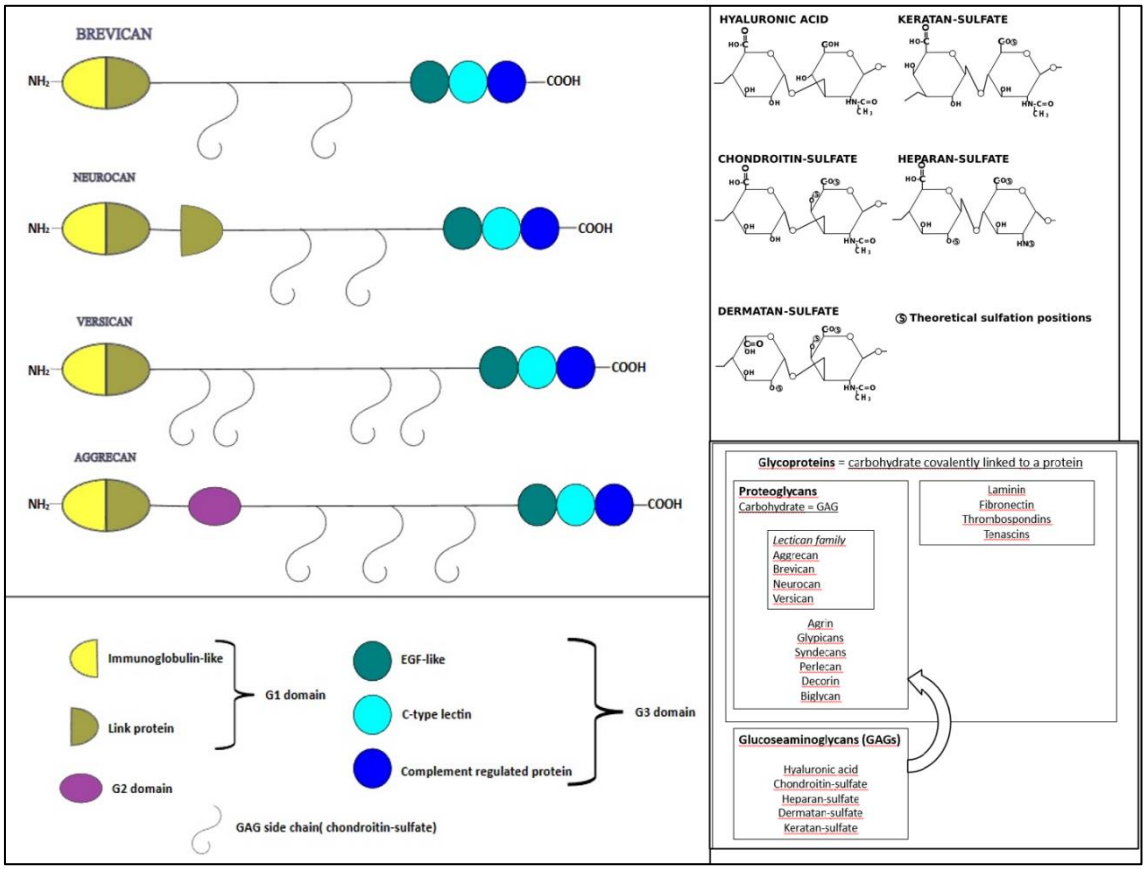

Figure 1.: Constituents of the extracellular matrix in the central nervous system. Glycoseaminoglycans are characteristic building blocks of proteoglycans (lecticans and non-lecticans). Non-proteoglycan glycoproteins include laminin, fibronectin, the tenascin and thrombospondin families.

The extracellular matrix is in permanent change, its remodeling and spatio-temporal variation in composition are mandatory to fulfil its function (41,42). Structural changes require the presence of efficient degradation mechanisms. In the central nervous system, several families of matrix-degrading enzymes are responsible for this delicate balance. The degradation process is continuous, essentially a slow turn-over, but select conditions like migration (20,43), inflammation (44–46), growth (21), cell division (47) or injury (48–50) can significantly accelerate this process. The most prominent family of demolishing enzymes are the zinc

endopeptidases called matrix metalloproteinases (51,52) which are regulated by members of metalloproteinases family (TIMP) (53). In addition, the tissue plasminogen activator (tPA) (54,55), the ADAMTS family (56) and the hyaluronidase/chondroitinase family (57,58) are also involved in matrix metabolism. Chondroitinase ABC, a bacterial enzyme used extensively *in vitro* and in animal studies (59–62), is not present in the nervous system of higher organisms.

The wealth of biochemical matrix components diffusely fills the intercellular space amongst the neuropil but may appear in condensed form around the somatodendritic compartment of certain neurons to form perineuronal nets. These structures account for about 2% of the extracellular matrix in the central nervous system (63). Perineuronal nets were first described by light microscopy in the 19th century (64) and their ultrastructure was studied several decades later (65). The first approach to unveil their composition happened in the 1960s when they were visualized by the periodic acid Schiff (PAS) reaction (65). Later, the development of biochemical methods significantly improved our knowledge about the exact composition of perineuronal nets (typically aggrecan - hyaluronic acid – tenascin (66–70)). A large body of evidence suggests that the extracellular matrix, including its special form, the perineuronal nets, profoundly impact neuronal function under both physiological (71) and pathological (72–76) conditions.

A further distinct type of matrix assemblies was named axonal coats (68,77) which appear at the preterminal axon segment and the terminal boutons themselves (78) to establish a local scaffold for individual synapses. Its biochemical composition is unique since it contains brevican, unlike the postsynaptic, aggrecan-rich matrix assembly (66,77,79). Of note, a biochemically similarly composed extracellular matrix accumulate around the axon initial segment and the nodes of Ranvier (42,80,81), which helps signal conduction (80).

This dissertation does not investigate or focus on the extracellular matrix of the blood-brain barrier/neurovascular unit, which contain significant amounts of specialized extracellular matrix components.

In the following chapters of my thesis, I will focus on the matrix component chondroitin sulphate proteoglycan-5 (CSPG-5) first from a biochemical and then from a functional point of view, and discuss its co-occurrence with other molecules and its role in phenotypization.

1.2 CSPG-5

CSPG-5 is also known in the literature as neuroglycan-C (NGC) and CALEB (Chicken acidic leucine-rich EGF-like domain containing brain protein). It was first described in 1995 by

Watanabe et al. (82), who recognized it as a molecule associated with developing neurons and exclusively found in the central nervous system. Over the past nearly three decades, there have been numerous publications on the structure and function of CSPG-5, the most relevant of which are briefly summarized below.

1.2.1 Genetic code

The gene encoding CSPG-5 has been identified in mice on the 9F1 chromosomal band by *in situ* hybridization. In humans, it is located on the short arm of chromosome 3 (3p21.3) (83,84). In mice, it was first discovered that 3 splice variants exist, of which NGC I is predominantly present. Its structure is in significant agreement with its rat and human analogues (83,84). In the following chapters of my work, given its predominance in occurrence, all statements will refer to splice variant I, although it is important to note that all three splice variants are expressed in both the developing and mature central nervous system (based on data obtained from experiments in mouse cerebellum (83)).

1.2.2 Molecular structure

In a similar way to the first description by Watanabe et al. (82), Aono et al. also describe the structure of CSPG- 5 as follows (83). It consists of 539 amino acids including a signal peptide, proceeding outward from the intracellular C-terminal, we find the transmembrane domain, followed by a cysteine-rich EGF domain, a domain containing amino acids of acidic character, a region for joining side chains, the signal peptide, and finally the N-terminal. Domain structure is schematically illustrated in *Figure 2*.

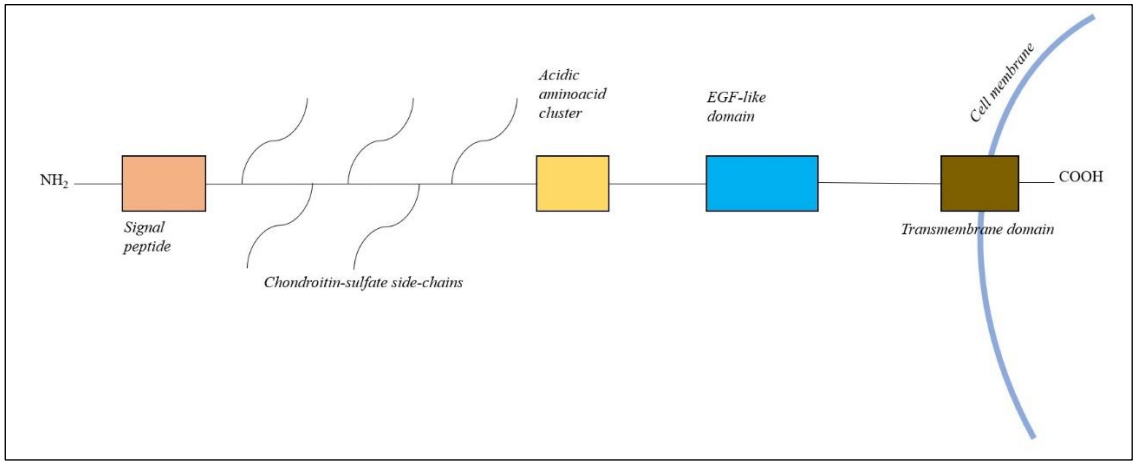

Figure 2.: Domain structure of CSPG-5. From C-terminus, outward: transmembrane domain, EGF-like domain, acidic amino acid rich domain, side-chain attachment site and a signal peptide.

1.2.3 Ectodomain shedding

Western-blot (WB) studies early after its discovery demonstrated that CSPG-5 was present in several different molecular weight forms. Not only applied chemicals (eg. chondroitinases) but age of the experimental animals also influenced the proportion of detectable heavier and lighter forms (83). Several authors have observed a 150 kDa molecule using Western blot, of which a band of 120 kDa mass can be obtained after applying chondroitinase ABC treatment (82–87) . This is the complete form of the protein. However, other authors besides Watanabe et al. report a 75 kDa band, which is considered to be the soluble form of the protein (82,86,88) . *In vivo*, the solubilized type is more abundant in young experimental animals (82,88). The process whereby the extracellular domains of a membrane-bound protein detach is called ectodomain shedding. *In vivo*, proteolytic enzymes with an affinity for the acidic amino acid domain of CSPG-5 may catalyze this process (82).

1.2.4 Part-time proteoglycan - definition and function

The term “part-time proteoglycan” refers to the phenomenon that the chondroitin sulphate side-chain content of some members of the proteoglycan family can vary over a wide range. They might even become completely depleted, drastically altering not only their structure but also their function. It is known from the work of Aono et al. and other authors that CSPG-5 can also be considered as a part-time proteoglycan (85,89).

During the development of the cerebellum and retina, CSPG-5 gradually loses its GAG side chains. This coincides with critical developmental milestones, specifically the closure of the "critical period." In this context, the critical period refers to the synaptogenesis of climbing fibers and mossy fibers in the cerebellum, as well as the synaptogenesis and dendritic branching of the inner layers of the retina, respectively (83,90).

1.2.5 Function

Previous descriptions (e.g., but not limited to the work of Watanabe (82), Aono (83,85), Nakanishi (87)) clearly define CSPG-5 as a molecule characteristic for the developing nervous system. CSPG-5 was originally described as being most typical for the early days of postnatal life, at least in the rat. Its expression has been observed to increase in the rat neocortex from the second half of embryonic life until day 20 of postnatal life, after which it decreases significantly and reaches levels typical for adult life. On that basis alone, it was logical to speculate that CSPG-5 mainly contributes to the development of the central nervous system and is of little importance after the closure of the critical period (82).

