

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

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Organization of extracellular matrix macromolecules in  
the vestibular nuclear complex of the rat and frog, and  
their possible role during compensation

by **BOTOND GAÁL**

SUPERVISOR: PROF. DR. KLÁRA MATESZ



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF DENTAL SCIENCES

DEBRECEN, 2014

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The Examination takes place in the Lecture Hall 210 of the Faculty of Dentistry, University of Debrecen; November 10, 2014, 11:30 am.

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# INTRODUCTION

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## EXTRACELLULAR MATRIX IN THE CENTRAL NERVOUS SYSTEM

In the central nervous system (CNS), the intercellular space between neurons and glial elements is approx. 10-20% of the total brain volume. This extracellular gap contains the network of extracellular matrix (ECM) molecules providing the working environment for neurons. The ECM molecules are produced intracellularly and secreted to form a dense network of proteins and carbohydrate-like glycans.

### Overview on the function of ECM molecules in the CNS

- (1) In the adult nervous system the ECM molecules are involved in *trafficking soluble and membrane-bound molecules*.
- (2) By accumulating around neuron cell bodies, proximal dendritic segments, axon hillock or preterminal axons, the ECM is an *important stabilizer of synaptic connections*.
- (3) ECM molecules *influence neuronal activity* during normal and pathological conditions, and neuronal activity influences ECM structure.
- (4) The different molecular composition of ECM is associated to several membrane bound receptors or synthases, thus anchored to the cytoskeleton, enabling *involvement in signaling cascades*.
- (5) Since the past decade ECM molecules are considered as the *fourth component of synapses*, termed as 'tetrapartite' synapses.
- (6) *During development*, specific ECM components are secreted in spatial and temporal timing, to *facilitate the migration and engagement of newly born neurons*.
- (7) Clinically important to note that in CNS gliomas the perivascular or periaxonal invasion of *tumor cells is facilitated by the modified ECM*.

Molecules of the ECM are present in various extracellular compartments of the brain tissue.

### Organization of ECM in the CNS

Diffuse and condensed forms of ECM are present in different interneuronal compartments of the CNS, as listed below:

- (1) Basement membrane is a sheet-like plate that lies under endothelial cells of the cerebrovascular system, and provides barrier between vessels and CNS parenchyme.
- (2) The perineuronal net (PNN) is a dense layer of mesh-like network, built up by accumulations of matrix molecules around the neuronal soma, proximal segments of dendrites, and the axons' initial segments. The PNN, if labeled, demarcates the neuron somas, proximal parts of neuronal processes, and can be well distinguished from the less condensed neuropil. Principal molecular constituents are the hyaluronan (HA), chondroitin sulfate proteoglycans (CSPG), tenascin-R and various link proteins.
- (3) The neural interstitial matrix consists of ECM molecules in the interneuronal compartment of the parenchyme that are not in close relation to the basement membranes or perineuronal nets, but considered as diffuse matrix in the neuropil.
- (4) Recently, a new compartment at the preterminal segment of axons and boutons has been distinguished, enwrapped by ECM accumulation, termed as the axonal coat (AC).
- (5) The nodal ECM accumulation forms in the nodes of Ranvier, composed of brevican, versican, tenascin-R and link proteins. The excess negative charge serves ion reservoir function for saltatory action potential propagation.

## **Molecules of ECM in the CNS**

### ***Hyaluronan***

The HA is a non-sulfated polymer of repeating dimers composed of D-glucuronic acid and N-acetylglucosamine, producing chains up to 25,000 dimers in length. Despite the simple basic structure, hyaluronan forms complex secondary and tertiary structures, with differing size and conformation being the key organizer of ECM, especially in the PNN. HA is a GAG that does not bind to other proteins covalently, thus doesn't build proteoglycans. It is ubiquiter in most of the tissues in various amounts, and its structure is conservative in vertebrates. It carries strong negative charge that traps cations, water, or other extracellular trafficked molecules.

### ***Chondroitin sulfate proteoglycans***

CSPGs are built up of a core protein of variable composition, wearing covalently bound chondroitin sulfate glucosaminoglycans in various number.

The chondroitin sulfate GAG chains are long linear molecules; they are formed by repeating disaccharide units of D-glucuronic acid and N-acetyl galactosamin, linked by  $\beta$ -glycosidic bonds. Although the family of CSPGs gathers a number of various molecule types, the present work concentrates only on lecticans, the HA binding CSPGs (aggrecan, versican, neurocan, and brevican).

#### *Aggrecan*

The aggrecan isolated from tissues is polydisperse due to its giant molecular size ranging at 1-3 MDa molecular weight, because of variations in the number and length of GAGs attached. It consists of a linear core protein, 300 kDa in size, with glycosylated serine residues. The core protein has 3 globular domains, the G1, G2 and G3. It is only the aggrecan that carries the G2 domain. The core protein's N-terminal domain, also called G1, establishes the HA bond, while the C-terminal domain, or G3, may bind to tenascins to form ternary structure. GAG chains are connected at the long segment between G2 and G3 domains. Aggrecan chains bind two types of GAGs, the chondroitin sulfates and keratan sulfates.

