THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

ROLES OF THE EXTRACELLULAR MACROMOLECULES IN DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

by

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Debrecen, 2008

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1. Introduction

Neurons are postmitotic cells therefore cell death following renewal of cells in the central nervous system is extremely limited. Moreover, CNS is a sensitive tissue for factors of microenvironment including oxygen and nutrients which result difficulties in regeneration process after damage of the CNS caused by injury or by other disorders. The key for understanding of the basic mechanisms during regeneration is to learn more about the fine details of normal development of the CNS. Although the main landmarks of the developing nervous system are more or less clear, but the molecular mechanisms behind of these processes is started to be understood due to the modern molecular biology techniques, recently. The nervous system develops from the neuroectoderm a part of the dorsal ectoderm that can be found parallel with the long axis of the embryo called neural plate. The term to express this process is the neurulation including the formation of neural plate from ectoderm and neural tube by folding of the lateral aspect of the neural plate. The following cell proliferation, migration and differentiation in the neural tube occur simultaneously. Characteristic feature of the developing CNS including the spinal cord is that proliferating cells mainly can be found close to the lumen while differentiating cells are found laterally from this zone. Therefore the radial migration is the main way of the migrating neurons using the processes of the radial glial cells as a bolster generating layers of neurons in the CNS. Some groups of cells migrate on another pathway generating a subpopulation of neurons in the CNS called nucleus. Accordingly, the CNS is a highly organized structure where all of the neurons have individual destinations required for subsequent development of functional connections via growing axons of neurons**.**

The extracellular matrix (ECM) is present in each animal tissue including that of the CNS. This complex structure contains different macromolecules which interact with each other, with cell membrane receptors, ions and water molecules. The structure of ECM varies during development. Although there are much *in vitro* experimental data on the role of ECM in neuronal differentiation, relevant *in vivo* data are scarce. One of the major peculiar component of the ECM is hyaluronan (HA) that first occurs in the vertebrates during evolution. This large polysaccharide is synthesized by membrane bound HA synthases (HAS1, 2 and 3), and extracellularly it can be bound to cell membrane receptors (CD44, RHAMM), as well as to the lectican proteoglycans (aggrecan, brevican, versican and neurocan) and high amount of water. HA is thought to be involved in many essential functions during development such as cell migration, morphogenesis, matrix assembly, regulation of gene expression. Up to date little is known about the presence and function of HA in the developing CNS. Although HA and HA binding proteins (hyalectins) are abundant in the CNS and they are reported to play important roles in the neuronal development, little is known about the exact function of these molecules. HA can act through lecticans by serving an organized ECM around migrating neurons or HA receptors via HA mediated signaling pathways regulating proliferation, differentiation and neurite outgrowth.

Chicken embryos is an excellent model for *in vivo* investigation of the development of the nervous system because they are commercially available and easy to handle. Therefore most of the data of the developing CNS are corresponding to the chicken embryo.

Regeneration of nerves in the central nervous system is a complex biological phenomenon. To study the mechanisms involved in nervous tissue regeneration embryos and lower vertebrates has been used earlier. The major advantage of these *in vivo* animal models that their CNS are naturally able to regenerate.

2. Aims of the study

Data from the cell-ECM interactions in the CNS are scanty. Extracellular macromolecules around the neurons play important role in development and regeneration of the nervous system. We focused on detecting changes of hyaluronan in two different *in vivo* experimental models. In the first model, we investigated the expression pattern of HA and the molecules binding to HA in embryonic development of chicken spinal cord to provide new data for understanding the possible roles of these molecules during neuronal differentiation. These data may also be important for better understanding the basic mechanisms during the neuronal regeneration. Connection to the first, in the second model we investigated the changes in the ECM structure of nervous tissue during regeneration in frog.

