CENTRAL CONNECTIONS AND REGENERATION OF THE VESTIBULAR SYSTEM

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Debrecen, 2005

1. INTRODUCTION

The vestibular system has an important role in the control of posture and movements of the body and it mediates these motor activities through a network of receptors and neural elements. Maintenance of position of body upon the stimulus of vestibular apparatus and proprioceptive receptors requires a continuous flow of information from the peripheral receptors to the vestibular nuclei of the brainstem. The processing of information takes place very rapidly in these nuclei and their output is involved in the regulation of the tones of muscles, in the maintenance of compensatory eye movements and in the modification of different vegetative function. The vestibular system appears in a very early period of phylogenesis and its highly conserved structural and functional organization is recognizable in the lower and higher vertebrates. Receptors sensitive for linear acceleration are the otolith or macular organs located in the saccule and utricle and, in lower vertebrates in the lagaena. Ampullary receptors of the semicircular canals are sensitive for the rotational movements in any conceivable plane. The vestibular receptor cells are innervated by the peripheral processes of the vestibular ganglion cells, whereas the central processes terminate in the superior, medial, lateral and descending vestibular nuclei. The secondary vestibular neurons located in these nuclei are in connection with different areas of the central nervous system (CNS).

Numerous experiments were performed to study the ascending and descending fiber connections of the individual vestibular nuclei, which convey their most versatile function, but unfortunately the different functions of these nuclei are known only to a limited extent. The functional differences between the vestibular nuclei can probably be explained by their different input from each vestibular receptor, and by their different connections with the structures of CNS. Little is known about the reciprocal connection of the vestibular nuclei with their termination areas. In order to understand the neural mechanism of the vestibular compensation it is necessary to be acquainted with the morphological background on the connections of individual vestibular nuclei. The normal map of these connections may serve as a basis to follow any changes caused by vestibular lesion and may give information about the involvement of the different nuclei in the mechanism of the subsequent vestibular compensation. In lower vertebrates the compensation is followed by regeneration of the lesioned fibers. To understand the morphological and physiological changes during the vestibular regeneration, the knowledge of normal connections at light- and electron microscopic level is inevitable.

It has been recently suggested that the macromolecules of the extracellular matrix (ECM) play an important role during the development and organization of the nervous system. It has been observed that qualitative and quantitative differences in the distribution of these molecules can modify the regenerative capacity of the nervous system. The possible role of the ECM in neural regeneration was previously examined in *in vitro* experiments. Taking advantage of the regenerative capacity of the frog vestibular nerve, the changes in the expression pattern of ECM molecules and their receptors, the alterations in the signal transmission mechanisms and the changes in the expression of neurotransmitters can be examined in *in vivo* studies. The expected results may provide new insights into fundamental mechanisms of neural regeneration and plasticity.

2. AIMS OF THE STUDY

2.1. Central connections of the vestibular nuclei

- Investigation of the antero- and retrograde connections of the descending vestibular nucleus (DVN) in the rat in order to map its central connections.
- Investigation of fine structure of terminals of superior vestibular nucleus (SVN) with the oculomotor and red nuclei in the rat.

2.2. Distribution of the extracellular matrix (ECM) macromolecules and their possible receptor, dystrophin-glycoprotein complex (DGC), in the nervous system of the frog

- Identification of distribution pattern of the hyaluronan, laminin, tenascin-C, fibronectin and phosphacan in the nervous system of the frog.
- Investigation of distribution pattern of the subunits of DGC, an ECM receptor, in the frog nervous system.

2.3. Possible role of hyaluronan and laminin in the vestibular regeneration

- Investigation of the changes in the distribution pattern of hyaluronan and laminin following axotomy of the vestibulocochlear nerve in the frog.

3. MATERIALS AND METHODS

3.1 Animals used in experiments

The studies on the central connections of the vestibular nuclei were carried out on Wistar rats under urethane anesthesia. The experiments on the distribution pattern of ECM macromolecules and DGC and studies on the vestibular regeneration were performed on common water frogs (*Rana esculenta*) under tricain-methane-sulphonate (MS-222) anesthesia in accordance with state regulations.

3.2 Tracing methods

For mapping the antero- and retrograde central connections of the descending vestibular nucleus (DVN) the neurobiotin, taken up by axon terminals and dendrites, was applied as a neuronal tracer. For electron microscopic analysis of the axon terminals of the superior vestibular nucleus in the oculomotor and red nucleus the *Phaseolus vulgaris* leucoagglutinin (PHA-L) tracing method, described by Gerfen and Sawchenko (*1984*), was performed. The PHA-L is applied as an anterograde tracer in mammalian species.