#### *Brevican*

Like in all lecticans, the brevican core protein comprises of an N-terminal globular G1 domain, being able to bind HA, and a C-terminal domain carrying a G3 domain. Only a small number of chondroitin sulfates (1-5) attach the core proteins. Brevican represents a molecular weight of 140 kDa.

After aggrecan, brevican is the most abundant ECM molecule in the CNS, which contributes to PNN formation. Its expression is specific in the nervous system, and it is particularly present at perisynaptic sites, and it has also been shown to surround axon initial segments *in vivo* and *in vitro*. Brevican may accumulate in a selected group of the Ranvier nodes.

### *Neurocan*

The expression of neurocan is CNS specific, and shows the general structure of other CSPGs, having the N-terminal G1 domain with the hyaluronan binding globular module and a double Ig-like sequence.

The full length neurocan (245 kDa) is only present in the developing nervous system, but during maturation the central part of the core protein is cleaved. After cleavage, a 150 kDa N-terminal fragment (recognized by antibody 1F6) and a 130 kDa C-terminal fragment (recognized by antibody 1D1) remains in the extracellular space. Neurocan accumulates in PNNs, but also accumulates in nodes of Ranvier.

### *Versican*

Versican is present in various other soft tissues. The core protein structure follows the general assembly of CSPG lecticans, for it binds to HA by its globular G1 domain found on the N-terminal, and at the C-terminal it has two EGF modules, a C-type lectin, and a complement regulatory region. The middle region of the core is encoded by two large exons, in which RNA splicing specifies the chondroitin sulfate attachment regions, determining the types of GAGs binding.

The V2 versican is the dominantly present isoform in the adult CNS, carrying 5-8  $\alpha$ GAGs, with the molecular weight of approx. 400kDa. Versican V2 has a characteristic punctuate immunohistochemical occurrence. Specific places of accumulations are perisynaptic spaces, myelinated tracts, or nodes of Ranvier, alongside with brevican, tenascin-R and link proteins.

## ***Glycoproteins***

### *Tenascins*

Tenascin units are able to bind one another, thus form large multimeric structures, which can also connect to C-terminals of CSPGs. They represent a well conserved structure across phylogenesis. The family of tenascins gathers tenascin-C, -R, -X, -W, (and -N) molecules.

Tenascin-R has 3 subunits, each having 4,5 EGF and 9 fibronectin-III repeats, and has two splice variants with 180 kDa and 160 kDa molecular weights per subunit. Tenascins in the

CNS bind to C-terminal domains of CSPGs, forming the HA-CSPG-TN-R ternary complex, as well as to cell surface ligands, mediated by the fibronectin-III modules.

### *Link proteins*

Link proteins (LP) belong to the family of glycoproteins that establish binding between lecticans and HA in the ECM, stabilizing their aggregates. The group of LPs counts four members: the HAPLN1 (hyaluronan and proteoglycan link protein 1); HAPLN2; HAPLN3 (not in CNS); and HAPLN4.

They have 3 modules: Ig like fold at the N-terminal, and two proteoglycan tandem repeats. The molecular weight is 41, 44 or 48 kDa. In absence of link proteins ECM aggregates are unstable, PNNs don't form.

## **VESTIBULAR NUCLEAR COMPLEX**

The vestibular system is responsible for sensation of head movements and position, and regulates posture, eye movements, and gaze, and adjusts the tone of antigravity muscles for the balance of the body. It influences autonomic regulation, and has extended connections with cerebellar, thalamic, and spinal areas.

The peripheral parts of the vestibular system are found in the inner ear. Sensory hair cells in the *cristae ampullares*, *maculae* of utricle and saccule (and *lagena* in frogs) provide information on head motions. Sensory stimulus is received and propagated by bipolar neurons of the vestibular ganglion (Scarpa), and transmitted into the vestibular nuclei in the brainstem.

### **Vestibular nuclear complex of the rat**

The vestibular nuclear complex (VNC) of rats show similarities with other mammalian species, due to the relatively conservative organization of the vestibular system across phylogenesis. There are four nuclei in the brainstem receiving primary input from inner ear organs: *superior, lateral, medial, and descending vestibular nuclei*.

*Superior vestibular nucleus (SVN)*: Medium-sized neurons provide two-third of SVN, and only a minor group of large or giant neurons is present. Large neurons are located

centrally, medium and small-sized cells are found mostly in the peripheral part of the nucleus.

The large-sized neurons establish inhibitory synapses with the oculomotor and trochlear neurons, the small-sized neurons project to the cerebellum and reticular formation and establish commissural connections with the contralateral vestibular nuclei.

*Lateral vestibular nucleus (LVN)*: LVN gathers the largest perikarya within the VNC, nearly half of neurons being large or giant sized, and only a minor portion is small-sized, evenly distributed throughout the nucleus. The rostral part of LVN receives afferents from the utricle and cristae ampullares, and project to the oculomotor nuclei, and establish reciprocal connection with cerebellum. The caudal part of the LVN receives spinal afferents, relayed in the inferior olive or the reticular formation. The entire nucleus projects to the spinal cord.