The goals of the study were the followings:

1. Detection and computer assisted image analysis of the distribution pattern and level of HA around various neuron groups of chicken embryonic spinal cord in different developmental stages.
2. Using RT-PCR, detection of expression level of different HAS enzymes in developing chicken spinal cord from same stages what we used for detection of HA.
3. Showing of expression of HA-binding lecticans and non HA-binding phosphacan associated with the changing of distribution pattern of HA and analyis of some major candidate of lectians using RT-PCR methods.
4. In *in vivo* experiments, detection of the changes in the expression pattern and level of HA with computer assisted image analysis after transection of the vestibulocochlear nerve of the frog.

3. Materials and methods

3.1. Animals

3.1.1. Chicken in vivo model

Investigating of the ECM molecules during development of the spinal cord was performed by using chicken embryos. The samples were collected from 8 (embryonic hour 20) to 39 (embryonic day 8) stages of development according to Hamburger and Hamilton (HH).

3.1.2. Frog in vivo model

The nerve regeneration experiments were performed on 96 adult common water frogs, *Rana esculenta*. Under MS 222 anesthesia (0.01%, tricaine methane-sulfonate, Sigma-Aldrich, St Louis, MO, USA) the brainstem was exposed from a ventral approach by an incision of the mucosa on the roof of the oral cavity and by opening the bony capsule of the cranium. In one group of animals (n = 45 of which 27 survived), the brainstem and the root of the eighth cranial nerve were exposed, cleaned and cut using a fine razor blade between the brainstem and the vestibulocochlear ganglion. Special care was taken to protect the blood supply of the nerve and brainstem. The nerve stumps were reunited by aligning the cut ends, and the animals were allowed to recover and were kept at 12 °C in a refrigerator until the end of survival time. Nine different survival periods were investigated, from 3 days to 12 weeks. A sham operated group of frogs (n = 27) underwent the same microsurgery as the operated animals with the exception of the trans-section of eight cranial nerve and these animals were allowed to survive for the same periods as the treated ones. Intact animals (n = 5) without any microsurgery were used for statistical analysis of data obtained from optical density measurements. Two or three operated frogs were re-anesthetized at the end of each survival period and then perfused transcardially with physiological saline solution. On the 6th, 8th and 12th postoperative weeks, some of the operated animals (n = 19 of which 12 survived) were re-anesthetized as described above, the vestibulocochlear nerve was cut with a scalpel distally to the ganglion and Neurobiotin crystals (Vector Laboratories) were placed on the stump of the nerve.

The protocols of these studies was reviewed and approved by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary according to the national and EU laws [European Communities Council Directive of 24 November 1986 (86/609/EEC)], and they were properly carried out under the control of the University's Guidelines for Animal Experimentation. License number: 22/ 2005 DE MÁB.

3.2. Histochemistry

3.2.1. Sample preparation

3.2.1.1 Chicken *in vivo* model

The histochemical reactions were performed in 5-µm-thick paraffin embedded histological sections. For immobilizing the water soluble HA in tissues, the embryos were fixed in alcohol based Sainte Marie fixative (99% absolute ethanol and 1% glacial acetic acid) for overnight at 4 ℃. After washed in 70% ethanol, the embryos embedded in paraffin and transverse sections were made from the lumbar segments.

3.2.1.2 Frog *in vivo* model

Two or three operated frogs were re-anesthetized at the end of each survival period and then perfused transcardially with physiological saline solution. The brainstem with the attached vestibulocochlear nerve was removed and after the meninges were replaced, immersed into Sainte-Marie's fixative for overnight at 4 °C with the same procedure than in the case of chicken embryos. After fixation and dehydration, tissue samples were embedded in paraffin at 54 °C, and 10-µm thick horizontal sections were cut.

3.2.2. Detection of HA in histological sections

HA was detected by using a biotinylated HA-binding complex in 5 µg/ml concentration (bHABC, kindly provided by R. Tammi and M. Tammi, Department of Anatomy, University of Kuopio, Kuopio, Finland) The probe specifically recognize and bind to the decasaccharide unit of the HA polysaccharide and originally derived from the HA binding G1 domain of the bovine aggrecan. Before hyaluronan detection the slides were deparaffinated, treated with 1% hydrogen peroxide and with 1% BSA for 30–30 min. The sections then were incubated with the bHABC solution for overnight at 4 °C. The reaction was visualized either by avidin-biotin complex, H_2O_2 substrate and DAB chromogen (Vector Laboratories Ltd., England), or with Streptavidin-Alexa555 (2 µg/ml, Invitrogen Corporation, Carlsbad, CA, USA) for fluorescence microscopy.