Under urethane anesthesia the head of the animal was fixed in a stereotactic holder, the cranial cavity was opened and a glass micropipette filled with a solution of tracer was introduced into the appropriate vestibular nucleus according to the coordinates of Paxinos and Watson (*1998*). Positive direct current of 5 μA was used for the injection with a pulse duration of 7 second, followed by 3 second intervals for a period of 15-20 minutes. After a survival period of 8-14 days the animals were re-anaesthetised and transcardially perfused. Cross sections of diencephalon, brainstem and the spinal cord were made at a thickness of 60 μm by using a vibrotome. Sections were incubated with avidine-biotin complex (ABC) and the reaction was visualized by applying Ni-diaminobezoline (Ni-DAB). The labeled fibers, terminals and retrogradely labeled cells were drawn with Neurolucida equipment.

3.3 Electron microscopical experiments

During preembedding procedures, 60 μm sections of the midbrain containing the mesencephalon from the PHA-L labeled animals were incubated with biotinylated goat-anti-PHA-L (1:2000) on 4ºC and subsequently with avidin-biotin peroxidase complex. The immunoreaction was completed with a DAB chromogen reaction. Following incubation in postfixative of 0.5% osmium tetroxide the sections were flat embedded in Araldite (Durkupan ACM). During postembedding procedures selected areas containing the oculomotor and red nucleus were re-embedded, serial ultrathin sections were cut and

collected on Formvar-coated nickel grid. Sections on every second grid were processed for GABA immunohistochemistry using a rabbit-anti-GABA (1:1000). The secondary immunogold conjugate goat-anti-rabbit IgG (1:20) was connected to these complexes in order to visualize them in EM (golden granules diameter: 20 nm). Ultrathin sections were stained with uranyl acetate and lead-citrate and examined with Jeol 1010 electron microscope.

3.4 Immunohistochemical identification of the extracellular matrix (ECM) macromolecules and the subunits of an ECM receptor, dystrophin-glycoprotein complex (DGC)

The experiments were performed on common water frogs, *Rana esculenta*. Under MS 222 anesthesia (0.01%), the animals were transcardially perfused with physiological solution. The brain and the spinal cord with the attached cranial and spinal nerves were removed and immersed into Sainte-Marie's fixative (99 ml of 96% ethanol, 1ml of glacial acetic acid). The specimens were embedded in paraffin and either horizontal or transverse sections were made at a thickness of 10 μm. Following dewaxing hyaluronan (HA) probe and different antibodies were used to detect the macromolecules of the ECM and DGC subunits. For detection of HA, a specific biotinylated hyaluronan-binding probe (bHABC) (kindly provided by R. Tammi and M. Tammi, Department of Anatomy, University of Kuopio, Kuopio, Finland) was used. This probe is made of the biotinilated hyaluronan-binding site of aggrecan, and makes possible to detect the polysaccharide chain of hyaluronan. The negative controls included sections incubated without bHABC or with bHABC but after treating the sections with *Streptomyces* hyaluronidase. For positive controls, we used sternal cartilage of frog. For immunohistochemical detection of other ECM molecules, the following primary antibodies were used overnight at 4 ºC: mouse anti-human tenascin C (1:4000), mouse anti-chicken phosphacan (1:40), mouse anti-human fibronectin (1:400), rabbit anti-mouse laminin (1:200). The signal was visualized with ABC complex and DAB chromogen. The negative controls were performed by omitting the primary antibody. For positive control of laminin, tenascin-C and fibronectin the frog kidney was used and in case of the phosphacan reaction the cerebellum of the frog and rat were examined.

To detect the dystrophin and beta-dystroglycan, the mouse-anti-dystrophin (Dys2, 1:25) and rabbit-anti-β-dystroglycan (BDG, 1:200) were used as primary antibodies. The secondary antibodies were the biotinilated goat-anti-mouse IgG (1:500) and biotinilated goat-anti-rabbit IgG (1:500), respectively. The signal was visualized with ABC complex

and DAB chromogen. For fluorescent and laser scanning confocal microscopical analysis, Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were used as the secondary antibodies.