*Medial vestibular nucleus (MVN)*: The large and medium sized neurons form the magnocellular part, located ventrolaterally in the MVN. The primary input is from the semicircular canals and cerebellum, and its efferents project to oculomotor neurons, and caudally give rise to the vestibulo-spinal pathways. The parvocellular part is located in the dorsomedial position of the nucleus, receiving input from the otolith organs, sending projections to the inferior olive. It also has reciprocal connection with the cerebellum and spinal cord.

*Descending vestibular nucleus (DVN)*: Approx. two-thirds of neurons are medium sized, and only a minority belongs to large-or giant sizes. There is great difference in neuron morphology and function, between the rostral and caudal parts of the nucleus. The rostrally located large and giant cells project to the eye movement nuclei, and to the spinal cord. The small neurons in the caudal part are in connection with the nucleus of solitary tract, the dorsal motor nucleus of vagus, and ventrolateral medulla, suggesting influence on cardiovascular, respiratory, and digestive regulation.

## **Vestibular nuclear complex of the frog**

Although the frog's VNC is not entirely characterized until today, but still the afferentation from the sensory end organs and the central projections show considerable overlap with mammalian species. The structural organization of frog VNC corresponds with the mammals, composed of four nuclei, the superior, lateral, medial, and descending vestibular nuclei.

The lateral part of SVN, rostral part of both LVN and MVN project to the oculomotor nuclei, the medial and lateral vestibulo-spinal tracts originate in the caudal part of LVN, MVN and DVN. Extensive cerebellar connection is established, most probably regulating vestibulo-spinal and vestibulo-ocular circuits. Indirect experimental evidence suggests the conserved functional properties of frog VNC, being comparable to mammals.

## **PLASTICITY OF THE NERVOUS SYSTEM, COMPENSATION**

It has long been stated that functional circuits of the brain continuously change throughout life with experience, which is termed as plasticity of the brain. It has been shown that ECM molecules are involved in the regulation of synaptic plasticity, therefore being essential components of learning and memory. Thus, turnover of ECM, as a degradable stabilizer of neural microcircuits, is a promoter of structural and functional plasticity.

The term compensation collects a number of simultaneous processes setting on soon after an injury of a CNS pathway. Neuronal circuits have the ability to take over the function of a lesioned area, involving a series of synaptic plastic events, which will eventually change the prelesional functional map of the area. Compensation processes are crucial elements of neurological rehabilitation.

In the background of vestibular compensation *many possible neural mechanisms* are suggested, both on the *presynaptic and postsynaptic* components of interneuronal connections. We may expect changes of ECM composition in the VNC following unilateral labyrinthectomy (UL), especially in the structure and molecular composition of PNNs.

# AIMS

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## **1, In intact rat VNC:**

\* Provide a comprehensive mapping of ECM molecular composition in each nuclei and subnuclei of the rat VNC, by using histochemical and immunohistochemical methods. ECM molecules to be labeled are: hyaluronan; CSPGs generally; and specifically: aggrecan, versican, neurocan, brevican; tenascin-R; and HAPLN1.

\* Evaluate the intensities of PNN staining in each nucleus and subnucleus of the rat VNC, by semiquantitative scoring in case of each studied reaction.

\* Illustrate the distribution of PNN-bearing neurons in nuclei and subnuclei of the rat VNC by NeuroLucida reconstruction.

## **2, In rat VNC following unilateral labyrinthectomy:**

\* Deafferentation of VNC by UL, then follow the changes of HA and CSPGs staining pattern/intensity on 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> postoperative days, in the LVN of rat.

\* Observe whether amelioration of symptoms caused by UL shows temporal correspondence with reestablishment of PNNs around neurons of LVN.

## **3, In intact frog VNC:**

\* To make a comprehensive mapping of ECM molecules in the VNC of the frog, by labeling HA, TN-R, CSPGs generally, and aggrecan.

# MATERIALS and METHODS

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Experimental protocols were revised and licensed by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary, considering national laws and EU regulations [European Communities Council Directive of 24 November 1986 (86/609/EEC)], and was properly conducted under the control of the University's Guidelines for Animal Experimentation (license number: 11/2011/DEMAB).

For purposes of ECM mapping in the VNC, we used female adult Wistar rats (12-14-week old; 250-300 g; n=6), from Charles River Laboratory (Strain CrI: WI); and adult water frogs (n=12) (*Ranaesculenta* L.), taken from natural fishpond environment. For unilateral labyrinthectomy, to investigate alterations of ECM expression during compensation in subnuclei of the VNC, we used adult male Wistar rats (n= 9).

