Analysis of perisynaptic extracellular matrix components in the brain

Summary of PhD thesis

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1. Introduction

1.1. Extracellular matrix

The extracellular matrix is a network of proteins and complex carbohydrate molecules present between cells, influencing cellular functions and signal transduction. While initially believed to be absent in the central nervous system, research over the past few decades revealed its significance in neuronal functions.

Central nervous system extracellular matrix takes various forms, including the diffuse neuropil, perineuronal nets, and axonal coats. It plays a crucial role in providing structural integrity, protecting neurons from stress and pathological processes, and regulating synaptic functions. The extracellular matrix also contributes to the blood-brain barrier, shaping metabolism, and signal transduction. In addition, alterations in the structure or composition of central nervous system extracellular matrix can impact tumor growth, tissue reaction to injuries or neurodegeneration.

The composition of the CNS ECM is unique, primarily consisting of glycoproteins, including proteoglycans like aggrecan, versican, neurocan, and brevican (lectican family). Several families of matrix-degrading enzymes are responsible for ECM remodeling, including matrix metalloproteinases and tissue plasminogen activator.

1.2.Chondroitin sulfate proteoglycan-5

Chondroitin sulfate proteoglycan-5 (CSPG-5), also known as neuroglycan-C (NGC) or CALEB is predominantly found in the CNS and is considered a part-time proteoglycan due to the variability of its chondroitin sulfate side-chain content. It is a transmembrane 150 kDa protein capable of ectodomain shedding which produces a 75 kDa soluble fragment. CSPG-5 was discovered in 1995 and believed to be associated exclusively to the developing nervous system. The different forms containing varying amounts of carbohydrate side chains and different molecular weights exert different physiological effects; in general, CSPG-5 was associated to neuritogenesis, synaptogenesis and formation of neuronal connections.

1.3.Calcium-binding proteins, secretagogin

Calcium binding proteins can be categorized as calcium buffers or calcium sensors and play a role in calcium homeostasis and neuron characterization. Secretagogin belongs in the latter group. It is detected in various organ systems, including the nervous and neuroendocrine systems, and has been linked to vesicle exocytosis. Secretagogin appears early during embryogenesis, persists into adulthood, and it’s distribution among vertebrate brainstem nuclei is largely conserved evolutionally.

Based on the example of parvalbumin positive GABAergic interneurons that are usually covered in a Wisteria floribunda

agglutinin positive „classical" perineuronal net, we aimed to detect any overlap between classical perineuronal nets and secretagogin positivity in the vertebrate brainstem.

Overall, my thesis focuses on the central nervous system extracellular matrix, emphasizing CSPG-5's role in synaptogenesis, maintenance of synapses and plasticity. I also examined the co-occurrence of secretagogin and perineuronal nets in known secretagogin positive loci of the vertebrate brainstem.

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 postembryonic 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?

vi. What role might CSPG-5 play in the human brain?

2.2. Phenotyping the extracellular matrix structure and its calcium-binding protein content

i. Which secretagogin positive cell groups in the brainstem are covered by an extracellular matrix envelope?

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.Samples

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. Primary cortical neuron and astrocyte cultures, as well as a human neuroblastoma cell line, SH-SY5Y were used. Some neuron cultures were treated with glial-conditioned medium. SH-SY5Y cells were transfected with green fluorescent protein-tagged rat-CSPG-5 plasmid DNA. With the help of the Human Brain Tissue Bank and Laboratory of Semmelweis University, I used brain tissue samples isolated by the micropunch technique. 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.

3.2.Immunhistochemistry

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 (30 μm) in the coronal plane for multiple immunolabelling. Samples for electron microscopy analysis were first sectioned with a vibratome at 50 μm thickness, immunolabelled, postfixed, contrasted, buffered and flat-embedded. The primary somatosensory cortex was identified using light microscope, excised and subsequently re-embedded for ultrasectioning at 100 nm thickness.

Using select combinations of antibodies, chromogenic or multiple immunofluorescence histochemistry were performed. Chromogenic labeling was used for samples destined for electron microscopy.