Based on the thorough studies of Aono et al. (85), we know that the density of the chondroitin sulfate side chains of CSPG-5 can alter its function. In general, as the nervous system ages, the average glycosylation of CSPG-5 increases, so we can hypothesize a correlation between maturation and the amount of side chains. Nevertheless, an opposite process is taking place in the cerebellum (83). In a mouse model, CSPG-5 is only present around Purkinje cells, and Western blot experiments confirmed that it is present in proteoglycan form during the initial phase of development. Presumably, its presence around the cell body and thick dendrites plays a role in determining the location of climbing fiber and parallel fiber synapses. After synaptogenesis, it is present in the mature cerebellum without side chains. In neuron culture, CSPG-5 concentrates around dendritic filopodia, from which neurites and postsynaptic spikes are formed later (90). This may also suggest a role in neuritogenesis and synaptogenesis. In particular, it is known from studies of Nakanishi et al. (87) that different subunits of the CSPG-5 molecule may have different physiological effects. *In vivo*, the ectodomain shedding mentioned above also results in fragments of different lengths and domain contents, with a possibility to exert spatially and temporally different effects. When the whole CSPG-5 ectodomain is added to neuronal cultures, longer neurites are formed per unit time than in control, mediated by the protein kinase C and phosphoinositide 3-kinase pathways.

Both the acidic aminoacid-containing domain and the EGF-like domain of CSPG-5 appear to promote neurite outgrowth, but while the former leads to the formation of a larger number of identical neurites, the latter promotes the outgrowth of one dominant neurite. This suggests that the two domains act through different pathways. It is possible that the acidic aminoacid domain promotes neurite outgrowth in cooperation with other extracellular matrix macromolecules, such as tenascins. Both fragments promote neurite outgrowth in excitatory neurons, but only the EGF-like domain fragment is able to influence GABA positive neurons (87). Based on the work of Nakanashi et al., it appears that the EGF-like domain does not act via EGF receptor-mediated signal transduction, but by other means.

Both soluble (shed) and membrane-bound forms of CSPG-5 are thought to contribute to the formation of neuronal connections (87,90). According to Nakanishi et al, NGC (CSPG-5) is concentrated on budding neurites and dendritic filopodia. CSPG-5 sheds its ectodomain from the filopodia, which acts on the growing axon and promotes its extension toward the filopodia. After the axon tip meets the filopodium, synaptogenesis occurs. Dendritic filopodia differentiate into postsynapses, while the axonal tip becomes the presynaptic compartment.

1.3 Calcium binding proteins, including secretagogin, phenotypization

Calcium binding proteins classify as calcium buffers – typically like parvalbumin – or as calcium sensors - like synaptotagmin, calmodulin or S100. The structure of calcium buffers does not change upon calcium binding, while calcium sensors undergo conformational changes that can regulate signaling cascades. Calcium-binding proteins have both an important physiological role through the regulation of calcium homeostasis (91) and provide a useful tool to characterize neuron populations (92). Whilst calcium binding proteins chelate excess calcium ions in the cell, calcium sensor proteins trigger signaling cascades upon calcium binding (91,93) Calcium binding proteins may exert dual effect though, to individually varying degrees. Thus, in the brain parvalbumin typically acts as a calcium binding protein, whereas calbindin and calretinin can fulfil both tasks, with calretinin functioning mainly as a calcium sensor. In addition to these three most abundantly occurring calcium binding proteins in the brain, we investigated secretagogin, a calcium sensor protein or coincidence detector (94).

Secretagogin was first described in the pancreas by Wagner et al. (95) as a 32 kDa intracellular protein with 6 EF-hand domains, whose genetic code in humans is localized to chromosome 6 (6p22.1-22.3). It was later detected in almost all organ systems, including the developing and adult nervous system (96) and the neuroendocrine system (95). Although its structure suggests that it could bind up to 6 calcium ions, it appears that in higher eukaryotes, calcium binding occurs at a ratio of 1:4 under physiological conditions (97). As mentioned above, secretagogin does not exhibit typical features of calcium buffers, but is rather a calcium sensor or coincidence-detector, and its role in vesicle exocytosis has been suggested (97,98).

As we have shown in our previous work with my colleague, Peter Zahola, secretagogin immunopositive brainstem regions are evolutionarily conserved in vertebrates (based on analyses of rat, mouse, chicken and human samples). Secretagogin turned out to be an excellent „molecular guide" through the entire ontogenesis given the fact that it appears early in embryonic development and persists into adulthood (99). . It also shows high functional conservativity throughout the phylogenesis.

Series of previous studies highlighted that – in addition to morphological and intracellular neurochemical descriptions – characterization of the pericellular environment provide useful information to understand cell function (100). Indeed, extracellular matrix can influence and maintain neuronal function in different ways. The most widely known example is that parvalbumin-positive GABAergic interneurons are characterized by fast-spiking, fast waveforms and are also often covered by Wisteria floribunda agglutinin (WFA)-labelled / PAS-

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positive perineuronal nets (101); presumably, the matrix envelope supports active metabolism required for dynamic function (102). Further, extracellular matrix assemblies can protect against oxidative stress and excitotoxicity (18,75). Nevertheless, the exact role of extracellular matrix around many neurons remain unexplored and need further research. In this work, I also investigate the interrelation between extracellular matrix and calcium sensor proteins in the brainstem.

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2 Objectives

My research aimed to answer the questions raised below.

2.1 CSPG-5

i. Does the CSPG-5-containing extracellular matrix persist in the brain during the postembryonic stage of development?
ii. What are the amounts and proportions of CSPG-5-containing extracellular matrix in different brain regions and what might be the significance of these?
iii. What is the morphological relationship between the CSPG-5-containing extracellular matrix, the synapse and the classical perineuronal net?
iv. In general, around which synapse types does the CSPG-5 containing extracellular matrix appear? What significance might this have for signal transduction?
v. Is CSPG-5 produced by neurons or glia cells?
vi. What role might CSPG-5 play in the human brain?

2.2 Phenotyping by extracellular matrix structure and calcium-binding protein content

i. Which secretagogin positive cell groups in the brainstem are covered by an extracellular matrix?
ii. Is it possible to assume that there is a correlation between the calcium-binding protein content of a given neuron and the composition of the surrounding extracellular matrix?
iii. Can the analysis of the composition of the surrounding matrix in other species and/or in other brain areas, represent an improvement in the categorization of neuron populations?

3 Materials and Methods

3.1 Animal experiments

A total of 30 male Wistar rats, 6 one-day-old, 3 three-day-old, 6 seven-day-old, 6 fourteen-day-old and 9 twelve-week-old animals were used. In addition, 20 rat embryos from 3 litters were used for *in vitro* experiments. Further, 3 male 12-weeks old mice and 3 14-day-old chicken (*Gallus domesticus*) were sacrificed and their brains processed for immunohistochemistry (IHC) studies as detailed below. Food and water were continuously available to the animals, they lived in a light-dark cycle changing every 12 hours in a room with a humidity of about 55%.

3.1.1 Perfusion and sectioning

During perfusion fixation the experimental animals were anaesthetized (Ketamine 50 mg/kg bw, Xylasine 4 mg/kg bw) and after opening their chest, perfusion fluid was transcardially introduced into the circulation. The perfusion fluid contained 4% (wt/vol) paraformaldehyde dissolved in 0.1 M phosphate buffer (PB; pH = 7.4). For samples destined for electron microscopy, the perfusion fluid also contained 0.5% glutaraldehyde. Fixed brains were removed from the skull, cryoprotected in 20% sucrose (in 0.1M PB) and processed for further histological analysis as described below. Transcardial perfusion was approved by the Ethical Review Boards of Semmelweis University and conformed to the 2010/63/EU European Communities Council Directive.

Sections of the rat and mouse brainstem (containing the microcellular tegmental nucleus, locus coeruleus, spinal trigeminal nucleus and dorsal nucleus of vagus nerve) and forebrain (containing the hippocampus and the primary somatosensory cortex) were prepared. Fixed and cryoprotected brains were sectioned on a cryostat in the coronal plane thick coronal sections (30 μm) for multiple immunolabelling. Samples for electron microscopy analysis were first sectioned with a vibratome at 50 μm thickness, immunolabelled, postfixed, contrasted and buffered in 1% osmium tetroxide at 22-24 °C for 1 hour and flat-embedded in Durcupan ACM (Fluka). The primary somatosensory cortex was identified using light microscope and excised then re-embedded for ultrasectioning at 100 nm thickness. Sections were collected on single-slot nickel grids coated with Formvar.

3.1.2 Non-fixed brains

The dams were overdosed and decapitated using a guillotine, and the embryos were subsequently removed by opening the abdominal wall and uterus. Embryos were immediately decapitated and their heads were snap-frozen (qPCR analysis) or alternatively transferred into ice-cold medium (DMEM - primary cortical culture experiments).

3.2 Human samples

With the help of the Human Brain Tissue Bank and Laboratory of Semmelweis University, I used brain tissue samples isolated by micropunch (103). Anterior and posterior cingulate cortices and the entorhinal cortex were included in the analysis. Our samples derived from the brains of 7 individuals who died by suicide and from 7 age-matched otherwise healthy controls. Sample data are available in *Table 1*.

Table 1: human samples (cause of death, age at the time of death, sex and post-mortem delay data for each specimen)

Identification number	Cause of death/ status	Age (years)	Sex	Post-mortem delay(hours)
#205	other/ control	66	male	4,5
#215	other/ control	44	female	5
#216	other/ control	53	male	5
#220	other/ control	63	male	3,5
#223	other/ control	61	male	5
#156	other/ control	47	male	1
#228	other/ control	27	male	8
#105	suicide	42	female	3
#139	suicide	79	male	4
#175	suicide	49	female	6
#176	suicide	35	male	2
#234	suicide	31	male	7
#066	suicide	58	male	4
#138	suicide	52	male	3

The average weight of samples was 266 mg. In the control population, the mean age was 51.7 years, and the mean post-mortem delay (time from death to removal and freezing of the sample) was 4.5 hours. Mean age in the suicide population was 49.4 years and with a postmortem delay of 4.1 hours.

Tissues were obtained and used in compliance with the Declaration of Helsinki and following institutional guidelines (Regional and Institutional Committee of Science and Research Ethics of Semmelweis University (TUKEB 84/2014)).

3.3 Cell cultures

3.3.1 Primary cortical neuron and glia cultures

Primary neuronal and glial cultures were prepared from the neocortex of embryonic day 20 (E20) rat embryos. After isolation of neocortex, cells were enzymatically dissociated and plated at a density of 200,000 cells/well in 6-well plates. Neurons were maintained in a 1:1 mixture of DMEM:F12 supplemented with B27 (2% vol/vol), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml) (all products of Invitrogen). Astrocytes were grown in DMEM based plus 10% foetal bovine serum, L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100 ug/ml). Neurons were alternatively treated with glial-conditioned medium from the fourth day after being plated. This means that half of their daily changed culture medium was the used medium taken from the astrocyte culture. Experiments were carried out in triplicates.

3.3.2 SH-SY5Y cell line

SH-SY5Y is a human neuroblastoma cell line. The cells were maintained in DMEM-GlutaMax medium containing 10% foetal bovine serum, to which penicillin (100 U/ml) and streptomycin (100 ug/ml) were added. All chemicals required for the solution were Invitrogen products. Cells were plated on poly-D-lysine covered coverslips at a density of 150,000 cells/well. From day 2, cells were differentiated for 4 days in DMEM:F12 based medium with GlutaMax supplement containing 1% foetal bovine serum, and antibiotics (as described above) also containing 10 uM retinoic acid (Sigma R2625). At the end of the process, the cells were transfected with 0.5 ug/ 500 ul mGFP-tagged rat-CSPG-5 plasmid DNA (Origene CAT#: RR200364L2 Lenti ORF clone of CSPG-5 mGFP-tagged ORF-rat chondroitin sulfate proteoglycan 5, transcript variant 1 (NM_019284)) using jetPRIME transfection reagent (CAT#114-07 Polyplus

transfection) according to the instructions of the manufacturer. After additional 3 days, coverslips were immunostained (104) and inspected using Zeiss LSM 780, as described below.