## **Histochemistry**

The biotinylated *Wisteria floribunda* agglutinin (WFA) lectin (bWFA, Sigma-Aldrich, L1516) is a general marker of most of the CSPGs, by recognizing N-acetylgalactosamin. The biotinylated Hyaluronan Binding Protein (kindly provided by R&M Tammi, University of Kuopio, Kuopio, Finland) is the isolated HA binding N-terminal G1 domain of bovine nasal cartilage aggrecan, that specifically recognizes HA. The same histochemical staining was used in LVN of labyrinthectomized rats and vestibular nuclei of frogs.

## **Immunohistochemistry**

For specifically detecting the local expression of ECM molecules we used the following primary antibodies and immunohistochemical (IHC) protocols: anti-aggrecan (Millipore, AB1031); anti-versican (DSHB, 12C5); anti-neurocan (DSHB, 1F6); anti-brevican (BD Biosciences, 610894); anti-tenascin-R (R&D Systems, AF3865); and anti-HAPLN1 (R&D Systems, AF2608). Primary antibodies used on frogs were the anti-CSPG clone Cat-301 (Chemicon-Millipore, MAB5284) and anti-tenascin-R (R&D Systems, AF3865).

## **Visualization**

Following bHABP and bWFA primary markers, sections were incubated with ExtrAvidin Peroxidase (Sigma-Aldrich) followed by 3, 3'-diaminobenzidine-tetrahydrochloride(DAB; Sigma-Aldrich), for conventional light microscopy.

For IHC, the secondary antibodies were the biotinylated goat-anti-rabbit IgG (aggrecan, Vector Laboratories, BA-1000); biotinylated horse-anti-mouse IgG (versican, neurocan, brevican, Vector, BA-2000); or biotinylated rabbit-anti-goat IgG (TN-R, HAPLN1, Vector Laboratories, BA-5000). On rat tissues taken from labyrinthectomized animals visualization was by Streptavidin Alexa 555 (Invitrogen, S32355).

For the ECM mapping on frog we used the secondary reagents of Streptavidin Alexa 488 (bHABP, Invitrogen, S32354); Streptavidin Alexa 555 (bWFA, Invitrogen, S32355); Alexa Fluor 647 anti-goat IgG (TN-R, Invitrogen, A21469); and Fluorescein anti-mouse IgG (Cat-301, Vector, FI-2000).

## **Semiquantitative analysis of results**

In the rat VNC the intensity of PNN staining was semiquantified by subjective scoring of each studied reaction, and nucleus of the VNC. Scorings of PNN intensities are as follows: - : no staining; +: weak staining; ++: moderate staining;+++: strong staining; ++++: very strong staining. The characteristic punctuate appearance of versican staining suggested separate symbolizing.

## **NeuroLucida reconstruction**

In rat, to further describe and compare the distribution differences of PNN-bearing neurons between the nuclei of the VNC, a NeuroLucida reconstruction was made on all nuclei and subnuclei of the VNC, separately for each of the reactions. Representative sections were chosen for reconstruction, being characteristic for each subnucleus. The reconstruction was drawn in NeuroLucida 8.0 program

## **Unilateral labyrinthectomy**

UL was applied to unilaterally deafferentate the VNC. We found the ventrolateral approach of the middle ear cavity the best to keep neck muscles, facial nerve, parotid gland intact, and to expose the tympanic bulla for further interventions. After opening skin behind the

left ear and pressing parotid gland forward, sternomastoid muscle was retracted caudally until preparation reached the posterior belly of digastric muscle, then the tympanic bulla becomes palpable, which is analogue of the middle ear cavity. After opening the bulla with small malleotome, the promontory becomes visible, then through it the inner ear becomes accessible by breaking bone with sword shaped scalpel.

After the lesioned animals returned to awake state, they produced static (ocular, postural) and dynamic vestibular symptoms, due to wide range of vestibular connections. After UL, operated animals survived for 1, 3, or 7 days. Within 7 days static signs almost entirely ameliorized, and dynamic disturbances gradually settled.

# RESULTS

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## DISTRIBUTION OF EXTRACELLULAR MATRIX MOLECULES IN THE VESTIBULAR NUCLEAR COMPLEX OF THE RAT

### *Superior vestibular nucleus*

The intensity and staining pattern of the reactions showed regional differences within the cross section of SVN, but in the rostrocaudal extension no difference was seen. In the central part of the nucleus PNN was present around the large and giant sized neurons, with all studied reactions. Staining was most intense with the anti-aggrecan and anti-brevican reactions, but wasn't as strong with HA, TN-R, WFA, versican, and was the weakest with neurocan, and HAPLN1 reactions. With aggrecan antibody, patch-like immunoreactive spots were seen in perisomatic location. With the anti-versican reaction, in addition to continuous PNN, we also observed heavily stained dots in the pericellular area. The majority of small and medium sized neurons were surrounded by PNNs with variable staining intensity for each reaction, experienced both in the central and peripheral parts of the nucleus, but a minority of small and medium neurons weren't covered by perineuronal ECM condensation.

A Neurolucida reconstruction indicates that large and giant size neurons bear PNNs, positioned centrally in the SVN.