3.5 Experiments on vestibular regeneration

Under MS 222 anesthesia the brainstem of the frog was exposed from a ventral approach by an incision of the mucosa on the roof of oral cavity and by opening the bony capsule of cranium. The root of the eighth cranial nerve was exposed, cleaned and sharply transsectioned approximately 1 mm distal to the brainstem, but proximal to the vestibulo-cochlear ganglia. The nerve stumps were reunited by reposition of the cut ends and animals were allowed to recover. After a period of survival from 3 days up to 12 weeks the frogs were re-anesthetized and the brainstem with the eighth cranial nerve was fixed, embedded and sectioned as described above in 3.4. The expression pattern of the HA and laminin was examined at the lesioned and intact sides. The regenerated fibers were detected by using neurobiotin as a tracer.

In order to follow the quantitative changes in the distribution pattern of HA during vestibular regeneration the area-integrated mean optical density of the transitional zone (TZ) of the vestibulocochlear nerve, and of the medial (MVN) and lateral (LVN) vestibular nuclei was measured separately on the operated and non-operated side by using Image J software. Analyses of measured data were performed with different statistical methods. Two-way ANOVA test was performed to avoid measurement-errors and to examine the possibility of significant differences between sections or between animals. Paired T-test was applied to compare the changes in the intensity of HA-probe between two different survival times. Fisher-exact test was used to evaluate the direction of the changes in intensity between the operated and non-operated side. In order to compare the HA-probe intensity and the direction of changes in intensity between structures the values of the HA-probe intensity and values obtained from a sham operated animal were normalized. The criteria to test the normal distribution were the following: (1) $0{,}9 < \text{median/mean} < 1{,}1$ and (2) $3 \cdot \text{standard deviation} < \text{mean}$. The equality of variances was controlled by using F-test.

4. RESULTS AND DISCUSSION

4.1 The central connections of the descending vestibular nucleus (DVN)

Following neurobiotin injection into the DVN the tracer diffused in an area of 200 μm in the rostral part of the nucleus showing a round-shaped black spot. The axons, terminals and perikarya were also visible in black color.

4.1.1 Anterograde projections

The rostralmost projection from the DVN was detected in the diencephalon bilaterally, mainly in the ipsilateral side, and the termination areas were in the thalamic ventral posteromedial nucleus and zona incerta. This result corresponds to those findings that the posteroventral nucleus of the thalamus is the primary termination area of the vestibular fibers, and this area with the zona incerta is an integrative center for somatic and vestibular inputs playing an important role in the maintenance of the balance and posture.

In the midbrain, the fibers of DVN traveled in the medial longitudinal fascicle and reached the contralateral interstitial nucleus of Cajal and the nucleus Darkschewitch. The fibers terminated among the motoneurons of oculomotor and trochlear nuclei bilaterally. In the mesencephalic nuclei related to the eye movements the terminals of DVN origin were found in less number than the terminals of superior (SVN) and medial (MVN) vestibular nuclei. This founding is in agreement with previous results indicating a minor role of DVN in the control of vertical eye movements, compared to SVN and MVN. We have demonstrated for the first time the connection of the DVN with the magno- and parvocellular parts of the red nucleus, which is an important center of the motor-coordinating system. The red nucleus receives cortical and cerebellar fibers and projects to the spinal cord, inferior olive and the other parts of the brainstem. The parvocellular part receives inputs from the cerebellum, and its main output, the rubrothalamic tract terminates in the motor, premotor and parietal parts of the cerebral cortex. The cortical areas are in reciprocal connection with the red nucleus. The rubrospinal pathway takes its origin from the magnocellular part of the red nucleus. Our result indicates that in addition to the well-known indirect control, the vestibular system has a direct influence on the function of the red nucleus.

At the level of pons the ipsilateral fibers of the DVN were followed mainly to the territory of lateral vestibular nucleus (LVN), MVN, and SVN establishing the so-called intrinsic connections of the vestibular nuclei. Another group of fibers terminated in the spinal nucleus of trigeminal nerve (nspV), in the paragigantocellular and intermedier areas

of the reticular formation. The medially running fibers reached the bilateral abducens nucleus with an ipsilateral dominance demonstrating the involvement of the DVN in the coordination of horizontal eye movements. Several commissural fibers were followed to all the contralateral vestibular nuclei similarly to the commissural fibers described previously at the MVN. The commissural fibers play an important role in the compensatory processes following vestibular axotomy. The contralateral termination areas in the reticular formation were similar to that of the ipsilateral side.