3.4 Immunohistochemistry

Immunohistochemistry was carried out according to previously published protocols (69,99,105–108). Using select combinations of antibodies, chromogenic or multiple immunofluorescence histochemistry was performed. The list of antibodies used is detailed in *Table 2*. Chromogenic labeling was used for samples destined for electron microscopy. Solutions for washing and antibody dilution were similar for multiple immunofluorescence and chromogenic labeling, except that we omitted Triton X-100 for the latter. Free-floating sections were rinsed in 0.1M PB and, where appropriate, pre-treated with 0.3% Triton X-100 for 1 h at room temperature to enhance antibody penetration (69,104,106,109–115). We suppressed non-specific reactivity by incubating our sections in a solution of 5% normal donkey serum (Jackson), 2% bovine serum albumin (Sigma) and 0.3% Triton X-100 for 1 hour at room temperature. Sections were treated with the primary antibodies detailed in *Table 2* in refrigerator (4 °C) for 16-72 hours. Primary antibody solutions were diluted in 0.1M PB which also contained 0.1% normal donkey serum and 0.3% Triton X-100. After extensive rinsing in 0.1M PB, sections were processed by using immunofluorescence or chromogenic detection. In single-labeling experiments for electron microscopic investigations, specimens were exposed to biotinylated anti-rabbit IgG raised in donkey (1:1000 (Jackson), at room temperature, for 2 hours) followed by pre-formed avidin-biotin complex also incorporating horseradish peroxidase for 1 hour at room temperature. Immunosignals were visualized by 3,3'-diaminobenzidine (0,025%, Sigma) as chromogenic agent, intensified by nickel-ammonium sulfate (0,05%, Merck) in the presence of 0,001% hydrogen peroxide as substrate (dissolved in 0,05M Tris buffer, pH 8,0). In multiple immunofluorescence labelling experiments, immunoreactivities were revealed by carbocyanine (Cy)2, 3 or 5-tagged secondary antibodies raised in donkey (1:200, Jackson, at room temperature, for 2 h). Glass-mounted sections were coverslipped with glycerol/gelatin (Sigma).

Table 2: List of primary antibodies used for immunolabelling (targeted molecule, source of antibody, host, clonality, applied dilution in immunhistochemistry and Western-blot studies, reference). Abbreviations: GAPDH - glyceraldehyde-3-phosphate dehydrogenase, MAP2 – microtubule-associated protein, TH – thyrosine-hydroxylase, VGLUT - vesicular glutamate transporter, VAChT - vesicular acetyl-choline transporter, VGAT - vesicular GABA transporter, VMAT- vesicular monoamine transporter

Target	Source	Host	Mono/polyclonal	Dilution used for IHC	Dilution used for WB	Reference
beta-actin	Sigma	mouse	monoclonal	n.a.	1:10 000	(61)
CSPG-5	kind gift from the Human Atlas Project (HPA071779)	rabbit	polyclonal	1:2000	1:5000	(33)
GAPDH	Abcam	mouse	monoclonal	n.a.	1:10 000	(86)
MAP2	Sigma	mouse	monoclonal	1:200	n.a.	(61)
Synaptophysin	Synaptic Systems	rabbit	polyclonal	1:1000	1:5000	(86)
TH	MERCK/Millipore	mouse	polyclonal	1:1000	n.a.	(89)
VGLUT-1	Synaptic Systems	guinea pig	polyclonal	1:2000	n.a.	(88)
VAChT	Synaptic Systems	guinea pig	polyclonal	1:2000	n.a.	(87)
VGAT	Synaptic Systems	mouse	monoclonal	1:1000	n.a.	(85)
VMAT-1	MERCK/Millipore	mouse	monoclonal	1:1000	n.a.	(33)
secretagogin	R&D Systems	goat	polyclonal	1:100	n.a.	(113)
WFA	Sigma	lectin	lectin	25 µg/ml	n.a.	(61)

3.5 Microscopy

3.5.1 Confocal laser scanning microscopy

A 780LSM confocal laser scanning microscope (Zeiss) was used to investigate samples with multiple immunofluorescence labeling. The optical zoom ranged from 1x to 3x at

63x primary magnification (Plan-Apochromat 63x/1,40), and pinhole setting limited signal detection to 0,5-0,7 μm optical thickness. Emission spectra for each dye were limited as follows: Cy5 (650-720 nm), Cy3 (560-610 nm) and Cy2 (505-530 nm).

3.5.2 Electron microscopy

Sections processed for electron microscopy were inspected and images acquired on a Jeol 1200 EMX electron microscope. Primary magnification ranged from 20,000x to 80,000x.

3.6 Protein and RNA analysis

3.6.1 Western-blotting

Protein samples from human subjects as well as from neocortical cultures were homogenized in TNE buffer containing 0.5% Triton X-100 (Sigma), 1% octyl-β-D-glucopyranoside (Calbiochem), 5mM NaF, 100 μM Na_3VO_4 and a combination of protease inhibitors (CompleteTM, Roche) by using a tissue grinder. Cell debris and nuclei were pelleted by centrifugation (800 g, 30 min at 4 °C). Protein concentrations were determined by the Bradford's colorimetric method (116). Samples were diluted to a final protein concentration of 2 μg/μl and denatured in 5× Laemmli buffer. Human samples, synaptosomal samples and primary culture samples were then analyzed by SDS-PAGE on 8% or 10% resolving gels. Following transfer onto Immobilon-FL polyvinylidene difluoride membranes (Millipore), membrane-bound protein samples were blocked in 3% bovine serum albumine and 0.5% Tween-20 diluted in TBS for 1.5 h, and finally exposed to primary antibodies overnight at 4 °C. Appropriate combinations of HRP-conjugated secondary antibodies were used for signal detection (Jackson; from goat, rabbit or mouse hosts; 1:10,000, 1 h; see: *Table 2*). We considered an approximately 170 kDa (85) and approximately 110 kDa (86) heavy band in our analysis. Blots were scanned on a Bio-Rad XRS^+ imaging system and subsequently quantified with the Image Lab 3.01 software (Bio-Rad Laboratories). For loading control β-actin (1:10.000; Sigma) or GAPDH (1:10,000; Abcam) were used. For antibody validation we processed rat brain, liver and kidney tissues from 12 week old rats (n=2) as well as human brain tissue. Tissues were processed and samples analyzed similarly to human brain samples as described.

3.6.2 Preparation of synaptosomal fractions

Adult rat neocortices (n = 2) were homogenized in 0.32 M sucrose-containing HEPES buffer (composition in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 5 HEPES.

pH 7.4). The homogenate was centrifuged (10 min, 600g at 4-8 °C) and the supernatant repeatedly cleared (by centrifugation at 20 000g, 4°C for 30 min in concentrated, 1,3 M sucrose-containing HEPES buffer) (117). The pellet was then resuspended in 100 µl protease inhibitor-containing TNE-based homogenizing buffer (described in section *Western blotting*).

3.6.3 qPCR

P1, P3, P7 and P14 (postnatal day 1, 3, 7 and 14) rat brains (n = 3 each) were dissected on ice to isolate the medial prefrontal cortex, primary somatosensory cortex, cerebellum and hippocampus. Samples were snap frozen in liquid nitrogen and stored in -75 °C until processing. RNA was extracted using the RNeasy Mini Kit (Qiagen) with a DNase I step performed to eliminate traces of genomic DNA and were reverse transcribed using a high capacity cDNA reverse transcription kit (Bio-Rad), all in accordance to manufacturers' instructions. RNA from primary neuronal and glial cultures was collected and reverse transcribed using the same reagents. Reactions were performed after an initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, denaturation, annealing, and extension at calculated temperatures (60 s), and a dissociation step from 60 to 95 °C with 0.5 °C steps for 10 s each (CFX96; Bio-Rad) with primer pairs amplifying short fragments for each gene. Samples without reverse transcriptase or template served as negative controls. Expression levels obtained for each sample in parallel assays were normalized to the housekeeping gene encoding GAPDH. All experiments were carried out in triplicates. Gene stability was analyzed on preformed Droplet Digital PCR (ddPCR™) plates (Bio-Rad) and by using the CFX96 Maestro software.

3.7 Quantitative analysis

3.7.1 In Western-blotting experiments

Chemoluminescence of individual samples were compared. Data were analyzed using the Statistica Software Package version 13.2 (StatSoft, Dell). Pairwise comparisons on independent samples (in WB experiments) were carried out by applying Students' t-test. Data were expressed as mean +/- standard error of mean. Statistical significance was defined as $p < 0.05$.

3.7.2 Estimation of the CSPG-5-containing matrix prevalence around different synapse types

Immunostained samples from the dorsal hippocampus were used, 15 for each staining type. Fives sections containing the dorsal hippocampus from six-series cryosection files were taken from 3 rats each. Images were taken from the pyramidal layer of the CA2 region using the 63x oil-immersion lens (Plan-Apochromat 63x/140, as described above) for magnification with a pinhole setting of 0.7 mm optical thickness. VGLUT-1, VGAT, TH, VMAT1 or VAChT immunoreactive profiles larger than 500 nm in their smallest diameter were counted manually in 900-1100 μm^2 sized regions of interest using the ZEN software. The region of interest was positioned into the center of the image, altogether 15 regions of interest per staining were taken for analysis. The proportions of CSPG-5 bearing VGLUT-1-, VGAT-, TH-, VMAT-1- or VAChT- immunoreactive profiles were calculated on each section, their average calculated in each animal. The method for calculating their proportions was (number of CSPG-5 bearing VGLUT-1, VGAT, TH, VMAT-1 or VAChT immunoreactive profiles) / (all VGLUT-1, VGAT, TH, VMAT-1 or VAChT immunoreactive profiles), for example in case of VGLUT-1, (number of CSPG-5 bearing VGLUT-1 immunoreactive profiles) / (number of VGLUT-1 immunoreactive profiles in total). The final value was presented as the average +/- standard error of the mean of the three average values of the individual animals.

3.7.3 Gene stability analysis in qPCR studies

The CFX96 Maestro software was used for analysis. The CFX Maestro software is based on the GeNorm algorithm, details of which are described in Vandesompele's study (118). Briefly, the ratio of a pair of reference genes and all pairwise variations are calculated across all samples. The M value is the arithmetic mean of all pairwise variations. The lower the M value, the more stable the reference gene, where stability is defined as (Ln(1/Avg M)).

4 Results

4.1 CSPG-5 accumulated in perineuronal nets around pyramidal cells of the hippocampus during postnatal development of rats

CSPG-5, similarly to other extracellular matrix components diffusely fills the brain extracellular matrix, and only a minority of its quantity forms compact assemblies around cells (63). We examined the hippocampal formation of young rats of different postnatal ages to assess whether CSPG-5^+ matrix forms any distinguishable matrix assemblies during the first two weeks of life (Figure 3., a-c_1). In the CA1 field, CSPG-5 containing matrix appeared as a diffuse network without any well definable phenotype at postnatal day 1 (P1, Figure 3., a-a_1). On day 3 (P3) however it started to faintly outline somata of pyramidal cells (Figure 3., b-b_1). By postnatal day 14 (P14, Figure 3., c-c_1), CSPG-5^+ extracellular matrix outlined cell bodies in the stratum pyramidale clearly and had a phenotypic appearance of perineuronal nets.

4.2 CSPG-5 expression shows temporal and regional differences during postnatal development in rats

We investigated the expression of CSPG-5 at the transcriptional level at postnatal days 1, 3, 7 and 14 in different brain regions (Figure 3., d). This allowed us to explore the temporal dynamic of this extracellular matrix components' expression across different brain areas. In the medial prefrontal cortex, CSPG-5 expression showed a gradual, approximately three-fold increase from P1 to P14. On the other hand, expression levels in the primary somatosensory cortex showed a transient initial increase during the first week of life, followed by a marked decline after P7. In the cerebellum, CSPG-5 mRNA levels increased to over fourfold until P14 compered to P1 with a marked peak at P3. Finally, in the hippocampus, we observed a significant, ten-fold increase during the first week and this expression plateaued throughout the second postnatal week.

To connect our observations across different areas, we also performed an experiment to compare CSPG-5 mRNA levels among the aforementioned brain regions at P14 (Figure 3, e). We found lower expression levels in the somatosensory cortex and cerebellum compared to the medial prefrontal cortex and hippocampus.