### *Lateral vestibular nucleus*

Opposite to regional differences experienced in SVN, no regional distinction was seen in the staining pattern of the entire LVN. The nucleus is rich in giant and large sized neurons, which were characteristically surrounded by PNNs, whereas the medium and small sized neurons varied in having or lacking perineuronal nets around their somas. The strongest staining intensity was seen with aggrecan and brevican reactions, considered most abundant in PNNs from the CSPG family. The hyaluronan, TN-R, WFA, and neurocan staining appeared strong, but considerably fainter than aggrecan reaction, and the weakest was the HAPLN1, regarding only the PNNs. Comparing versican reaction with the one seen in SVN, in LVN a different pattern was shown, in which no continuous perisomatic ring showed, just few heavily stained dots surrounding the soma.

Neurolucida reconstruction demonstrated the centrally located PNN-bearing large, and giant sized neurons, showing no regional differences in the entire rostrocaudal extent.

#### *Medial vestibular nucleus*

MVN is divided into two well distinguishable subpopulations of neurons, forming the periventricular parvocellular, and ventrolaterally oriented magnocellular subdivisions.

The magnocellular part mostly contains medium- and large-sized neurons, which were characteristically surrounded by PNN, stained strongest for HA, aggrecan, brevican, and TN-R. Slightly weaker staining appeared for WFA and neurocan, the faintest staining occurred with HAPLN1 reaction. As described in LVN, the aggrecan reaction produced some occasionally seen heavily immunoreactive patch like patterns. Versican reaction resulted in dense dot-like staining, localized around cell bodies.

In the parvocellular part, where mostly small sized neurons are present, cell bodies are rarely covered by PNNs. In those few cases, where PNN was identifiable, only HA and TN-R appeared to be positive. Only a few PNN ensheathed neurons were labeled with aggrecan, brevican and WFA staining, and no PNN was recognizable with neurocan and HAPLN1.

Neurolucida reconstructions clearly show the difference in the number of PNN-bearing cells among the MVN MC and MVN PC.

#### *Descending vestibular nucleus*

DVN can be subdivided into rostral and caudal subnuclei. In the rostral part PNNs were widely observed around giant, large, and medium size neurons. The aggrecan and WFA staining produced the strongest staining intensity, then TN-R followed, and the weakest was by HA, neurocan and HAPLN1. Compared to the other nuclei, versican produced much fainter immunostaining, but still with the characteristic dot appearance. The brevican immunoreactions appeared weakest, with no or very faint reaction. The small-sized neurons were mostly not covered by PNN, only the WFA, aggrecan and the TN-R reaction was positive.

A very different ECM distribution and PNN construction was observed in the caudal part of the nucleus. Staining was generally fainter showing the strongest reactions for HA, WFA, versican, and TN-R. PNNs were sporadically visible in weak staining intensity, showing positivity only with versican and TN-R reactions. In case of the other reactions, no PNN was observed in the caudal part.

NeuroLucida reconstruction supports the results on histological sections.

### *Semiquantitative analysis*

The semiquantitative analysis of PNN staining intensities seems to support the morphological results, suggesting that those large and giant size neurons, found in the rostral part of the VNC, are coated with strong PNN. Those neurons transmit the fast response to vestibular stimulus to oculomotor nuclei and cervical spinal cord. Neurons found in the caudal part of the VNC are mostly medium or small sized, and participate in the tonic regulation of motor and autonomic responses, or establish commissural spinal and interneuronal connections.

## **ALTERATIONS OF HA AND CSPGs LEVELS IN THE PNNs OF LVN FOLLOWING UNILATERAL LABYRINTHECTOMY**

On the **first** postoperative day HA staining almost entirely disappeared in the PNN on the operated side, but there wasn't severe change in the intact side LVN. On the **third** day the HA staining in the LVN of the operated side further faded, PNN wasn't recognizable, whereas in the unoperated side the staining intensity was similar to the first stage. After **seven** days of survival intensity of HA staining increased in the PNN on the operated side, PNNs were again recognizable and were rebuilt to appearance slightly under controls. The neuropil staining still showed increased HA expression on the 7<sup>th</sup> day, as it was seen in the 3<sup>rd</sup> postoperative day.

The staining of CSPGs in the PNN by WFA lectin revealed severe fading of PNN labeling on the operated side, suggesting gradual decrease of CSPG levels in the PNN, whereas on the intact side PNNs were still present. Intensity of staining was much fainter in the neuropil as well on the operated side, on the **first** postoperative day in the LVN. On the **third** day of

survival the overall staining pattern did not differ from the one seen on first day, both in the PNN and neuropil. By the **seventh** postoperative day the CSPGs reaccumulated in the PNN, and weak staining of neuropil followed similar dynamics.