The fibers in caudal direction were found in the medial longitudinal fascicle and terminated in the following structures of the medulla oblongata: nucleus gracilis and cuneatus, the nspV, the nucleus of the solitary tract, nucleus prepositus hypoglossi, the ventral and dorsal parts of the reticular formation, and the caudal part of MVN and DVN. Our results suggest that the DVN may influence the activity of the vegetative nervous system and may control the motor coordination through the reticular formation. The nucleus prepositus hypoglossi is connected with the central cervical nucleus of the spinal cord and with the cerebellum and plays an important role in the integration of the spinal proprioceptive and vestibular input. The connections between the DVN and medullary sensory nuclei suggest that the vestibular input can modulate the processing of sensory information mediated by these nuclei. The inferior olive received only a few fibers and terminals from the DVN. In accordance with the previous results the descending fibers of DVN were followed to the thoracic level of the spinal cord running in each funiculus of the white matter. Most of the descending fibers were found in the ipsilateral anterior funiculus and in the anterior part of lateral funiculus. The major termination area of these fibers was at the cervical level of the spinal cord and the terminals were distributed mainly in Rexed laminae VIII and IX, and in a smaller amount in laminae II–VII. The central cervical nucleus was richly supplied from the DVN ipsilaterally. Our results suggest that the descending fibers of the medial longitudinal fascicle are responsible for the appropriate compensatory movements of the head during the changes in the position of the body. We propose that the motoneurons of the axial muscles located in the medial part of ventral horn are under a direct control of the DVN.

4.1.2 Retrograde projections

Neurobiotin was used to label the retrograde connections of the DVN. We could not detect any retrogradely labeled neurons at the level of diencephalon and mesencephalon. In the rostral rhombencephalon the bilateral vestibular nuclei, the parvocellular, magnocellular and intermedier part of the reticular formation, the nucleus prepositus

hypoglossi and the spinal nucleus of the trigeminal nerve contained retrogradelly labeled neurons originating from the DVN. In the caudal rhombencephalon the retrogradely neurons were situated in the nucleus gracilis and cuneatus, the spinal nucleus of the trigeminal nerve, the nucleus of the solitary tract, the nucleus prepositus hypoglossi and in the intermedier, parvocellular and gigantocellular part of the reticular formation. There were no retrogradely labeled neurons at the level of spinal cord. The coincidence of retrograde labeled cells with vestibular receptive areas suggests reciprocal interconnections between these structures and the DVN.

4.2 Connections of the superior vestibular nucleus (SVN) with the oculomotor and red nucleus

Following PHA-L injections of the SVN, labeling was found in different structures of the nervous system, including the oculomotor nucleus and the magnocellular part of the red nucleus. The majority of PHA-L-labeled boutons displayed very similar distribution patterns in the oculomotor and red nucleus appearing in close contact with the cell bodies and proximal dendrites. At ultrastructural level, the PHA-L-labeled boutons were almost exclusively in presynaptic position in both nuclei. The boutons were engaged in symmetrical synaptic connections. Of the 166 PHA-L-labeled boutons counted in the oculomotor nucleus, 133 established axodendritic, 29 axosomatic and 4 axoaxonic contacts. In the red nucleus these numbers were 47, 16 and 2, respectively. The immunostaining for GABA revealed immunogold particles in the majority of PHA-L-labeled terminals of SVN origin in both the oculomotor nucleus, the postsynaptic elements were negative for GABA reaction. These results corroborate to those earlier physiological experiments, which revealed that most of the inhibitory vestibular neurons associated with the vertical canal-related vestibulo-ocular reflex were located in the SVN. We have demonstrated for the first time that the axons of SVN origin established symmetrical, mainly GABAergic synaptic contacts on the somata and proximal dendrites within the magnocellular part of the red nucleus. The red nucleus has widespread connections with different parts of the central nervous system and plays an important role in the coordination of the motor activity. According to our results, the SVN may directly modify the activity of the cortico-rubral and cerebello-rubral pathways through inhibition of the neurons in the red nucleus.

4.3 The distribution of extracellular matrix (ECM) macromolecules in the frog nervous system

In our recent study we described for the first time the presence of hyaluronan and phosphacan in the adult frog nervous system. We gave also a detailed map of the expression of tenascin-C, fibronectin and laminin in the frog nervous system.