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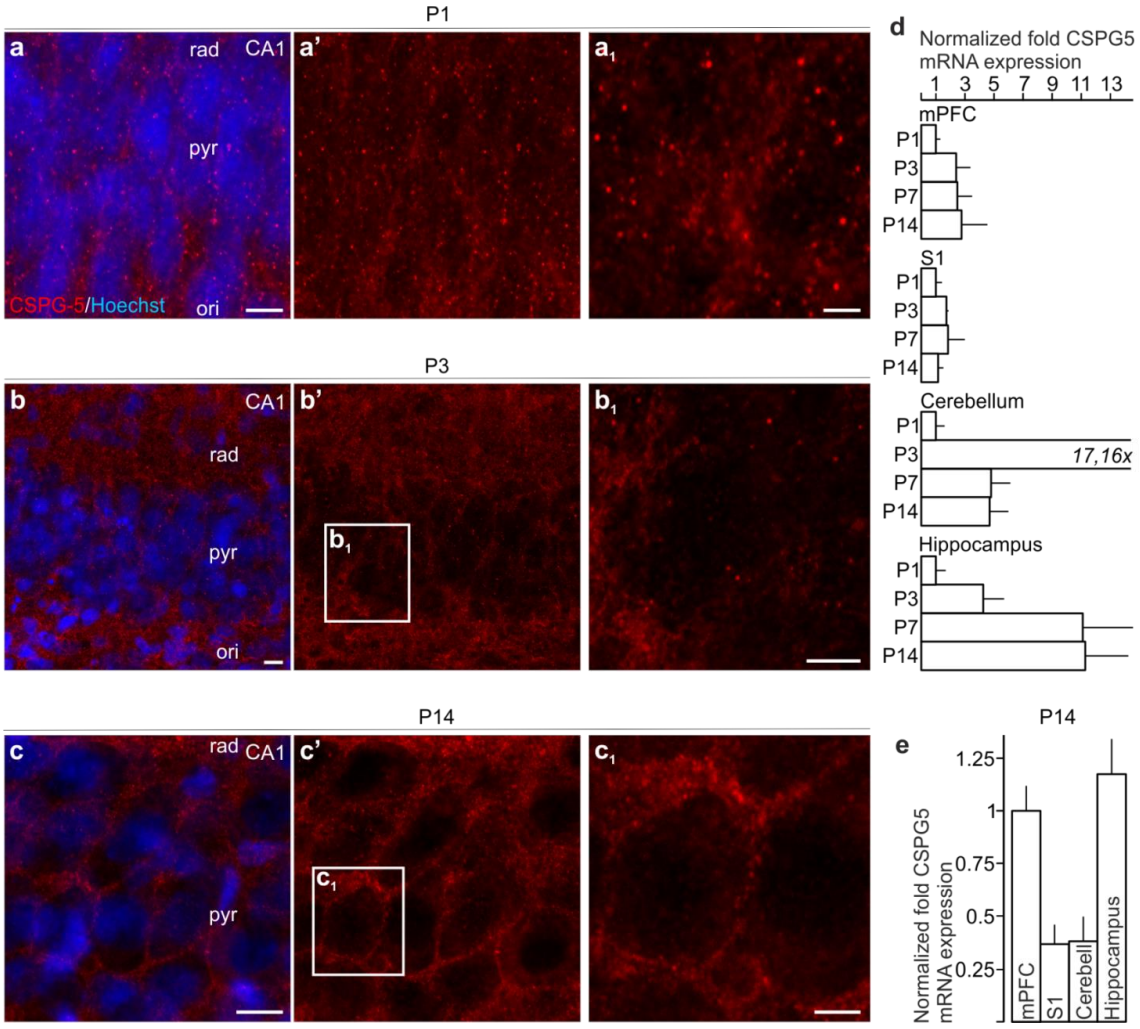

Figure 3.: During the first two weeks of postnatal development of the rat, CSPG-5 gradually accumulated in the extracellular matrix sheath of young neurons. (a-a$_1$) At postnatal day (P1), diffuse CSPG-5 immunopositivity appeared in the Cornu Ammonis 1 (CA1) region of the hippocampus. (b-b$_1$) At P3 CSPG-5$^+$ matrix started to outline pyramidal cell bodies in CA1. (c-c$_1$) By P14, CSPG-5 containing matrix developed into a mature perineuronal phenotype. (d) Medial prefrontal cortex (mPFC), primary somatosensory cortex (S1), cerebellum and hippocampus normalized fold CSPG-5 mRNA expressions on postnatal day 1, 3, 7 and 14. e) Normalized fold CSPG-5 mRNA expression showed significant difference in various cortical regions. .Scale bars 10µm (a, b, c), 5µm (a$_1$,b$_1$,c$_1$). Figure 3 was published in Cellular Signalling (https://doi.org/10.1016/j.cellsig.2020.109710).

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4.3 CSPG-5 formed ring-shaped assemblies at the border of „classical" perineuronal nets

Observing ring-like accumulations of CSPG-5$^+$ matrix around pyramidal cells that resembled mature perineuronal net phenotype in the developing rat led us to carry out further research in order to determine the relationship between CSPG-5$^+$ matrix and „classical" perineuronal nets as well as pyramidal cells in the adult rat brain. We chose to examine the hippocampus (Figure 4) and the primary somatosensory cortex (Figure 5). We defined „classical" perineuronal net as a WFA-reactive matrix accumulation that has been known to ensheath neo- and allocortical neurons in a typical distribution pattern (119,120). WFA is a lectin, commonly used in histological studies, that typically labels a distal N-acetylgalactoseamine of the chondroitin-sulfate side-chains (64).

According to our findings, both the hippocampal stratum pyramidale and the somatosensory cortex harbored a specific CSPG-5$^+$ matrix phenotype. It surrounded neurons (Figure 5 a_1', a_2', b_1'), but accumulated in the peripheral domain of WFA$^+$ perineuronal nets (best visualized in Figure 4 b_1 and Figure 5 a_2''', b_1'''). It appeared adjacent to the inner layer of perineuronal nets and formed tiny ring-like structures (Figure 4 b_1, Figure a_2-a_2''',b_1'''). We typically detected this second coat around the somata of the neurons with faintly labelled perineuronal nets (Figure 5 a_1-a_1'').

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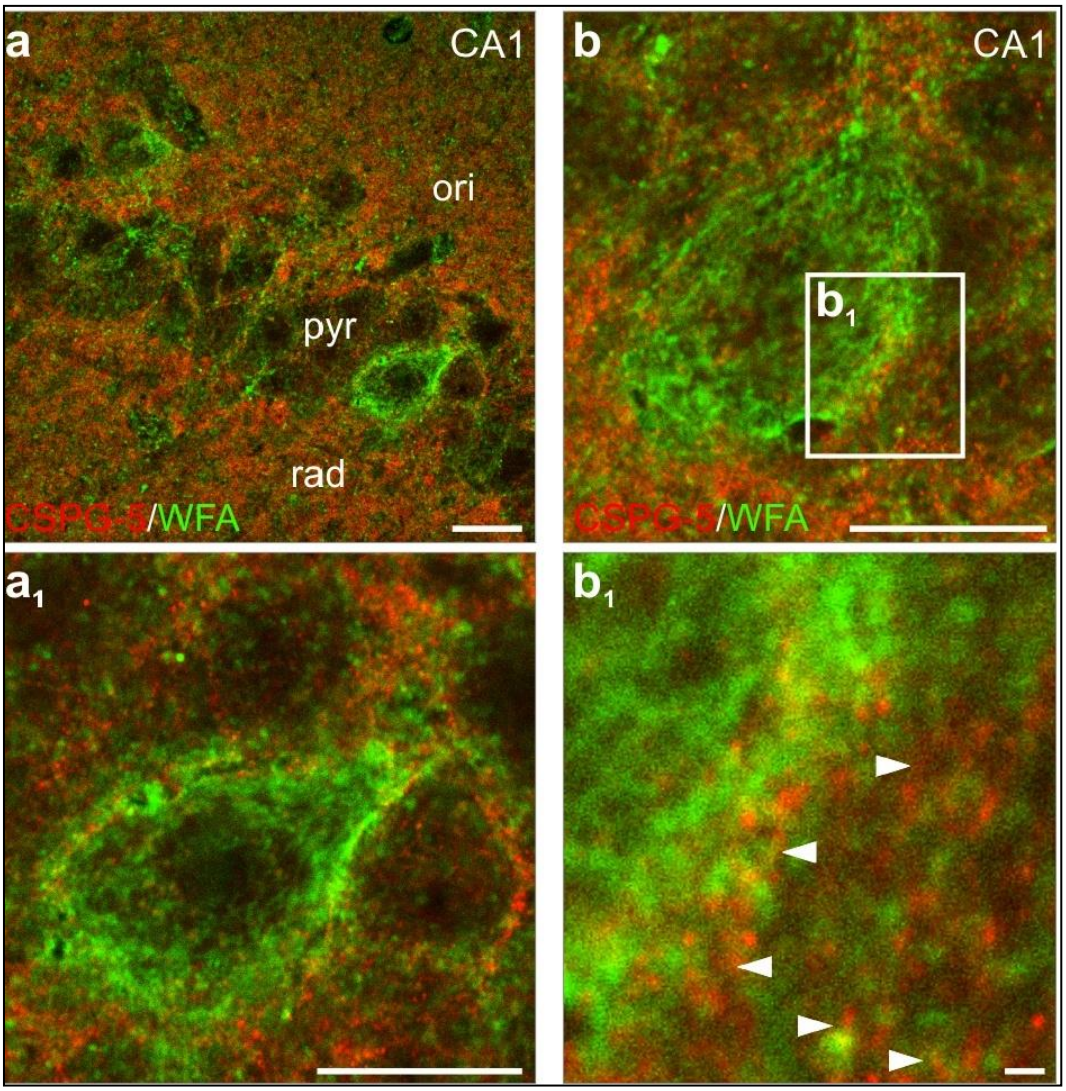

Figure 4.: (a) In the hippocampus of the adult rat, CSPG-5 was present as part of the diffuse neuropil in strata oriens and radiatum. (b, a_1) CSPG-5^+ matrix was more organized around pyramidal cell bodies. (b_1) Here, it appeared as tiny matrix assemblies (indicated by arrowheads) in the peripheral domain of WFA^+ perineuronal nets. Scale bars 10μm (a, a_1, b), 3μm (b_1). Figure 4 was published in Cellular Signalling as part of a separate figure. (https://doi.org/10.1016/j.cellsig.2020.109710)

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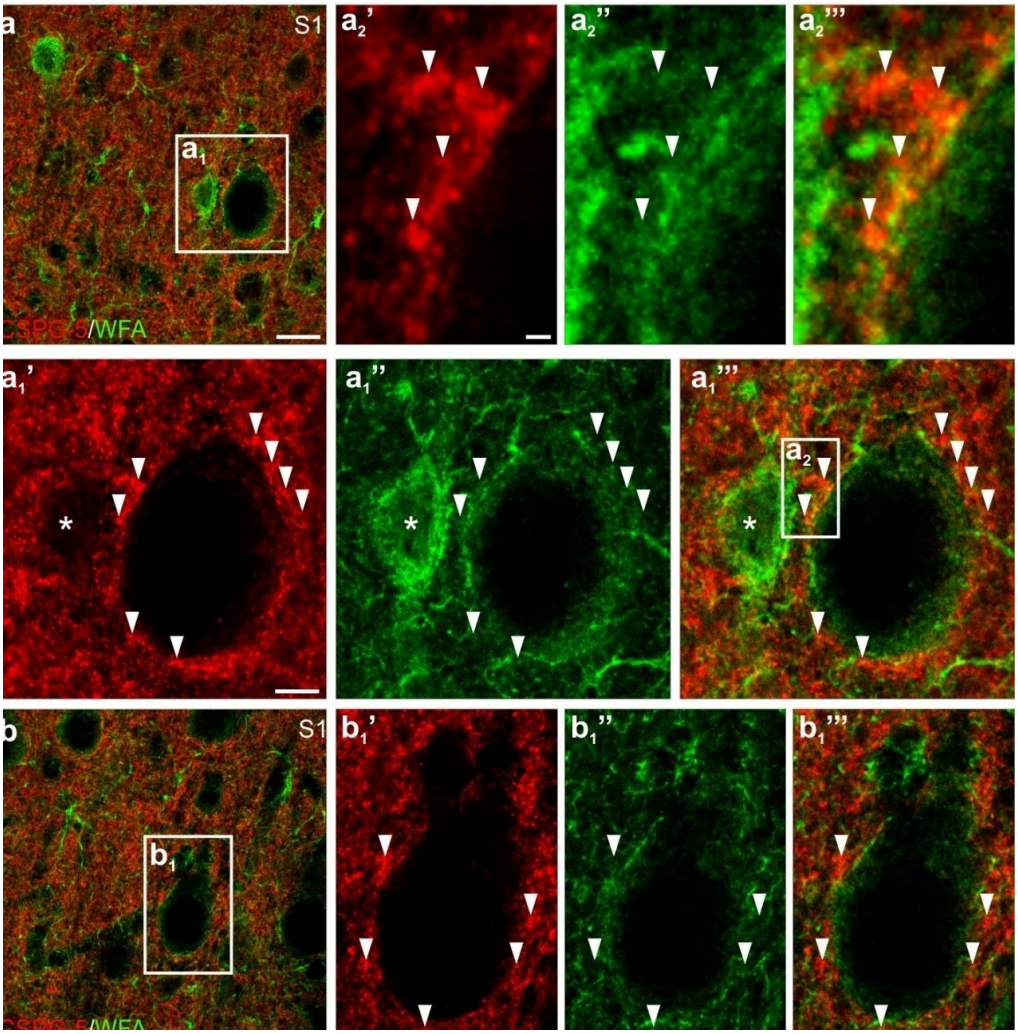