## **IN THE FROG PNNs FORM ONLY IN LVN AND MVN, AND SHOW POSITIVITY FOR HA**

### *Superior vestibular nucleus*

The bHABPrevealed a faint or medium signal intensity in NVS. PNNs were not shown at all, only a discontinuous ECM accumulation was seen around perikarya. The TN-R reaction was completely negative, both in the neuropil and perineuronally. No PNNs were recognized in case of WFA reaction, and in the neuropil signals were few and sporadic. With WFA, only some ovoid or circular patterns were labeled, distant from neuron somas having diameter of approx 1,5-17  $\mu\text{m}$ . Those, most probably, represent nodal ECM accumulations. Similar staining was shown by Cat-301 labeling and, some cytoplasmic granules were also observed with Cat-301

### *Lateral vestibular nucleus*

HA staining showed to be most intensive in the LVN, and a continuous PNN was seen around neurons. Interestingly, only HA was present in PNNs of LVN, and some TN-R signal was also seen perisomatically, although continuous PNN wasn't demarcated by TN-R staining. The bWFA didn't mark PNNs, unexpectedly, but the ovoid or round patterns did appear in the neuropil of LVN. With Cat-301 labeling, only cytoplasmic immunoreactive granules were seen, but not PNNs.

### *Medial vestibular nucleus*

There was PNN seen in MVN, but only with the HA reaction. Intensity of labeling was much below the one seen in LVN, nevertheless we may state that PNNs in the frog's vestibular nuclear complex positivity only for HA, and was only present in LVN and MVN. TN-R labeling was negative perineuronally, as well as in the neuropil or in nodes. WFA labeling was only seen in the neuropil, most probably labeling the nodal ECM. With Cat-301 antibody, no cytoplasmic granules showed, only some patches, believed being nodal ECM.

*Descending vestibular nucleus*

Some diffuse patterning was seen in the neuropil with the bHABP labeling, but PNNs weren't identified. There was very weak or even no TN-R signal. Perineuronal patterns didn't appear in case of WFA staining, and even less nodal patterns could be seen. With the Cat-301, very few cytoplasmic granules were identified, and no perisomatic staining could be seen. Neuropil was very faint or even negative.

# DISCUSSION

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## ORGANIZATION AND FUNCTION OF ECM IN THE VESTIBULAR NUCLEI OF THE RAT

### *Superior vestibular nucleus*

**The presence of PNN around neurons in the SVN correlated with the neuronal size, consequently experienced throughout the entire SVN. This also may indicate that neurons found in functionally different regions of the SVN are associated with different PNN staining patterns.** The large-sized neurons establish inhibitory connections with the oculomotor and trochlear nuclei neurons through the ipsilateral medial longitudinal fasciculus containing glycine (horizontal) or GABA (vertical) inhibitory neurotransmitters. They are critical components forming the vestibulo-ocular reflex, while the middle- and small-sized neurons establish commissural connections, or regulate the tone of extraocular muscles for gaze fixation.

### *Lateral vestibular nucleus*

The LVN mostly contains large- and giant-sized neurons, which were all ensheathed by thick perineuronal nets, and **there was no variability experienced in the PNN staining patterns throughout the nucleus, despite the rostrocaudal segregation of function, hodology, and neurochemical properties of LVN.** The rostral portion of LVN coordinates eye movements, while the caudal portion has spinal connections. Differences in distribution of neuronal sizes weren't experienced in the rostrocaudal extension. The possible explanation remains to be elucidated.

**Presence of PNN was in association with neuron sizes, as seen in SVN.**

### *Medial vestibular nucleus*

We found that the staining patterns of PNNs appeared regionally different in the subdivisions of the MVN. Results also suggest that differences are due to the cytoarchitectonical and hodological properties of the neurons composing subnuclei. **We found that PNN ensheathed the large- and medium-sized neurons in the ventrolateral magnocellular portion of the nucleus,** with all eight reactions studied,

although in various intensities, **whereas in the parvocellular part, located periventricularly, no or just few PNNs were labeled.**

Since there are morphologically, hodologically, and thus physiologically different neuron populations composing the MVN, interpreting results demands the identification of neurons according to these parameters.

Neurons of MVN were earlier categorized in mouse, rat, and guinea pig into two major types. Considering the localization of distinct neuron groups in MVN, it is likely that 'type B' neurons are surrounded by PNN. On the other hand the 'type A' subpopulation, having rare or no PNN, represent the parvocellular division of MVN, involved in cerebellar, spinal, and perhaps autonomic regulations. Neurons of magnocellular division are either glutamate-ergic or glycin-ergic and give origin to the vestibulo-ocular and vestibulo-spinal pathways. Parvocellular neurons have reciprocal connections with spinal cord and cerebellum.

**Thus we may suggest, that middle- and large-sized excitatory and inhibitory neurons of MVN are PNN ensheathed mostly located in the MVN MC division, whereas just minority of MVN PC were PNN covered.**

#### *Descending vestibular nucleus*

In the DVN we found that there is considerable difference in the staining of ECM pattern between the rostral and caudal parts of the nucleus. Characteristic appearance of DVN includes rostrocaudally running fiber bundles, which weren't recognizable with of all reactions, possibly due to local expression differences of ECM molecules.