3.2.3. Immunohistochemistry

3.2.3.1. Detection of proliferating and differentiating cells in developing chicken spinal cord.

The proliferating cells were detected by monoclonal antibody against PCNA (1:500, Chemicon, Temecula, CA, USA). The proliferating cells are expressing PCNA (proliferating cell nuclei antigen) in the S phase where acts as a processivity factor for DNA polymerase delta in eukaryotic cells. For detection of different neuronal precursor cells in the embryonic spinal cord monoclonal antibodies were used against their specific homeobox genes. Along this line the postmitotic interneurons showed by their Lim 1 and Lim 2 homeodomain expression (1:100, DSHB, Iowa City, IA, USA). To find the earliest motoneuron precursor cells according to their MNR2 homeodomain expression a monoclonal antibody (1:50, DSHB, Iowa City, IA, USA) were used. The paraffin embedded sections were deparrafinized and rehydrated and then incubated with the primary antibody solutions in PBS for overnight at 4 °C that was followed by repeated rinsing for 10-10 minutes. For fluorescent microscopy

anti-mouse IgG-Alexa488 (1:500, Invitrogen Corporation, Carlsbad, CA, USA) was applied as secondary antibody. Following repeated rinsings, sections were mounted with Vectashield with DAPI (Vector Laboratories Ltd., England).

3.2.3.2. Detection of the intermedier filaments in developing chicken spinal cord.

The perikarya and processes of differentiated neurons were labeled by using anti-tubulin (1:100, Chemicon, Temecula, CA, USA) since tubulin expressed in the cytoskeleton of the cytoplasm of differentiated neurons. For fluorescent microscopy the histochemical procedure was the same method as described above. The immunohistochemistry was combined with fluorescent bHABC histochemistry in all cases.

For selective labeling of the axons of neurons antibodies to neurofilament protein were used. The 68 kDa neurofilament labeled by monoclonal antibody (1:200, Sigma-Aldrich, St Louis, MO, USA) was characteristic for the stages around HH23 while in the older embryos were expressing the 200 kDa neurofilament protein showed by immunohistochemistry (1:200, Sigma-Aldrich, St Louis, MO, USA). The histochemical procedure was the same method as described above.

3.2.3.3. Detections of chondroitin-sulphates and phosphacan in developing chicken spinal cord.

All of the HA binding proteoglycan called lectican core protein are glycosylated with chondroitin-sulphate (CS) side chains. Therefore we showed all of them by using monoclonal antibody to CS (1:500, Sigma-Aldrich, St Louis, MO, USA) in histological sections. Phosphacan a non HA binding CSPG was detected by a monoclonal antibody against the core protein (1:100, Chemicon, Temecula, CA, USA). The sections were incubated with the first antibodies for overnight at 4 °C. For fluorescent visualizing the reaction anti-mouse IgM-Alexa488 or Alexa-568 (1:500, Invitrogen Corporation, Carlsbad, CA, USA) was applied as secondary antibody. Following repeated rinsings, sections were mounted with Vectashield with DAPI (Vector Laboratories Ltd., England).

3.3. RNA based experiments

3.3.1. Purification of RNA from isolated chicken spinal cords

The spinal cord from different developmental ages (HH16, HH23, HH28, HH34 and HH39) was isolated by dissection under stereomicroscope. The embryos were kept in PBS treated with ProtectRNA (Sigma-Aldrich, St Louis, MO, USA) during dissection. We used Qiagen Micro Kit (Qiagen GmbH, Hilden, Germany) according to the supplier's protocol for purifying of totalRNA from 10-15 mg spinal cord samples.