Figure 5.: Primary somatosensory cortex of the adult rat. (a-a1, b) CSPG-5+ matrix assemblies appeared around pyramidal cell bodies. (a2, b1) They were outside but seemingly adjacent to WFA+ „classical" perineuronal nets (relationship indicated by arrowheads), very similarly to the arrangement observed in the hippocampus. . Asterisk in a1'-a1''' denotes a soma surrounded by a thick WFA+ perineuronal net, which lacks CSPG-5+ profiles in its surrounding. Figure 5 was published in Cellular Signalling as part of a separate figure (https://doi.org/10.1016/j.cellsig.2020.109710).

4.4 CSPG-5 as part of the synaptic compartment

Since CSPG-5+ profiles seemed to form small, ring-shaped matrix assemblies, it was tempting to speculate that they were closely related to synapses. Therefore, we investigated the interrelation of CSPG-5+ extracellular matrix assemblies to the synaptic compartment. To assess this relation at the protein-level, we prepared synaptosomal

fraction from neocortices of adult rats. By Western-blot analysis we indentified a 170 and a 110 kDa molecular weight band which typically resembled the 150 and 120 kDa bands described previously (for details, please refer to section *Ectodomain shedding*). We found that CSPG-5 was present in cortical synaptosomes (4.74.7).

Continuing our experiment, we examined CSPG-5$^+$ matrix at the ultrastructural level (Figure 6, c-e). CSPG-5 enwrapped synaptic sites, expanding both into the pre- and postsynaptic compartments.

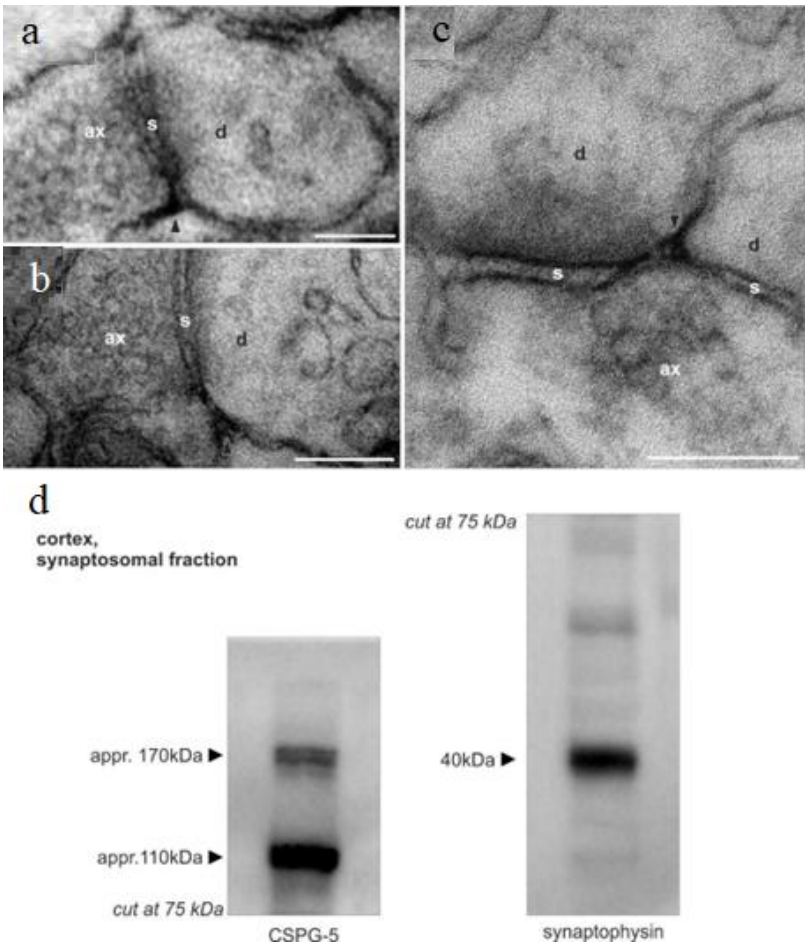

Figure 6.: CSPG-5 was associated to synapses. (a-c) Electron micrographs prove the presence of CSPG-5$^+$ matrix (indicated by arrowheads) both in the presynaptic and in the postsynaptic compartment. (d) Western-blotting showed rat neocortical synaptosomal fraction to be CSPG-5 immunoreactive. Abbreviations: ax-axon, d-dendrite, s-synapse. Scale bars: 100 nm (a-b), 250 nm (c). Figure 6 was published in Cellular Signalling (https://doi.org/10.1016/j.cellsig.2020.109710)

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4.5 Specific axon terminals bear CSPG-5$^+$ matrix in the rat allocortex

Next, we investigated if CSPG-5$^+$ matrix specifically associated to select synaptic types. Again, we used the adult rat hippocampal formation for immunohistochemical investigations. Allocortical VGLUT-1$^+$ mossy fiber terminals in the CA3 region were densely surrounded by CSPG-5$^+$ profiles (Figure 7 a-a$_2$'''). However, CSPG-5$^+$ assemblies were not restricted to excitatory synapses: in stratum pyramidale of CA2, both VGLUT-1$^+$ excitatory (Figure 7 b, b') and VGAT$^+$ inhibitory (Figure 7 c, c') axon terminals were surrounded by similar extracellular matrix accumulations.

On the other hand, cholinergic terminals (labelled by VAChT) showed little to no association to CSPG-5$^+$ extracellular matrix (Figure 7 d-d$_1$'''). Similarly, mono-aminergic terminals (identified by their TH-immunoreactivity) remained naked from (Figure 7, e$_2$'-e$_2$'''), or poorly covered by CSPG-5$^+$ perisynaptic matrix (Figure 7 e$_1$',e$_1$''', f, f').

The ratio of matrix-surrounded immunoreactive boutons in stratum pyramidale of the CA2 region was estimated and we found that 88.9% ± 4.7% (mean ± s.e.m.) of the VGLUT1$^+$ terminals, 78.6% ± 4.8% of theVGAT$^+$ terminals, 35.4% ± 4.2% of the TH$^+$ terminals and 24.5% ± 2.2% of the VAChT$^+$ terminals were surrounded by CSPG-5$^+$ extracellular matrix. In addition, considering that TH-immunoreactivity is not fully specific to axon terminals, we specifically labelled mono-aminergic terminals by using the anti-vesicular monoamine transporter antibody VMAT1 (Figure 7, g-g") and approximated that 30.2% ± 5.7% of them were associated to CSPG-5$^+$ extracellular matrix.

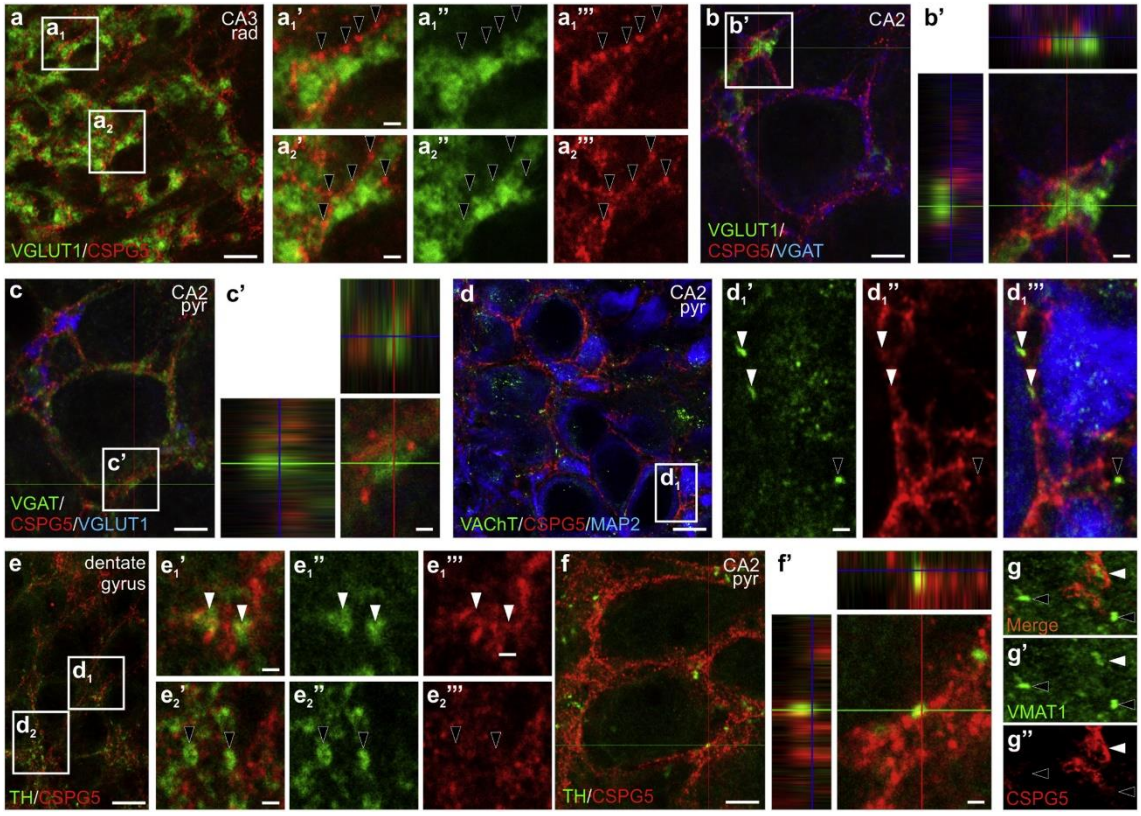

Figure 7.: Terminals of specific afferents are enwrapped by CSPG-5$^+$ extracellular matrix. (a) VGLUT1$^+$ mossy fiber terminals in the stratum radiatum of the CA3 hippocampal region are surrounded by CSPG-5$^+$ extracellular matrix. (b, b' and c, c') Orthogonal view of VGLUT1$^+$ and VGAT$^+$ profiles, respectively, surrounded by CSPG-5$^+$ matrix. (d-d1'', e-f', g-g'') In turn, VAChT$^+$, TH$^+$ and VMAT1$^+$ terminals were associated to no (black arrowheads) or only weakly labelled (white arrowheads) CSPG-5$^+$ extracellular matrix assembly. Abbreviations CSPG-5 chondroitin sulfate proteoglycan type 5, MAP2 microtubule-associated protein 2, TH tyrosine hydroxylase, VAChT vesicular acetylcholine transporter, VGAT vesicular GABA transporter, VGLUT1 vesicular glutamate transporter type 1, VMAT1 vesicular monoamine transporter type 1. Scale bars 10 µm (e), 5 µm (d), 3 µm (a, b, c, f), 1 µm (a2', b', d1', e2', f'). Figure 7 was published in Cellular Signalling (https://doi.org/10.1016/j.cellsig.2020.109710)

4.6 Neuronal CSPG-5 production

If CSPG-5 is an integral part of the synaptic compartment, it is highly likely that either neurons or glial cells (or both) contribute to its production. To investigate the production of CSPG-5 *in vitro*, we took advantage of primary cortical cultures from embryonal rat

neocortices (day 20 of *in utero* development – E20). Our experiments showed that neurons expressed CSPG-5 by 4 days *in vitro* (DIV) already and expression levels increased markedly by 10 DIV (Figure 8 a-a'). At the same time, we could detect only minimal CSPG-5 production in glial cultures. Further, adding glial-conditioned medium to primary neuronal cultures virtually attenuated CSPG-5 expression.