**In the rostral part the numerous large- and giant-sized neurons** have parallel function with the LVN neurons, **participating in the balance, posture, and head movements** by extended vestibulo-spinal connections, and also by being part of the vestibulo-ocular circuit. **Those neurons are all surrounded by strong PNNs.**

In the caudal part of DVN immunostaining was generally very weak. **Small and medium sized neurons are primarily involved in autonomic action,** which due to their

hodological and physiological properties **don't accumulate PNN around somas**, and is in concert with previous reports. Their efferent fibers target the brainstem's autonomic regions, related to respiratory, cardiovascular, and gastrointestinal functions.

### **VESTIBULAR COMPENSATION IS ACCOMPANIED WITH TEMPORARY ALTERATIONS OF HA AND CSPG LEVELS IN THE POSTOPERATIVE PERIOD**

**Our experiment was the first to reveal that amelioration of symptoms corresponds to the reestablishment of PNNs, suggesting the possible involvement of HA and CSPGs in vestibular compensation.** Our, yet unpublished, data suggests that all lecticans, aggrecan, brevican, neurocan, and versican, are deeply involved in the phenomenon seen with WFA.

Even though there is, most probably, no axonal rewiring in the VNC during the postoperative period, severe alterations can be expected in the synaptic transmission efficacy, due to the imbalance between bilateral VNCs. **Besides intracellular events, changes in the ECM is triggered, experienced in the PNN as the decrease of HA and WFA staining intensities.** Plastic synaptic modifications for the remodeling of resting membrane potentials demands structural “loosening” of synaptic elements in order to change physiologically and morphologically for adaptation to new circumstances, induced by deafferentation. Plastic changes of synapses involve two kinds of plastic alterations, (i) the synaptic plasticity, which is the activity-dependent changes in the efficacy of synaptic transmission across existing synapses, but these require simultaneous (ii) anatomical plasticity, or structural plasticity, during which a new anatomical arrangement of connections develop either by new synapse formation or modification of existing ones.

The turnover of intercellular space macromolecules is also in synchrony with the found results. The turnover of sulfated GAGs is approximately 24 h in the brain, by the rapid facilitation of extracellular MMPs, and decrease of synthetic enzyme activities. Similarly, the half life of HA in the intercellular space ranges between less than 1 to several days, which is in accordance with the experienced results after UL.

In the LVN, the giant sized synapses, or calyces of Held, are present. In the trapezoid body, these calyces are surrounded by brevican and aggrecan accumulations, which structural organization is most probably present in the VNC. **These prominent CSPGs give the majority of proteoglycans in the brains, thus we may expect their decreased presence in the background of our results.**

## **FUNCTION OF EXTRACELLULAR MATRIX MOLECULES IN THE VESTIBULAR NUCLEI OF THE FROG**

In the common water frog, our work was the first to describe the molecular composition of ECM in the VNC. This enables evolutionary comparison of taxons, still considering the relatively conserved organization of the vestibular system. **The most important result is that PNNs were exclusively found in the frog's LVN and MVN, which demonstrated positivity only for HA.** Despite the extended number of IHC reactions against various CSPGs, TN-R, and link proteins, we couldn't detect considerable signals in the PNNs. **This result is in strong contrast to findings in rat VNC, where CSPGs, TN-R, and HAPLN1 were present. However, similarly to observations in rat, a strict regional segregation was seen between nuclei of VNC, which also corresponded with function and connection specificities, or even with embryological origin of nuclei.** It is also known that the amphibian CNS has better regenerative and plastic capability than mammals, but the theoretical background of it is not well explored, so far. The HA dominant, but CSPG and TN-R poor matrix could be one explanation.

The HA rich PNNs were only experienced in the LVN and occasionally in the MVN, which is tempting to underline earlier remarks that these two nuclei are primarily involved areas in restoration of damaged vestibular function, also due to a wide range of connections. In amphibians not only functional compensation happens after vestibular damage, but the **HA rich milieu can promote anatomical repair as well, by axonal sprouting and neurogenesis, that will newly establish synaptic contacts.**

# SUMMARY

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We described for the first time the molecular composition and distribution of extracellular matrix (ECM) in the vestibular nuclear complex. Observations were carried out on intact and unilaterally labyrinthectomized rats, and on intact common water frogs.

Our main findings:

- In the vestibular nuclei of the rat, ECM molecules are expressed in an area dependent manner. All forms of ECM are present in rat's vestibular nuclei; diffusely in the neuropil, or in condensed forms as perineuronal net (PNN) or axonal coats, and accumulation in nodes of Ranvier.
- Formation of perineuronal nets is in association with the neuronal size, suggesting that giant and large neurons are ensheated, while medium and small neurons are mostly not covered by perineuronal accumulation of ECM.
- Considerable staining differences were seen, including both neuropil and perineuronal net patterns, between parts of the vestibular nuclei having different afferent and efferent connections. Thus, regional differences correlate with cytoarchitecture, hodological, and functional characters of each vestibular nucleus, and their subnuclei.
- In the rat severe alterations in staining patterns of hyaluronan and chondroitin sulfate proteoglycans occur around neurons of lateral vestibular nucleus following unilateral labyrinthectomy. This finding proves the possible role of ECM molecules in the induction of postlesional disorders.
- The temporal onset of functional and behavioral progress after unilateral labyrinthectomy corresponds with the time course of perineuronal net reestablishment, which suggests the role of matrix turnover in plastic changes of neuronal circuits and compensatory processes.
- In the frog vestibular nuclear complex perineuronal nets are only accumulated around neurons of lateral and medial vestibular nucleus. They are built only by hyaluronan, and devoid of chondroitin sulfate proteoglycans and glycoproteins, enabling better plastic and regenerative properties.
- Parallel with findings in rat, regional difference characterizes the expression of matrix molecules in frog's vestibular nuclei, associating with functional and connection properties.