In intact animals, the hyaluronan (HA) reaction was negative in the roots of spinal and cranial nerves, but appeared very intense in the transitional zone (TZ). The white matter showed a positive HA reaction throughout the neuraxis, having the most intense areas in the spinal cord and the brainstem, especially in the spinal tract of the trigeminal nerve, the vestibular tract and in the spinal ascending tracts. In the gray matter, the HA positivity appeared in fine granular form in the neuropil and it was also present around perikarya and dendrites of different neurons, indicating the presence of this molecule in the perineuronal net (PN). In the brainstem and spinal cord gray matter, the most intense HA reaction was detected in the following sites: around spinal and cranial nerve motoneurons, around neurons of the vestibulocochlear nuclei. Similar distribution pattern in higher vertebrates suggests a phylogenetically conserved distribution and function of HA in the nervous system. The permissive role of HA in fiber growth was established in the peripheral nervous system whereas its non-permissive function was suggested in the regeneration through the TZ of dorsal root of the rat. We have demonstrated the presence of HA around the perikarya and dendrites of the frog, in the perineuronal net (PN). According to the literature the HA of the PN is probably connected to the cells through its receptors, the CD44 and RHAMM.

Laminin immunoreactivity (IR) was very strong in the roots of cranial and spinal nerves and in their TZ. Apart from the meninges and basal lamina of blood vessels, no laminin IR was detected in the CNS. The distribution of laminin demonstrated in this study was similar to that found in higher vertebrates. It was suggested that he laminin has an important role in neuronal cell migration and axon growth, and it increases the regenerative capacity of the injured neurons.

Tenascin-C positivity showed a distribution pattern similar to that of the HA reaction. The tenascin-C IR was weak in the peripheral nerves and its intensity increased in the TZ and the white matter of the CNS. In the gray matter of the brainstem the tenascin-C IR was weakly positive in the neuropil and it was in contrast to the very strong IR detected in the PN of the vestibular nuclei. In mammals, tenascin-C and -R are found in the dorsal root transitional zone and their over-expression may results in failure of the axons to regenerate after dorsal root injury. Tenascin-C plays an important role in forming the

connections between chondroitin-sulphate proteoglycans of the PN, and its presence can inhibit the establishment of new synaptic connections in the adult. The tenascin is expressed in those areas of the CNS that have pronounced neural plasticity. It is re-expressed in adult nervous system following injuries and, by facilitating cell-migration, it promotes the regeneration of the CNS.

The phosphacan IR was weakly positive in the roots of cranial and spinal nerves, and a moderate reaction was detected in the TZ. In the white matter, phosphacan IR was very strong in the vestibular tract and in the PN of vestibular nuclei. In the spinal cord phosphacan IR was detected in the white matter and in the PN of motoneurons. In the neural regeneration both the permissive and also non-permissive role of the phosphacan was demonstrated because the chondroitin-sulphate side chains inhibit, whereas the core protein stimulates the growth of the axons.

The fibronectin IR was strong in the PNS and in the TZ, and a large part of the white matter showed a relatively strong and inhomogeneous IR, with the strongest expression in the commissural fibers. The gray matter displayed weak fibronectin IR in the neuropil, and the PN was barely recognizable. On the other hand, fibronectin IR was present in the cytoplasm of large and medium-sized neuron, without showing regional differences. Fibronectin plays an important role in the initiation of the proliferation of vestibular Schwann cells, which may explain its strong expression in the vestibulocochlear nerve. The explanation of the inhomogeneous distribution of fibronectin in the white matter is still not clear, but a possible conception could be that the distribution of glia cells in the territory of commissural fibers is different from other areas. During development of nervous system, the astrocytes facilitate the axon growth, which could be an effect of the fibronectin produced by these cells. Astrocytes loose this capability in the later stages of development. Other studies have demonstrated that the growth of CNS neurons is inhibited in the presence of fibronectin, and this could be a possible explanation for the presence of fibronectin in the PN. The two-way effect of fibronectin can be explained by the different distribution of its receptors. The presence of fibronectin in the cytoplasm of the neurons may refer to the production of fibronectin by these cells.

4.4 The distribution of the subunits of the dystrophin-glycoprotein complex (DGC) in the frog nervous system

Dystrophin (dys2) IR was negative in the PNS, while a strong IR was detected in the TZ. The white matter in the brainstem was negative, except its lateral part where the vestibulocerebellar and spinocerebellar tracts are situated. The dys2 IR was followed till

the granular layer of the cerebellum. The perikarya of the LVN showed a strong IR compared to the weak positivity of the MVN. The dorsal funiculus of the spinal cord showed a strong dys2 IR around the axons, while the gray matter was negative. Similarly to the results demonstrated recently on rat, the dystrophin can also be associated to the glia cells in the frog.

In the PNS the beta-dystroglycan (BDG) IR was stronger, compared to the white matter of CNS. In the territory of MVN and LVN the perikarya of the neurons displayed a strong IR and in a few cases the cytoplasm and proximal dendrites were also positive for BDG.