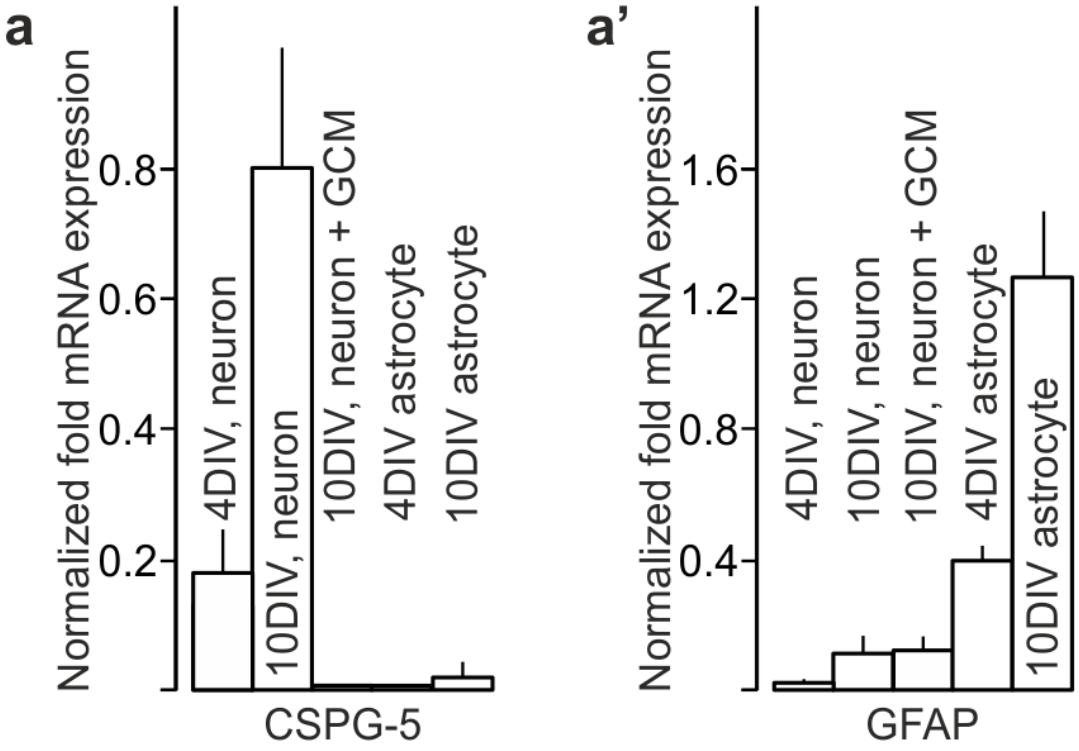

Figure 8.: Neurons produced CSPG-5. Glial conditioned medium inhibited CSPG-5 expression. (a, a') CSPG-5 and glial fibrillary acidic protein (GFAP) mRNA expression levels in primary cortical cultures produced in embryonal rat neocortices (on embryonic day 20 – E20). Neuronal, but less so glial cultures expressed CSPG-5. Glial conditioned medium transferred into the medium of neuronal cultures reduced CSPG-5 expression. Figure 8 was published in Cellular Signalling as part of a separate figure (https://doi.org/10.1016/j.cellsig.2020.109710).

4.7 CSPG-5^{+} matrix appeared around predilection points for synapse formation

After proving that CSPG-5 is produced by neurons we turned our attention to a human neuroblastoma cell line, SH-SY5Y, to further assess neuronal production of CSPG-5. We induced green fluorescent protein (GFP) tagged rat CSPG-5 plasmid DNA expression in

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plated neuroblastoma cells. On day 3 after transfection, GFP-labelled matrix showed a typical phenotypic appearance: it concentrated around the cell bodies (Figure 9, b, b_1) and neural endings (Figure 9, d-d_1). Furthermore, CSPG-5 typically assembled around neurite contact points (Figure 9 c-d_1'''). CSPG-5^+ did not appear around neurite shafts.

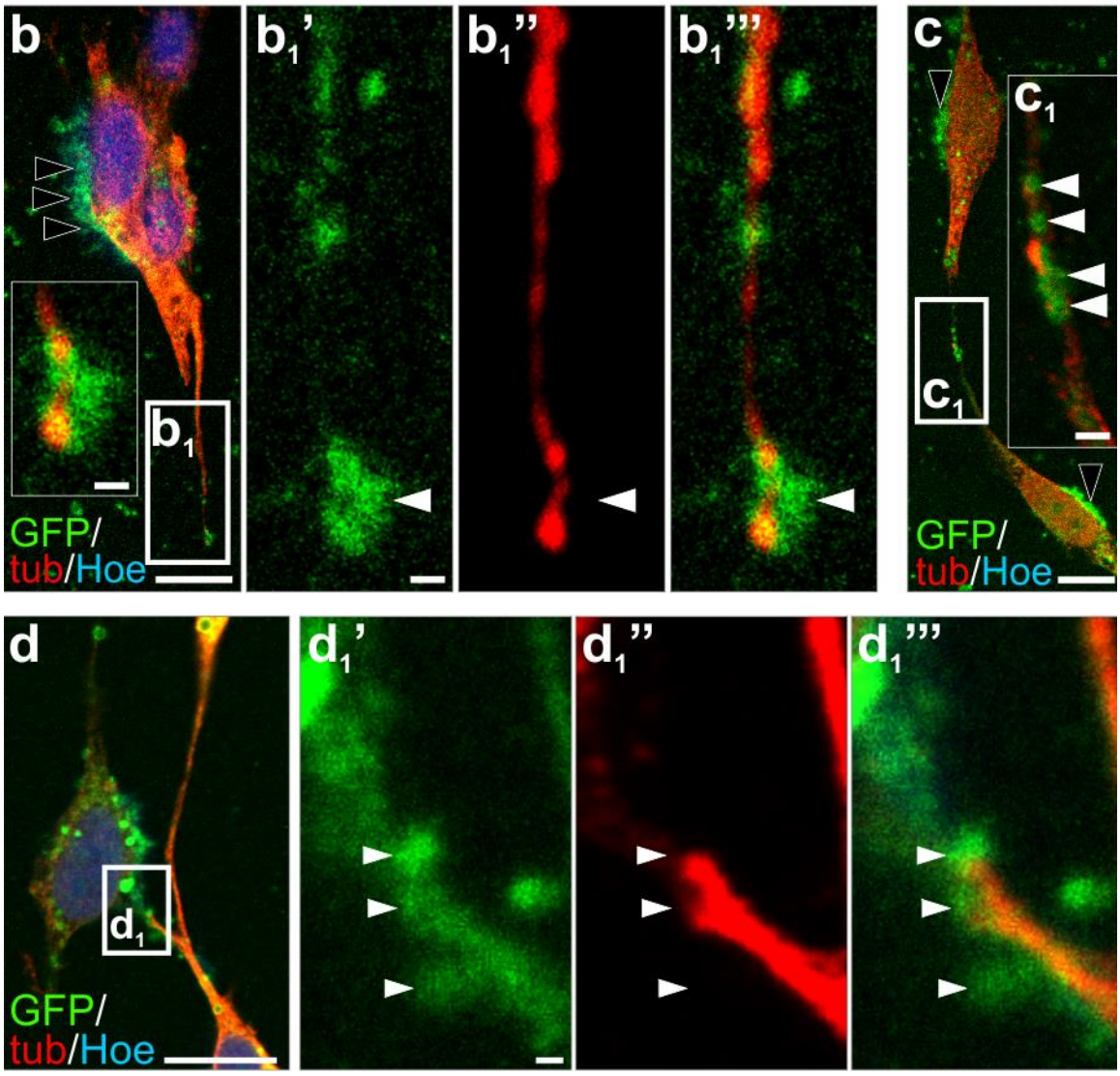

Figure 9.: Human neuroblastoma cells produced CSPG-5 which assembled around neurite terminals and neurite-cell contact points.(b-b_1''')Transfected β-III-tubulin$^+$ SH-SY5Y cells produced CSPG-5 after 3 days in vitro. The GFP-labelled proteoglycan appeared around the cell body (black arrowheads) and typically around the ending of neurites (white arrowheads). (c, c_1 and d-d_1''') Contact points between neurites of neighboring cells were selectively surrounded by CSPG-5^+ extracellular matrix. Abbreviations: tub-βIII-tubulin, GFP- green fluorescent protein, Hoe-Hoechst. Scale

bars: 10µm(b, c, d), 1µm(b_1',c_1,d_1',). . Figure 9 was published in Cellular Signalling as part of a separate figure (https://doi.org/10.1016/j.cellsig.2020.109710).

4.8 CSPG-5 expression shifted in select default mode network areas of suicide victims

Extracellular matrix composition has already been shown to change in psychiatric disorders, including mood disorders (121). Changes in extracellular matrix composition can fundamentally alter synaptic function (122). After recording high CSPG-5 expression in the medial prefrontal cortex and hippocampus of rat (as of section *CSPG-5 expression shows temporal and regional differences during postnatal development of rat),* we hypothesized that CSPG-5 expression might change under pathological conditions in brain regions relevant in mood control. We chose the anterior and posterior cingulate cortices as well as the entorhinal cortex of the human brain as representative areas of the default mode network (123). We referred to suicide as an extreme outcome of psychiatric disorders, especially mood disorders. Specimens obtained from brains of suicide victims showed decreased CSPG-5 protein levels in all three areas of interest compared to age-matched control (Figure 10, b).

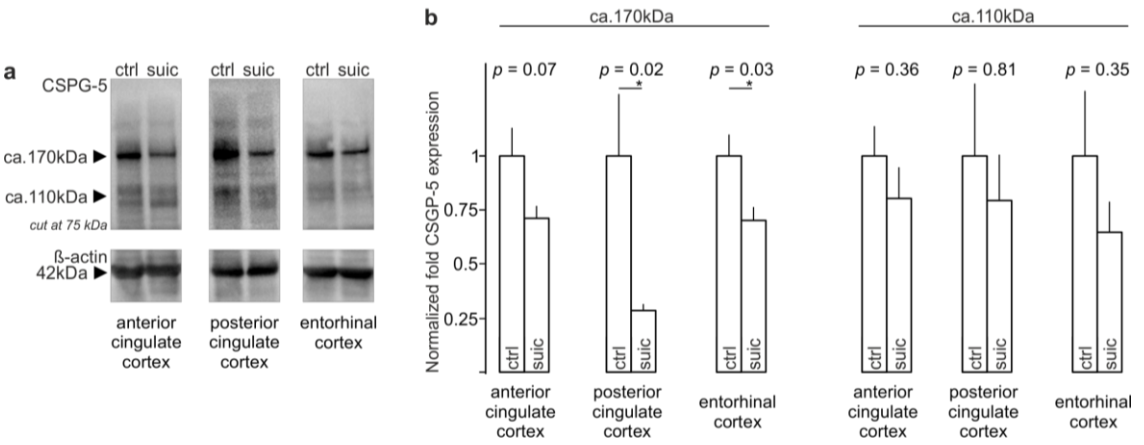

Figure 10.: CSPG-5 expression decreased in select default mode network areas of suicide victims. (a) Representative Western blots of CSPG-5-immunoreactivity in anterior cingulate, posterior cingulate and entorhinal cortices in suicide and age-matching control subjects (n= 7 each, altogether 42 samples). (b') CSPG-5 concentration showed a marked decrease in the anterior cingulate cortex, and a significant decrease in the posterior cingulate and entorhinal cortices in suicide victims (p< 0.05) when 170 kDa fragments were compared. CSPG-5 concentration decreased markedly, but not*

significantly, when 110 kDa fragments were compared. Abbreviations: ctrl control subjects, suic suicide subjects.). Figure 10 was published in Cellular Signalling (https://doi.org/10.1016/j.cellsig.2020.109710).