3.3.2. RT-PCR

Lectican and HAS expression were examined by RT-PCR from isolated chicken spinal cord samples. 2 µg of purified RNA were used for first strand cDNS synthesis by applying Qiagen Omniscript first strand synthesis cDNA kit (Qiagen GmbH, Hilden, Germany) according to the supplier's protocol. For amplification of PCR fragments we used 0,2 µg cDNS for each reactions. PCR reaction was carried out GoTaq polymerase (Promega, Mannheim, Germany). PCR conditions were as follows: 1× 94 °C for 2 minutes; 35× [95 °C for 50 seconds, 52-63 °C for 1 minutes, and 72 °C for 1 minutes], 72°C for 7 minutes. PCR products were separated in a 1.5% agarose gel and visualized with acridine-orange. Specific primers were selected by using Primer3 software on templates downloaded from NCBI. The sequences of primers used was following: Has2 (330) for 5'-GAGACGACAGGCATCTAACTAAC-3', rev 5'-AAGACTTTATCAGGCCCACTAA-3'; HAS3 (192) for 5'-CCAACAGACCCGCTGGAGCA-3', rev 5'-ACCGTCAACAGGAAGAGGAGGATG-3'; Aggrecan (304) for 5'-TGTTACATCGACAGGCTAAAGGG-3', rev 5'-AAGCGTGATGCCGTGACAGA-3'; Brevican (405) for 5'-GTGCCTCCTTGCCAGTCTTCCAG-3', rev 5'-GGTCCACCACGCCGTAGTTCCT-3'; Neurocan (269) for 5'-GGCGCTCGCTATGCACTGACCTT-3', rev 5'-TCCCGTGCGTAGCAGTAGACATCGTA-3'; GAPDH (366) for 5'-CTGCCCAGAACATCATCCCA-3', rev 5'-CACGGTTGCTGTATCCAAACTCAT-3'. Sizes of the amplified fragments are noted in brackets.

3.4. Computer assisted image analysis

3.4.1 Chicken in vivo model

Following bHABC-histochemistry the sections were used for image analysis. Digital images were captured by a common light microscope (Olympus AX 70, Japan) equipped with a cooled CCD camera (Olympus DP 70, Japan) using monochromatic light (λ = 492 ± 5 nm). After grayscale calibration, the intensity of HA-reaction, i.e. the relative local concentration of HA was measured in different regions of the lumbar spinal cord from stage HH23 as previously described. Double-labeled fluorescent images were captured by a Nikon Eclipse 800 (Japan) microscope equipped with a Spot Rt-slider (Diagnostic Instruments, Sterling Heights, MI, USA) CCD camera. Confocal imaging was achieved with a FluoView1000 confocal microscope (Olympus, Japan) suited with a He/Ne laser. Confocal stacks were processed using Image J software (NIH, Bethesda, MD). Acquired and presented images were representative of all the samples examined. For documentation, images were processed using Adobe PhotoShop software (Version 8.0, Adobe Systems Inc., San Jose, CA, USA). The total area of bHABC-histochemical staining was measured for each region of interest (ROI) using Image J software. Pictures were recorded from ten different serial sections and on each of them five independent ROIs (size 250 μm^2) were determined at three different locations (ventricular, intermediate, marginal zones), where the measurements were performed. After background subtraction the settings of the microscope and camera were kept constant during the study and area-integrated mean values were calculated. Finally the measured grey level values converted to optical density

values. Cell densities of the same microscopic regions from parallel sections were measured in hematoxylin-stained sections by the same set-up applying cell cytoplasm segmentation. Finally, using Microsoft Excel the ratio of the total area of segmented cells in each ROI compared to the total area of ROI were calculated and compared to each other at these three distinct regions of the lumbar spinal cord.