3.3.Protein analysis

We prepared synaptosomal fractions from the neocortex of adult rats using centrifugation and increasing concentrations of HEPES buffer.

Human samples, synaptosomal samples and primary culture samples were analyzed by Western blot. The same primary antibodies were used as in our immunhistochemistry experiments. We used HRP-conjugated secondary antibodies. An approximately 170 kDa and approximately 110 kDa heavy band were considered in our analysis.

3.4.RNA analysis

We compared different brain regions of rats (medial prefrontal cortex, hippocampus, somatosensory cortex and cerebellum) of

various ages (postnatal days 1, 3, 7 and 14) in terms of CSPG-5 expression. Additionally, we examined the level of CSPG-5 expression in primary cortical cultures using qPCR.

3.5.Imaging techniques

In addition to classical light microscopy, laser-scanning microscopy and electronmicroscopy were used.

4. Results

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

In the CA1 field of rat hippocampus, CSPG-5 containing matrix appeared as a diffuse network without any well definable phenotype at postnatal day 1. On day 3, however it started to faintly outline somata of pyramidal cells. By postnatal day 14, 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 of rats

We investigated CSPG-5 expression at different postnatal stages in various brain regions, revealing a gradual three-fold increase in the

medial prefrontal cortex from postnatal day 1 to 14, a transient initial increase followed by a decline after postnatal day 7 in the primary somatosensory cortex, a marked peak at postnatal day 3 and over fourfold increase in the cerebellum until postnatal day 14, and a significant ten-fold increase during the first week with plateaued expression in the hippocampus throughout the second postnatal week; additionally, the experiment showed lower CSPG-5 expression levels in the somatosensory cortex and cerebellum compared to the medial prefrontal cortex and hippocampus at P14.

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

According to our findings, both the hippocampal stratum pyramidale and the somatosensory cortex harbored a specific CSPG-5$^+$ matrix phenotype. It surrounded neurons but accumulated in the peripheral domain of Wisteria floribunda agglutinin$^+$ perineuronal nets. It appeared adjacent to the inner layer of perineuronal nets and formed tiny ring-like structures.

4.4. CSPG-5 as part of the synaptic compartment

By Western-blot analysis of synaptosomal fractions prepared from adult rats, we identified a 170 and a 110 kDa molecular weight band which typically resembled the 150 and 120 kDa bands described

previously. We found that CSPG-5 was present in cortical synaptosomes.

Continuing our experiment, we examined CSPG-5$^+$ matrix at the ultrastructural level by electron microscope. CSPG-5 enwrapped synaptic sites, expanding both into the pre- and postsynaptic compartments.

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. Examining the adult rat hippocampus by immunohistochemistry and confocal laser scanning microscopy, we found that CSPG-5 associated mainly to specific terminals (glutamatergic and GABAergic) and less so to cholinergic and monoaminergic synapses.

4.6. Neuronal CSPG-5 production

Our experiments with primary cortical cultures prepared from rat embryos showed that neurons expressed CSPG-5 by 4 days *in vitro* (DIV) already and expression levels increased markedly by 10 DIV. 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.

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

We induced green fluorescent protein (GFP) tagged rat CSPG-5 plasmid DNA expression in plated SH-SY5Y neuroblastoma cells. On day 3 after transfection, GFP-labelled matrix showed a typical phenotypic appearance: it concentrated around the cell bodies and neural endings, as well as around neurite contact points.

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

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. 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.

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

Secretagogin expression in the chicken brainstem showed largely similar distribution patterns compared to mouse, rat and human brains. The systematic immunohistochemical analysis of the mouse

and rat brainstem explored that secretagogin^{+} neurons remained largely uncovered by Wisteria floribunda agglutinin^{+} extracellular matrix.

5. Summary, conclusions:

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.

In conclusion, our experiments prove the following:

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 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.

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

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

6. Bibliography of the candidate's publications

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***Disclaimer**: My official name changed from Pintér Anna Veronika to Pintér Panka on 21.01.2022.*