4.9 Extracellular matrix around a newly discovered calcium-binding protein, secretagogin, -containing neurons

Our research group extensively investigated the distribution and role of a recently discovered calcium-binding protein, secretagogin, in the brainstem. In addition to the investigations made by the group in the rat and mouse brains, my contribution was to diagram occurence of secretagogin in the avian brain. Secretagogin expression in the chicken brainstem showed largely similar distribution patterns compared to mouse, rat and human brains (99). In detail, the vagal nucleus (Fig. 11 a, a_1), the ventrolateral medulla (Fig 11 b, b_1), the vestibular nuclei and the locus coeruleus (Fig 11 c), midbrain nuclei like the ventral tegmental area and periaqueductal grey (Fig 11 d-d_2) were typical secretagogin-expressing foci.

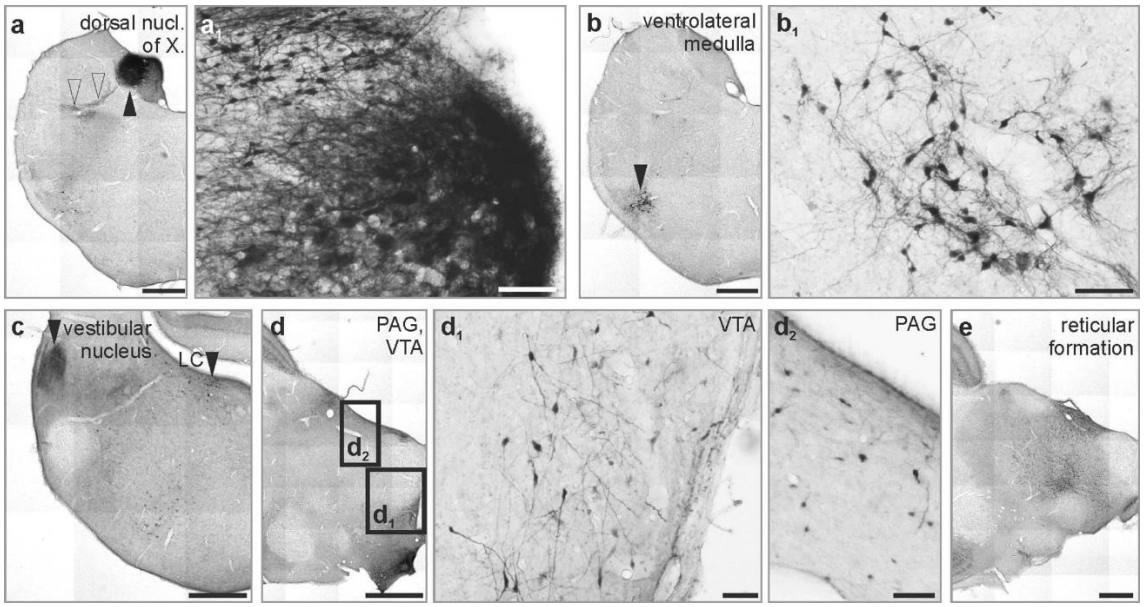

Figure 11.: Secretagogin expression in the avian brain stem. The immunoreactivity pattern of secretagogin in the chicken (Gallus domesticus) brain stem was similar to what was observed in mammalian species. In the medulla oblongata, immunoreactive neurons populated the dorsal nucleus of vagus (black arrowhead in a, open arrowheads indicate nerve fibres originating from the nucleus) and the ventrolateral medulla (a-b1). In the pons, immunoreactivity was typically confined to the vestibular area and the locus

coeruleus (c). In the midbrain, neurons of the periaq-ueductal grey matter, of the ventral tegmental area and scattered cells in the reticular formation showed secretagogin immunoreactivity (d-e). LC: locus coeruleus, PAG: periaqueductal grey, VTA: ventral tegmental area. Scale bars 1 mm (a–e), 50 µm (a1, b1, d1, d2). Figure 11 was published in Brain Structure and Function (https://doi.org/10.1007/s00429-019-01886-w).

Somatic expression of select calcium-binding proteins typically associate to the presence of perineuronal extracellular matrix, which is best exemplified by parvalbumin$^+$, less so by calretinin$^+$ neurons surrounded by perineuronal nets (69). The systematic immunohistochemical analysis of the mouse and rat brainstem explored that secretagogin$^+$ neurons remained largely uncovered by (WFA)$^+$ extracellular matrix (Fig. 12.). Of the regions examined (like the microcellular tegmental nucleus, locus coeruleus, spinal trigeminal nucleus, dorsal vagal complex where secretagogin$^+$ neurons typically accumulate in the vertebrate brain), only neurons in the spinal trigeminal nucleus were enwrapped by WFA$^+$ extracellular matrix (Fig. 12. a, a_1). Even in this case, secretagogin$^+$ neurons were surrounded by tiny speckles of extracellular matrix. In other regions, the extracellular matrix could be visualized by immunostaining, but cell bodies lacked the "classical" perineuronal net.

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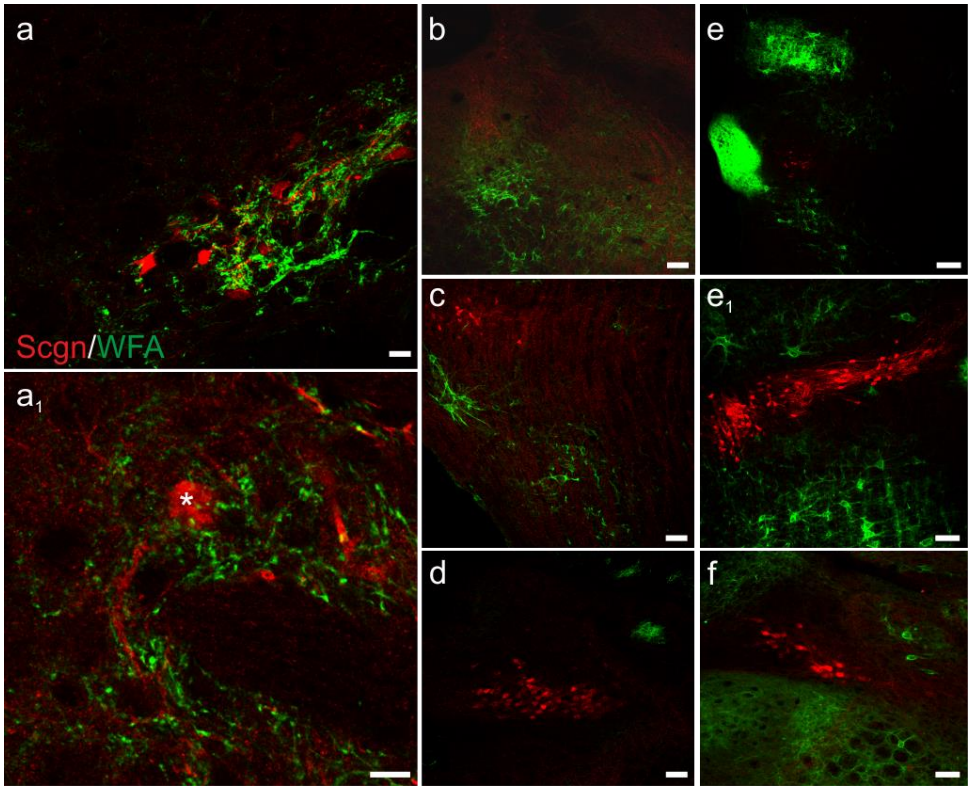

Figure 12.: Secretagogin was marked in red, WFA$^+$ extracellular matrix was marked in green. (a-a$_1$) Rat spinal trigeminal nuclei (a) at 40x magnification, (a$_1$) at 40x magnification and 2x optical zoom. Asterisk indicates a secretagogin$^+$ cell body overlaid by WFA$^+$ matrix. In the spinal trigeminal nucleus, extracellular matrix (perineuronal net) was observed to surround cell bodies. Other examined regions showed no significant juxtaposition. (b) Locus coeruleus, rat at 10x magnification. (c) Microcellular tegmental nucleus, rat at 20x magnification. (d) Dorsal vagal complex, rat. (e-e$_1$) Microcellular tegmental nucleus, mouse (e$_1$) at 20x magnification. (f) Dorsal nucleus of vagus nerve, mouse. Scale bars 10 µm (a, a$_1$), 30 µm (e$_1$, f), 50 µm (b, c), 100 µm (e).

5 Discussion

5.1 CSPG-5 and neuroplasticity, implications for human neuropsychiatric disorders

A growing body of evidence suggests that the extracellular matrix is an important regulator of neuronal adaptation and plasticity (122,124) and recent data suggested that its abnormal composition contribute to the pathogenesis of psychiatric disorders (121). Extracellular matrix elements that have been repeatedly implicated in psychiatric disorders include CSPGs, matrix metalloproteinases, and tPA. These matrix components have important roles not only in neuronal development but also in adult neuroplasticity, critically involving the medial prefrontal cortex (121).

CSPG-5 contributes to the formation and maintenance of synapses, as demonstrated by several previous studies. CSPG-5 is typically present in areas where basal and apical dendrites elaborate; it increases the complexity of developing spines and filopodia in the mouse cortex (125). Also, the role of CSPG-5 in neurite growth was addressed when this proteoglycan was identified in budding neurites of retinal ganglion cells *in vitro* (90) and implicated in its regulation via phosphatidylinositol 3-kinase and protein kinase C pathways (87). Further, the transitional appearance of CSPG-5 on Purkinje cells' proximal somatodendritic compartment suggested afferent specificity in adhesion and synaptogenesis of approaching fibers (83,89). These developmental actions are mediated via binding to the adhesion- regulating matrix molecules tenascin-R and tenascin-C (126,127) and activating a subtype of EGF receptor tyrosine kinases (128).

Here, we prove that CSPG-5 is also present in the adult – at least in the 12- week-old - rat brain, forming characteristic ring-like structures associated with the "classical" perineuronal network around mature synapses. This suggests that it remains involved in synaptic processes in adult life and may influence adult neuroplasticity. The latter is also supported by the fact that in the developing brain it reaches its maximum expression level at different ages, with an earlier peak in areas with less plasticity. In addition, it is present in higher concentrations in typically plastic areas, such as the hippocampus or the medial prefrontal cortex, at later ages compared to areas of more restricted plasticity, in our case the somatosensory cortex and cerebellum.

Our experiments also support the idea that CSPG-5, and more broadly the central nervous system extracellular matrix, play a role in development and regulation of neuronal

plasticity. Our experiments on human samples illustrate that changes in the extracellular matrix composition may have far-reaching consequences.

In our Western-blot studies, CSPG-5 content of samples from default mode network areas of suicide victims and healthy (from psychiatric point of view) controls were compared. We found significant decrease of CSPG-5 expression in the anterior cingulate, posterior cingulate and entorhinal cortices in suicide subjects when 170 kDa fragments were compared. CSPG-5 expression decreased markedly, but not significantly, when 110 kDa fragments were compared.

Suicide is one of the leading causes of death worldwide, in some cases without any detectable premorbidity, but often as a tragic outcome of psychiatric illness. In particular, it is most commonly associated with schizophrenia, mood disorders and substance use disorders (129).

In the following section, albeit not directly linked to CSPG-5 expression, I would like to briefly describe possible interactions between the extracellular matrix and the development of psychiatric disorders. By doing so, I would like to discuss that the extracellular matrix is highly relevant in both organic and functional psychiatric diseases. I will use schizophrenia and depression as examples, however a growing body of evidence supports the theoretic relationship between extracellular matrix abnormalities and substance use disorders (both alcohol and opioids, (130,131)).