3.4.2 Frog in vivo model

Detection of changes of the HA in frog vestibulocochlear nerve, and its entry zone as well as in the vestibular nuclei was performed by similar method as the chicken described above. Two-five digital images were taken from both sizes of the cross sections of the brainstem containing the eights cranial nerve per individual by using with a transmission light microscope (NIKON Eclipse 800) suited with a 12-bit Peltier-cooled digital camera (Spot Diagnostic Instruments, Sterling Heights, MI, USA) and a KAF 1400 charge coupled device (CCD) detector. Within the confines of the TZ, MVN and LVN the area of the region of interest (ROI) for all measurements was 400 μm2. The area-integrated mean optical density values (AIOD) were then calculated by weighing the intensities of pixels from the ROIs by the numbers of pixels in the ROI, and the AIODs were used as a measure of optical density of HA reaction.

3.5. Statistical analysis

The ratio differences between the three distinct regions of the lumbar spinal cord were tested by Mann-Whitney's U-test using PAST statistical software.

First we tested the dependence of the HA optical density in intact animals (n = 5) on the individual section and on the individual animal used. For these tests the solitary tract was chosen as a reference area for the reasons that it can be easily identified because of its clear-cut border and the HA reaction of the solitary tract showed the weakest intensity in the brainstem. Two-factor analysis of variance (ANOVA) test did not reveal any significant variability in optical density of HA reaction between intact controls either among different sections (n = 6–8/ animal, $P > 0.29$) from the same animal or among different animals (n = 5, $P > 0.34$). Therefore, we did not have to differentiate between individual sections taken from the same structure or between identical structures of different animals in this study allowing us to pool data for the structures studied (TZ, MVN, LVN) from all animals and sections. Statistical analysis also revealed that there was no significant difference between the sham operated group from each survival time point and the group of intact animals. Therefore for the best possible normalization and comparison of the data we used intact animals as a control for each survival time point.

Changes of HA reaction intensities during regeneration was tested with reaction optical density normalized by the mean control optical density. Mean control optical density was calculated from AIODs measured in three–six sections of identical structures of an operated animal incubated with

sections of intact animals in the same solution. Normality of frequency distributions of optical densities was tested by two criteria: (1) 0.9 < median/mean < 1.1 and (2) 3x standard deviation < mean. Equality of variances was checked by F test. Hyaluronan (HA) reaction densities at different postoperative days were compared by unpaired two-tailed t-tests, possible trends of changes in densities were tested by the slope of linear regression lines and the possible interrelation between the operated and un-operated sides was examined by the two-tailed Fisher's exact test. For statistical tests, MS Excel (Microsoft Corp. Redmond, WA, USA) and GraphPad softwares (GraphPad Software Inc. San Diego, CA, USA) were used.

4. Results and discussion

4.1. Age dependence distribution of HA in chicken embryos

4.1.1. Control of specificity of the bHABC histochemistry

We examined whether bHABC bind only to the HA both in frog and chicken. Using cross section of whole embryos, the developing cartilage of the chicken vertebrates and sternal cartilage of the frog showed intense HA signal at the the territorial and interterritorial matrix, which served as an excellent internal positive control. Three parallel negative control experiments of the bHABC histochemistry has been carried out: (1) histochemistry without the probe, (2) with the bHABC after the HA was digested by *Streptomyces* hyaluronidase and (3) with inactivated bHABC probe by added extra HA to the probe solution. No HA signal has been detected after these experiments.

4.1.2. Distribution of HA in the spinal cord of different stages of development

The HA was detectable in the spinal cord from HH8 stage and in almost all stages. However two definitive peaks in the reaction of HA according to the size of the positive area has been realized. The first peak could be found as early as HH24 stage (embryonic day 4), and the second peak could be detected in the older embryos where the highest relative area was found in the HH39 stage (embryonic day 8). There was no detecteble signal of HA reaction in only one stage (HH34) that was in the middle age between the two ages corresponding to the HA peaks. The most interesting stages were related to the highest HA positive areas. The HAS2 and HAS were responsible for accumulating HA in the spinal cord in all examined stages showed by RT-PCR.