Double-labeling experiments showed that the cytoplasm of cerebellar Purkinje cells were positive for BDG, while the vestibulocerebellar fibers, terminating on the granular cells, were dys2 IR positive. In the larger neurons of MVN, the BDG and dys2 IR co-localized, while the smaller cells showed IR only for BDG. The neuropil was positive only for dys2. In the peripheral part of vestibulocochlear nerve, the BDG positivity was detected around the axons, compared to the TZ and the CNS, where the vestibular fibers were double-labeled. The basal membrane of vessels and capillaries showed also double immunostaining. In earlier studies it was demonstrated that BDG co-localizes with dystrophin in many areas of the CNS in different species.

Dystrophin and beta-dystroglycan can be found in association of the synapses. The effects of these molecules on pre- and postsynaptic neurons can be modified by neurotransmitter activated matrix metalloproteinases (MMPs). MMPs can be activated by laminin, which may account the facilitating effects of laminin on regeneration and synaptogenesis. The laminin and the DGC may influence the activity of GABAergic synapses via this molecular connection.

4.5 Vestibular regeneration

Following vestibular deafferentation the animals showed characteristic symptoms typical for vestibular lesion, and up till the 6 weeks after the axotomy these symptoms gradually decreased.

4.5.1 Neurobiotin labeling of regenerated fibers

Neurobiotin, applied distally from the earlier axotomy of the eighth cranial nerve, was detected proximally to the lesion. Upon entering the brainstem the fibers gave rise to ascending and descending bundles, the regenerated fibers were fewer than in normal animals and they were located laterally to their normal trajectory. The majority of the

regenerated fibers reached the DVN and the LVN, while the number of the regenerated fibers in the MVN and SVN were less numerous.

4.5.2 Distribution of HA reaction in operated animals

From the third postoperative day the intensity of the HA reaction in the peripheral part of the eighth cranial nerve increased, and the maximum of the intensity was detected on the 42 day after the axotomy.

After 3 days of the axotomy the optical density of HA reaction in the TZ of the operated side was smaller than the normal value and there was a significant increase in the optical density between the 3-5 postoperative days ($p=0,042$, t-test). On the 5^{th} day after the axotomy the optical density of HA reaction in the TZ on both sides was bigger than the normal value. The highest optical density was measured at the 14 day after the axotomy both on the operated and intact side. During the period between 14-21 postoperative days the optical density of HA reaction diminished nearly to the value of control animals and no significant changes were detected between the 21-28 postoperative days. On the intact side the changes in the intensity of HA reaction were the same as on the operated side. In the TZ a barrier develops during the postembryonic life and it results in the inhibition of growth of peripheral axons into the CNS due to the presence of the chondroitin-sulphate proteoglycans (CS-PGs) produced by glia cells. The explanation of the irregular course in the intensity of HA reaction is still missing. We assume that the organization of the HA in the TZ is similar to the embryonic form, that is, the HA molecules do not form aggregate and the free HA, similarly to the embryonic life, might have a permissive role on the regeneration of the nerve fibers.

During the first few postoperative days the intensity of HA reaction in the PN of MVN decreased bilaterally and it was more pronounced on the side of lesion. The PN was indistinguishable from the neuropil these days. There was a noticeable increase in the optical density between the 3-5 days after the axotomy whereas it decreased significantly with the dominance of the operated side between the 5-7 postoperative days. In the following interval (between 7-21 postoperative days) no significant changes were detected. Twenty one days after the operation there was a rapid and pronounced increase in the optical density in the PN first on the intact and later on the operated side up till 42 postoperative day. The values of the optical density increased gradually in an interval between 7-14 after the axotomy on the operated side. There was no significant change in the optical density between the 42-84 postoperative days.

The changes in the values of the optical density in the PN of LVN showed similar tendency that was detected in the MVN. Three days after the axotomy the PN was undistinguishable from the surrounding neuropil either on the operated or intact side. By the 5 postoperative days the value of the optical density increased, but this change was also undetectable with microscopical investigations. Between the 5-7 postoperative days the optical density decreased significantly. During the interval of the 7-14 days no significant changes were measured in the optical density of the operated side. After the 14 postoperative days, especially from the 28 days, the optical density began to increase on the operated side. Between the 21-28 postoperative days the normalization of the values revealed that the optical density slightly exceeded the level of control animals both on the operated and unoperated sides, and the PN reappeared to postoperative day 28. The values of the optical density did not display significant changes between the 28-84 postoperative days, and microscopical investigation also didn't show any changes in intensity after postoperative day 42.