# LIST of *in extenso* PUBLICATIONS



UNIVERSITY OF DEBRECEN  
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PUBLICATIONS



Register number: DEENKÉTK/245/2014.  
Item number:  
Subject: Ph.D. List of Publications

Candidate: Botond Ágoston Gaál  
Neptun ID: SNBEKV  
Doctoral School: Doctoral School of Dental Sciences  
Mmt ID: 10037882

## List of publications related to the dissertation

1. Gaál, B., Rácz, É., Juhász, T., Holló, K., Matesz, C.: Distribution of extracellular matrix macromolecules in the vestibular nuclei and cerebellum of the frog, *Rana esculenta*. *Neuroscience*. 258, 162-173, 2014.  
DOI: <http://dx.doi.org/10.1016/j.neuroscience.2013.10.080>  
IF: 3.327 (2013)
2. Rácz, É., Gaál, B., Kecskés, S., Matesz, C.: Molecular composition of extracellular matrix in the vestibular nuclei of the rat. *Brain Struct. Funct.* 219 (4), 1385-1403, 2014.  
DOI: <http://dx.doi.org/10.1007/s00429-013-0575-x>  
IF: 4.567 (2013)
3. Deák, Á., Bácskai, T., Gaál, B., Rácz, É., Matesz, K.: Effect of unilateral labyrinthectomy on the molecular composition of perineuronal nets in the lateral vestibular nucleus of the rat. *Neurosci. Lett.* 513 (1), 1-5, 2012.  
DOI: <http://dx.doi.org/10.1016/j.neulet.2012.01.076>  
IF: 2.026

Total IF of journals (all publications): 9,92

Total IF of journals (publications related to the dissertation): 9,92

The Candidate's publication data submitted to the iDEa Tudóster have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

09 September, 2014



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# ACKNOWLEDGEMENTS

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The work, providing basis for the present thesis, was completely made in the Department of Anatomy, Histology, and Embryology, Faculty of Medicine, University of Debrecen. As an agricultural engineer it is a very special privilege for me to meet the PhD requirements of medical science. For this great opportunity I would like say special thank you to all who contributed to this PhD work.

Most importantly, I would like to express my highest gratefulness to my supervisor, Prof. Dr. Klára Matesz, for the endless support in scientific career and in person, motivation, scientific and technical consultations, confidence, and for creating such friendly and inspiring atmosphere in the lab, which is at least as important as scientific standards.

I would like to say special thank you to Prof. Dr. Miklós Antal, chairman of the Anatomy Department, for granting me opportunity in research and education. Academic teaching of anatomy became an important goal to me, and has given me countless wonderful experiences. I never want to forget the support given by Prof. Dr. László Módis, in conventional histological staining, immunohistochemistry, and scientific brainstorming, but before these the personal confidence, friendly chats, and motivation, inevitable for personality progress.

This work couldn't have come true without the contribution of Dr. Zoltán Mészár assistant professor and Dr. Éva Rácz assistant lecturer. From them I could learn details of histological preparation, immunohistochemistry, microsurgery, biochemical methods, image editing, and had many scientific discussions. Besides official duties, we became very good friends.

For the technical assistance I'm very thankful to Ms. Tímea Horváth, professionally managing the laboratory, and giving sophisticated technical solutions, even in the most challenging periods. For the first steps of my lab work I am also very thankful to Mrs. Erzsébet Gregori, Dr. Tímea Bácskai, and Dr. Ádám Deák, and at present Ms. Szilvia Kecskés, Dr. András Birinyi, and Dr. Tamás Juhász. I am also very thankful to all other members of the department for the kind help, continuously provided.

And at last, but above all, I am the most grateful for my dear parents, Prof. Dr. Botond Gaál theologian and Dr. Mária Czeglédy pediatric dentist, for the love and support during my studies and future career.

# FINANCIAL SUPPORT

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MTA-TKI 11008

MTA-TKI 255

OTKA Bridging Fund

OTKA K 67 64 1

TÁMOP-4.2.4B/2-11/1-2012-0001 **Campus Hungary**

TÁMOP 4.2.2/B-10/1-2010-0024 Scholarship for Predoctors

**TÁMOP 4.2.4. A/2-11-1-2012-0001:** This research was supported by the **European Union** and the **State of Hungary, co-financed by the European Social Fund** in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Program'.



Nemzeti  
**Kiválóság**  
Program