4.1.3. Layered distribution HA were detected from HH23 stage onwards

As it meantioned earlier the hyaluronan distribution pattern changed around stage HH23. A mediolaterally oriented pattern could be detected in the alar and basal plate in the ventricular, intermediate and marginal zones. The ventricular zone showed a weak HA signal, the strongest HA accumulation was detected in the intermediate zone and a moderate HA reaction was found in the marginal zone. These mediolateral patterns of HA staining were confirmed by optical densitometry. The cell density decreased along a mediolateral axis with the highest cell density around the central canal. This implies an increased extracellular space in the lateral part of the spinal cord of stage HH23 embryos that may be associated with the relative concentration of HA, since HA was present mainly in the extracellular space. After correction for density of the cells, the highest relative concentration of HA was measured in the intermediate zone. This finding assumes the specific effect of HA rather than its space filling role.

4.1.4. HA accumulates around various differentiating neurons

Using fluorescent double labeling, increased hyaluronan accumulation was detected in the intermediate zone where the numbers of PCNA-immunopositive proliferating cells were suddenly decreased compared to the ventricular zone. The differentiating neurons of Lim1,2 positivity and early cells with motoneuronal fate expressing the MNR2 homeodomain demarcated the HA reach area in stage HH23. Consequently the proliferating and differentiating cell populations were separated by an HA layer. The question whether the differentiating (Lim1,2 and MNR2 positive) cells producing HA or the proliferating cells in the S phase remains open. The question whether HA permissive for proliferation, differentiation or lateral migration of cells in the embryonic spinal cord it remains unclear. There are some data on the presence of HA in the developing CNS. These findings suggest that HA molecules may be required for differentiations of neurons. At this moment the exact molecular mechanism of the role of HA on the neuronal differentiation is poorly understood. It is very likely that the highly hydrated HA has a permissive role in the radial migration of spinal cord neurons. The involvement of HA in migration of neural precursor cells has been proposed by other researchers in the developing mouse cerebellum. Nevertheless, HA can act through CD44 or RHAMM pathways that activate small GTPases, resulting in cytoskeletal reorganization and lamellipodia formation in various cell types. Since neither CD44 nor RHAMM immunopositivities has been detected on these special locations, therefore the question whether this function may be due to the HA molecules alone or the complexes with other ECM molecules remains open. Because HAS2 and HAS3 also can bind to HA, we believe that HA involved in the regulation of migration and differentiation of nerve cells with an autocrine manner.

4.1.5. Distinct distribution profile of HA around the axons in the CNS and PNS

The development of the white matter was studied with antibodies against neurofilament in combination with bHABC histochemistry. Different distribution patterns of HA in the various funiculi both in early (HH23) and later (HH29) stages have been observed. The lateral funiculus showed a strong HA signal while the anterior funiculus exhibited a moderate reaction. The origins of the spinal cord cells of the lateral funiculus are also reach in HA. Moreover, the HA reach lateral funiculus may be related to the developing reticulospinal and spinocerebellar tracts indicating the permissive role of HA in growing axons of these pathways. The transitional zones were also HA negative. A distinct pericellular HA reaction was found around the motoneurons in HH29 and HH39 stages. The axons of peripheral nerves did not show any detectable HA signal opposite to the white matter and perikarya of the neurons including motoneurons. This may be due to the distinct glial cells between the CNS and peripheral nerves or due to the polarized HAS expression of the neurons. The later speculation is more likely because according to other researches the differentiated oligodendroglial cells are emerging first only later stages. There was a strong pericellular HA signal around the perikarya and the proximal

dendrites of the motoneurons in the HH39 stage. The end of the critical period of synaptogenesis is correlating with the increased pericellular accumulating of HA and lecticans, namely forming the perineuronal net. The perineuronal net is characteristic only in postnatal age in mammals. The fact that a hatched chicken already has been able to the step locomotion indicates that the synaptogenetic critical period of the motoneurons of the spinal cord occur earlier. This may explain the earlier perineuronal net we found around the motoneurons.