The Fisher-exact test showed that the changing in the optical density between the operated and unoperated sides are not independent events, indicating that the changing in the optical density on the operated side is the consequence of the axotomy.

The early decrease in the intensity of HA reaction in the vestibular nuclear complex, especially in the PNs of the MVN and LVN may indicate that changes in the chemical composition of PNs permits the reinnervation of neurons either by regenerating axons or by collaterals of the spinocerebellar tract. The disruption of its molecular composition suspends the repulsive effect of the PN and new synaptic connections may be established. The structure of the PN is restored at the terminal phase of regeneration, which may indicate the stabilization of new synaptic contacts. The bilateral changes of the HA reaction in the PN and the TZ can be explained by systemic effects, and/or by the increased expression of CD44 through the influence of commissural fibers, and/or by the modulation of HA-hyalectin connection on GABAergic neural transmission. These changes can be due to the different expression of hyaluronan-synthase isoforms producing HA molecules with different chain-length.

4.5.3 Changes in laminin expression following vestibular axotomy

Vestibular axotomy was followed by increased laminin IR bilaterally both in the peripheral part and in the TZ of the eighth cranial nerve. The reaction reached the highest intensity by the 5 postoperative day on the intact side. In the CNS, the laminin IR appeared bilaterally in the white matter of the brainstem, and the reaction was localized laterally to

the place of the normal vestibular tract. Positive laminin IR was also detected bilaterally in the neuropil of the vestibular nuclei, except in the MVN. Following this period the laminin IR decreased, and by the postoperative day 14 the IR showed the same distribution pattern in the PNS and the CNS as seen in unoperated animals.

The changes in the laminin distribution in the eighth cranial nerve and in the TZ suggest its permissive role during PNS regeneration. The role of laminin in the CNS regeneration is still not clear. The laminin may activate the tPA/plasminogen system, which leads to the formation of new synaptic contacts, and it is important in the formation of LTPs. The bilateral changes in the laminin expression can be explained by the activation of commissural fibers.

One of the possible explanations of the regeneration occurring in the CNS of lower vertebrates is that the inhibitory molecules are rapidly removed after the injury. Myelin debris, which contains inhibitors in mammals, is removed much slower rate and with fewer efficacies. Our results, in accordance to the literature, suggest that none of the ECM macromolecules can be determined as inhibitory or excitatory substances for the axon-regeneration, because their effects are largely dependent on the microenvironment and the receptor expression of their target cells. The role of these molecules has been previously investigated mainly in *in vitro* experiments, without studying their interactions. The *in vivo* studies may be more reliable for studying the role of the ECM macromolecules and their receptors during regeneration of nervous system.

5. SUMMARY

In the first part of our work we investigated the connections of the vestibular system in the rat at light and electron microscopic levels with different tracing methods. Using neurobiotin, we mapped the antero- and retrograde connections of the descending vestibular nucleus (DVN) in the central nervous system (CNS), confirming and supplementing previous results. We have for the first time demonstrated a connection between the DVN and the red nucleus. At the level of the rhombencephalon we described the connections of the DVN with the ipsi- and contralateral vestibular nuclei, the sensory nuclei of the brainstem and the reticular formation. The descending axons of the DVN could be found in every funiculus of the spinal cord. We also demonstrated the previously unknown retrograde connections of the DVN with the rhombencephalic areas. Using the tracer, Phaseolus vulgaris leucoagglutinin (PHA-L), we investigated the synaptic connections of the superior vestibular nucleus (SVN) with the oculomotor and red nuclei. In the oculomotor nucleus, GABA positive axon terminals originating from the SVN support the presence of inhibitory connections described earlier in physiological experiments. Similar connections to the red nucleus suggest that neurons of the SVN can alter the activity of the cortico-rubral and cerebello-rubral pathways.