Extracellular matrix has a dual role: it is involved in the formation of the nervous system from its earliest stages (132) and then contributes to the maintenance of the established structure and its transformation under controlled conditions. „Maintenance of order" can be interpreted at several levels, all of which are only separated here for didactic purposes.

i. at the cellular level, by maintaining an optimal microenvironment and securing cellular connections (4,17,18,71)
ii. at the synapse level, as postulated by the tetrapartite synapse model (to be discussed in more detail) (10,133)
iii. at the network level (134,135)

From the above, it is tempting to speculate that disruption of matrix function can have serious consequences upon not only individual cells but also upon large-scale brain

networks (132,136). Temporal order is also important: a different kind of pathology can arise if a connection or network failed to form, formed with disfunction, or is formed normally but subsequently becomes impaired.

Schizophrenia is a multifactorial functional mental health disorder, whose exact pathomechanism is currently unknown, but it is thought that both genetic predisposition and environmental factors play a role in its development. Amongst schizophrenia models network analysis-based ones attract increasing attention. According to these models, schizophrenia is a disconnectivity syndrome, with the composition and - therefore - function of certain central nervous system networks being altered (137,138). Other studies emphasize the significance of cell and synapse level alterations in the pathogenesis of schizophrenia, such as disturbances in cell migration, dysfunction of synaptogenesis or signal transduction (139,140). Extracellular matrix dysfunction might bring about or amplify these pathologic changes (141).

Actually, theories based on extracellular matrix and cellular dysfunction do not stand in sharp contrast to explanations based on network analysis, since dysfunctionality of individual network elements (cells, cell groups) or the pathological nature of their connections (neurites, synapses) likely lead to overall network function loss.

Post-stroke depression can be considered as an organic psychiatric disorder, in which the mental illness arises as a direct consequence of prior brain damage. Ischaemia alone or ischaemic-reperfusion injury impairs extracellular matrix and blood-brain barrier function (142,143) , and lead to activation of enzymes responsible for matrix degradation (144). As mentioned above, extracellular matrix alterations influence the formation, maintenance and function of neuronal network connections (134,135). In view of this, it is tempting to speculate that the damage associated with acute ischemic stroke triggers, among other things, structural and functional changes in the extracellular matrix that in the long term affect the connectivity of the whole brain. These connectivity changes lead to development of depression, as described by Egorova et al. (136).

Based on the two examples above, one can hypothesize a model in which psychiatric disorders stem from smaller-scale damage at the cellular or synapse level. These damages might be caused by either genetic or environmental factors, and some of them affect the

extracellular matrix. These lesions can add up and eventually cause dysfunction of brain networks.

In my thesis, the relationship between the default-mode network and CSPG-5 was investigated in more detail. The default-mode network, consisting of cortical areas on the medial convexity of the human brain, plays a critical role in shaping mood, creativity, self-representation and memory recall. Disturbance in their structure and/or function, including neurotransmitter release and receptor expression, and functional connectivity can result in suicidal ideation and attempts in various mental health disorders (123) . Three examples of major areas of the default mode network include the anterior cingulate cortex which recruits large cortical field associated with emotions and emotional balance (145), and the posterior cingulate and the entorhinal cortices relevant to self-referential processing and mentalization (146,147). These regions show different alterations in suicide subjects: expression of serotonin receptors (148) and expression of GABA and glutamate genes (149) increase in the anterior cingulate cortex, VGLUT1 and VGLUT2 expression decrease in the entorhinal cortex (150). Of note, both their functional connectivity and intrinsic activity change (147,151) which reflect altered synaptic function. We here showed that CSPG-5 expression in suicide subjects simultaneously decreased in all of these three major default mode network regions. We speculate that the reduced amount of CSPG-5 in perisynaptic matrix assemblies might contribute to an altered neuronal circuitry, possibly affecting functional connectivity and thus mood.

5.2 CSPG-5 metabolism, relationship between extracellular matrix and synapses

Our results suggest that neurons primarily contribute to their own CSPG-5-containing perisynaptic matrix production, since we could detect only minimal CSPG-5 mRNA expression in rat primary astrocytic culture. On the other hand, previous studies showed that astrocytes as well as neurons synthesize CSPG-5 in rat cortical culture (85). The discrepancy with this previous and our present results can be due to the difference of the applied embryonic age (E16 vs E20, respectively). It was interesting to observe that glial conditioned medium reduced CSPG-5 expression in primary neuronal cultures *in vitro*. We did not study the phenomenon further yet, but it raises the possibility that astrocytes actively influence CSPG-5 metabolism of neurons, hypothetically by a soluble factor.

Based on our experiments with a CSPG-5-transfected neuroblastoma cell line, CSPG-5 appears in a typical compartmentalization: in addition to the soma, neurite endings, but not shafts, were surrounded by labelled matrix *in vitro*. Further, CSPG-5 typically assembled around contact points between neurites. Taken together, these results suggest that CSPG-5 accumulates around predilection sites of synapse formation, even under experimental conditions. CSPG-5 has previously been identified in budding neurites of retinal ganglion cells (90) and on Purkinje cell's proximal somatodendritic compartment (83,89) which suggests its role in orchestrating specificity of neurite adhesion and synaptogenesis.

Nakanishi et al. described in great detail that different domains of the CSPG-5 molecule promote different aspects of neurite outgrowth and synaptogenesis (87). For example, addition of complete CSPG-5 ectodomain to neuronal cultures resulted in not just more, but also longer neurites, while the EGF-domain only supported the longitudinal growth of one given neurite and the acidic aminoacid domain caused the development of more neurites. Their study also describes a model according to which budding neurites (dendritic filopodia) on the future postsynaptic site shed CSPG-5 ectodomains as a result of depolarization. CSPG-5 ectodomain presumably affects the growing axon on the future presynaptic site, all in all, promoting synapse formation. We suggest that a similar process took place in our neuroblastoma experiment.

Based on primary cortical culture as well as neuroblastoma cell studies, we conclude that: *i*. neurons are able to produce CSPG-5 without the presence of astrocytes, *ii*. the sporadic presence of glial cells does not significantly alter CSPG-5 production in neuron cultures (it is well known that that some glial cells inevitably appear in primary neuron cultures, especially after multiple days *in vitro*, still 10 DIV primary neuronal culture cells were able to produce CSPG-5 in higher concentration than their 4 DIV counterparts), *iii*. on the other hand, application of glial-conditioned medium abolished neuronal CSPG-5 production *in vitro*.

Furthermore, in contrast to previous advances which associated CSPG-5 to the postsynaptic compartment (125), we argue that both pre- and postsynaptic domains take part in the establishment of the CSPG-5-containing perisynaptic matrix. This is reflected in the ultrastructure of the matrix-enwrapped synapse in the adult neocortex as well as by

the compartmentalization of the neuroblastoma cell line-produced CSPG-5 *in vitro* around soma and neurite endings which is typically enriched at neurite contact points.

5.3 Phenotyping of synaptic connections based on their extracellular matrix composition

According to our findings, CSPG-5$^+$ matrix assemblies typically surround glutamatergic and GABAergic, but less so cholinergic or monoaminergic afferents, which may reflect a morphological blueprint in synaptic functioning or development. In the cerebral cortex, acetylcholine and monoamines play a modulatory role and this is reflected in a different synaptic phenotype; thus, they may lack synaptic membrane specializations and instead of 1:1 neurotransmission the transmitters are released into the surrounding neuropil by volume transmission (152–155) . This is especially true in case of the prefrontal cortex where definitive synaptic junctions are formed only by 44% of cholinergic boutons, even though typically appositioned to spines or small dendrites (156). It is tempting to speculate that the lack of a perisynaptic matrix around modulatory synapses enables volume transmission.

5.4 Extracellular matrix around a hitherto unclassified calcium-binding protein, secretagogin-containing neuronal pool in the brainstem

Subcortical regions are known to contain less neurons covered by extracellular matrix, hence, perineuronal nets (19,67). In the second part of my work, I investigated the occurence of perineuronal nets around specific subset of brainstem neurons. These neurons express a recently discovered calcium-binding protein, secretagogin, which has been acknowledged as the „fourth musketeer of neuronal calcium binding proteins" for the classification of interneuron types (M.R. Celio, http://ejnblog.wordpress.com /2010/07/03/secretagogin-the-fourth-'musketeer/) For this, our research group comprehensively diagramed the distribution of secretagogin$^+$ neurons in the mouse, rat and human brain stem. As an independent personal contribution, and part of this thesis, my task was to complete an immunohistochemical mapping of secretagogin$^+$ neurons in the avian brain stem. It was via this dataset that the phylogenetical preservation of secretagogin expression in the vertebrate brainstem could be explored (99). Thus, secretagogin$^+$ neurons typically occurred i. in the brainstem nuclei which serve as a major vegetative command centres, such as the parabrachial or the solitary tract nucleus, ii.

populate relay stations in the pathway of special (vestibular and visual) senses, iii. outline the brainstem noradrenaline axis.

The relation of secretagogin$^+$ neurons to the extracellular matrix have not been investigated before. Therefore, I analyzed the presence of extracellular matrix assemblies around secretagogin$^+$ neurons in the mammalian brainstem. In rat and mouse samples, secretagogin$^+$ neurons remained typically naked from perineuronal matrix in all the secretagogin-foci of the brainstem; perisomatic matrix assemblies appeared only focally in the spinal trigeminal nucleus. Whilst the function of both secretagogin and perineuronal nets are yet to be fully understood, the absence of perineuronal matrix around secretagogin$^+$ neurons is defined during ontogeny, also since extracellular matrix plays a key role in neurodevelopment (157).

Extracellular matrix does not only refer to cellular fate (1,158), but, in case of an adult neuron, critically impacts metabolism and thereby cellular function (17,18,101).

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6 Conclusions

6.1 CSPG-5 expression in the postnatal mammalian brain

i. CSPG-5 expression is not restricted to the embryonic age but persists throughout early adulthood in the mammalian brain.

ii. Brain areas with plastic properties show higher CSPG-5 expression during later stages of development. Typically, young rats show higher CSPG-5 expression in the medial prefrontal cortex and hippocampus than in their somatosensory cortex and cerebellum.

iii. CSPG-5 is present at the synapse, it accumulates both in its pre-and postsynaptic compartments. It typically forms assemblies outside but adjacent to WFA^+ perineuronal nets.

iv. CSPG-5 appears around both excitatory and inhibitory synapses in the hippocampus. However, significantly more CSPG-5^+ matrix can be observed around specific than non-specific afferents. This suggests that CSPG-5 might be involved in the regulation of signal transduction at the synaptic level.

v. Neurons, but not glial cells produce CSPG-5 (at least in *in vitro*)

vi. CSPG-5 expression shifts in suicide victims. This suggests that CSPG-5 might be involved in the complex structure and connections of the default-mode network and reflects an impact in the pathogenesis of mood disorders / supports diagnostic power.

6.2 Distribution and pericellular matrix assemblies of secretagogin$^+$ neurons in the vertebrate brainstem

i. Secretagogin expression concentrates to relay, vegetative and stress centers of the vertebrate brainstem with remarkably preserved phylogeny.

ii. Secretagogin-containing brainstem neurons typically do not associate to pericellular matrix assemblies.

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7 Summary

The first part of my studies focused on extracellular matrix expression in the postnatal mammalian brain, with special focus on CSPG-5. Throughout various *in vivo and in vitro* experiments we showed that this proteoglycan persists in the postnatal brain, is an integral part of the pre- and postsynaptic compartment and likely shapes synapse formation and neuroplasticity. Including human samples in my research we proved that CSPG-5 concentration shifts in suicide subjects. Considering suicide as a tragic outcome of psychiatric disorders this finding suggests that CSPG-5 takes part in maintenance of the default mode network.

In the second part of my work, I explored the relation of secretagogin, a recently described calcium binding protein to the surrounding extracellular matrix. We demonstrated a phylogenetically preserved distribution pattern of secretagogin-containing cells in the vertebral brain with a typical absence of perineuronal matrix around them.

We discuss that the perineuronal matrix is a critical element in the central nervous system, since it shapes development and neuronal function.

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