4.2. Studying of the occurrence of the chondroitin-sulphate proteoglycans in developing spinal cord

4.2.1. Age dependence distribution of the chondroitin-sulphate

Since chondroitin-sulphate (CS) is binded to the core proteins of proteoglycans, using monoclonal antibody to CS all CSPGs occur in the developing nervous system could be detected. One large group of the CSPG is the lectican and the other is non HA-binding PGs such as the phosphacan. The distribution pattern of the CS immunoreactive area was considerable overlapped to the HA positive regions in all stages of development. These findings suggest that lecticans are present in those regions which were confirmed by RT-PCR as well. We found detectable level of aggrecan neurocan and brevican with an age dependent manner. The constant neurocan expression was characteristic in all stages examined while aggrecan and neurocan expressions were detectable only from HH23 stage.

Generally lecticans are bound to HA by the N-terminal G1 domain and to other ECM proteins (especially to tenascin) or cell adhesion molecules with the C-terminal end of the core proteins. The middle part of the core protein glycosylated with CS giving polyanionic character to the lecticans which important for regulating the concentration of ions around the cells. Since CS content of the lecticans can be variable, regulation of the core protein can also affect to the relative amount of the CS. The aggrecan contains the largest number of CS side chains while the neurocan and brevican have definitely less. Lecticans were described in neural crest that inhibited the cell migration, but data about the exact role of lecticans in CNS are scarce. The functions of CS around proliferating neurons as we have seen in HH23 stage are also not well characterized. According to the function in the neural crest they were supposed to be responsible for separating the proliferation cell zone from the others. Therefore the positive CS immunoreactions around the central canal maybe associated with inhibiting neuron precursor migration toward to the central canal. Near to the non permissive effect of lecticans to the migration of cells inhibition of cell proliferation were also detected. The involvement of the CS side chains of lectican in these functions was presumable because chondroitinase ABC treatment could suspend these functions. Functional correlation between CS and growth factors such as HB-GAM/PTN (heparin binding growth association molecule/pleitrophin) were proven by other researchers indicating that CSs bound mainly to lecticans always act in combination with other molecules.

4.2.2. The expression pattern of phosphacan in spinal cord of chicken embryos

Phosphacan is a member of non HA binding CSPG group which thought to be expressing only in the nervous system. Using chicken specific monoclonal antibody to phosphacan, we investigated the expression pattern of this CSPG in the spinal cord of different stages of development to learn more about the possible function of the phosphacan in neuronal development. The expression was detected as early as HH23 stage. The reaction was positive in the peripheral nerves, around the cells of the roof plate, in the intermediate zone of the alar plate and around the radial glial cells. The immunohistochemical reaction was also positive in the entry zone of primary afferents while we could not detect positive signal in the anterior commissure. The size of the immunopositive area was increasing in the spinal cord of older embryos. The signal was still well visible in the entry zone in the HH28 stage, while the signal was vanishing in the roof plate. The dorsal horn of the spinal cord grey matter of the HH35 stage embryos showed the weakest signal and the other area showed stronger immunopositive reaction especially around the cells surrounding the central canal. The spinal cord of the HH39 stage showed the strongest immunopositive signal, we could detect phosphacan around each cells. Only the dorsal horn was weaker compared to other regions that may was related to the forming of the perineuronal net. Generally perineuronal net is thought to stabilize synapses but inhibits newly forming connections. The peripheral nerves also were positive but the reaction was definitely stronger in the entry zone in all stages. Moreover in earlier stages, the phosphacan immunreaction was strong around proliferating neurons. It is thought that phosphacan can potentiate the mitogen activity of FGF2. We assume the same function of phosphacan in our system.

4.3. The effect of vestibular nerve section on the expression of the hyaluronan in the frog, *Rana esculenta.*

Following postganglionic lesion of the eighth cranial nerve, the changes in the expression of hyaluronan (HA), one of the extracellular matrix macromolecules, were examined in the medial (MVN) and lateral (LVN) vestibular nuclei and in the entry or transitional zone (TZ) of the nerve in the frog. HA was detected in different survival times by using a specific biotinylated hyaluronan-binding probe. HA expression was defined by the area-integrated optical density (AIOD), calculated from pixel intensities of digitally captured images. During the first postoperative days the perineuronal net (PN), a HA-rich area around the neurons, was not distinguishable from the surrounding neuropil in the MVN and LVN, characterized by a bilateral drop of AIOD specifically on the operated side. From postoperative day 14 onwards AIOD increased whilst the PN reorganized. In contrast, the AIOD wobbled up and down bilaterally without any trend in the TZ. Statistical analysis indicated that AIOD changes in the structures studied ran parallel bilaterally presumably because of the operation. Our results demonstrated for the first time that (1) the lesion of the eighth cranial nerve is accompanied by

the modification of AIOD reflected HA expression in the MVN, LVN and TZ, (2) different tendencies exist in the time course of AIOD in the structures studied and (3) these tendencies are similar on the intact and operated sides. Our findings may suggest an area dependent molecular mechanism of HA in the restoration of vestibular function.

5. Summary

One the major component of the extracellular matrix (ECM) is hyaluronan (HA) that is important for organization of other ECM molecules and in the regulation of cell-matrix communication.

We investigated the expression pattern of HA and HA-binding chondroitin-sulphate proteoglycans (CSPG) in spinal cord of chicken embryos in order that providing new data about the possible roles of these molecules in differentiating neurons and axon guidance and sprouting. Using biotynilated HA specific binding complex (bHABC) combined with immunofluorescent labeling of differentiating neurons in histological sections of spinal cords, HA was surrounding around neuroprogenitor cells before their postmitotic stage. These finding suggested the non-permissive role of HA in neuroprogenitor cell proliferation but it was possible that permissive for differentiation. We showed by RT-PCR two hyaluronan synthases HAS2 and 3 expressed by the developing spinal cord in each stages indicating that these molecules were producing HA and they might also were functioning as a HA receptor binding it the to the cell surface. By using RT-PCR and histotechnique, we also found a set of HA-binding CSPGs (lecticans) expressed by embryonic spinal cord. The expression pattern of these lecticans were largely overlapped with the HA reaction suggesting a functional relationship between the lecticans and HA to form HA-lectican complexes. Among lecticans the neurocan was expressed in earlier stage of development while the highly glycosylated lectican (aggrecan) was expressed by older embryos. Increasing of the relative amount of chondroitin-sulphate during differentiation of the spinal cord suggests that both chondroitin-sulphate and core protein has functional role in embryonic development. Another CSPG, phosphacan also were expressed by developing spinal cord. We found that phosphacan was accumulating around proliferating neurons and in the peripheral nerves and in their entry zone as well as in the white matter. Interestingly the axons in the grey matter did not contain phosphacan.

In the second part of our work we found changes in distribution of HA after transsection of the vestibulocochlear nerve in frogs. During regeneration HA decreased in the vestibular nuclei that received the primary afferents, including structural changes in the perineuronal net. These findings suggest a non-permissive role of HA to the axon sprouting in the central nervous system while the HA might be permissive in the peripheral nerves which indicated by the finding of increased HA relative amount during regeneration.

6. Publications

This thesis is built upon the following publications:

Meszar Z, Felszeghy S, Veress G, Matesz K, Szekely G, and Modis L. 2008. Hyaluronan accumulates around differentiating neurons in spinal cord of chicken embryos. Brain Res Bull 75:414-418. **IF: 1,684**

Halasi G, Wolf E, Bacskai T, Szekely G, Modis L, Szigeti ZM, **Meszar Z**, Felszeghy S, and Matesz C. 2007. The effect of vestibular nerve section on the expression of the hyaluronan in the frog, Rana esculenta. Brain Struct Funct 212:321-334. **IF: 1,277**

Other publications:

Felszeghy S, **Meszar Z**, Prehm P, and Modis L. 2005. The expression pattern of hyaluronan synthase during human tooth development. Arch Oral Biol 50:175-179. **IF: 1,288**

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