In the second part of our work we studied the distribution and role of the extracellular matrix (ECM) macromolecules in vestibular regeneration following vestibular nerve axotomy in the frog. We also analyzed the expression of ECM-receptor dystrophin-glycoprotein complex (DGC) subunits. We described for the first time in the nervous system of the frog the expression of hyaluronan (HA), using a specific binding-probe, and the distribution of phosphacan and DGC subunit dystrophin and beta-dystroglycan, using immunohistochemical methods. We gave a detailed map of the distribution of tenascin, fibronectin and laminin in the spinal cord and brainstem of the frog. We delineated the qualitative and quantitative changes in the expression of HA during vestibular regeneration. According to our results HA has a permissive role in the peripheral nervous system (PNS) and in the transitional zone (TZ) at the border between PNS and CNS during regeneration. The decreased expression of HA in the perineuronal net (PN) surrounding the vestibular neurons in the brainstem in the early phase of vestibular regeneration suggest that HA has a non-permissive role in the CNS, since the change in the distribution of HA can support the formation of new synaptic connections. We also showed an increased expression of laminin in the PNS, TZ and certain areas of the CNS during vestibular regeneration, indicating a permissive role of laminin as previously described.

6. PUBLICATIONS

THIS THESIS IS BASED ON THESE IN EXTENSO PUBLICATIONS

1. Matesz C, Bácskai T, Nagy É, Halasi G,Kulik Á: Efferent connections of the vestibular nuclei in the rat: A comparative neuromorphological study. Brain Res Bull. 57: 313-315. 2002. IF: 2.283
2. Halasi G, Bácskai T, Matesz C: Connections of the superior vestibular nucleus with the oculomotor and red nuclei in the rat: An electron microscopic study. Brain Res Bull. 66:532-5. 2005 IF: 2.283

OTHER IN EXTENSO PUBLICATIONS:

1. Matesz C, Modis L, Halasi G, Szigeti ZM, Felszeghy S, Bacskai T, Szekely G: Extracellular matrix molecules and their possible roles in the regeneration of frog nervous system. Brain Res Bull. 66:526-31. 2005 IF: 2.283
2. Rácz E, Bácskai T, Halasi G, Kovács E, Matesz C: Organization of dye-coupled cerebellar granule cells labeled from afferent vestibular and dorsal root fibers in the frog, *Rana esculenta.* Submitted to JCN.
3. Szigeti ZM, Matesz C, Szekely G, Felszeghy S, Bácskai T, Halasi G, Mészár Z, Modis L: Distribution of hyaluronic acid in the central nervous system of the frog. Submitted to JCN.
4. Halasi G, Bácskai T, Wolf E, Módis L, Székely G, Mészár Z, Szigeti ZM, Matesz C: Vestibular lesion-induced changes in the expression of the hyaluronan in the frog, *Rana esculenta*. In preparation Exp Brain Res.

Cumulative impact factor of published papers: 6,849; Independent citations: 4

THIS THESIS IS BASED ON THESE ABSTRACTS OF POSTER PRESENTATIONS:

1. Matesz C, Halasi G, Bácskai T: Antero- and retrograde connections of the descending vestibular nucleus in the rat. Congress of Hungarian Neuroscience Society (MITT), Szeged, 2001. Neurobiology.9. 228-229.

2. Matesz C, Bácskai T, Nagy É, Halasi G, Kulik A: Anterograde connections of the vestibular nuclei in the rat: A comparative neuromorphological study. 3rd European Conference on Comparative Neurobiology, Murcia, Spanyolország, 2001.

3. Bácskai T, Halasi G, Matesz C: Comparative study on the afferent connections of the lateral vestibular nucleus in the frog and rat. Congress of Hungarian Neuroscience Society (MITT), Budapest, 2002. Clinical Neurosci. 56:2.6.

4. Bácskai T, Halasi G, Matesz C: Electronmicroscopical studies on the mesencephalic termination areas of the rat vestibular nuclei. Congress of Hungarian Neuroscience Society (MITT), Balatonfüred, 2003. Clinical Neurosci. 56:2.

5. Matesz C, Módis L, Szigeti ZM, Felszeghy S, Bácskai T, Halasi G, Székely G: Extracellular matrix molecules in the nervous system of the frog. Congress of Hungarian Neuroscience Society (MITT), Balatonfüred, 2003. Clinical Neursci. 56:2 57.

6. Szigeti ZM, Módis L, Matesz C, Felszeghy S, Bácskai T, Halasi G, Székely G: Hyaluronan distribution pattern in the nervous system of the frog. Congress of Hungarian Neuroscience Society (MITT), Balatonfüred, 2003. Clinical Neurosci. 56:2 86-87.

7. Halasi G, Bácskai T, Módis L, Székely G, Matesz C: Vestibular lesion-induced changes in the expression of the extracellular matrix molecules in the frog. IBRO International Workshop, Budapest, 2004. Clinical Neurosci. 57: 1. 22.

8. Szigeti ZM, Matesz C, Bácskai T, Halasi G, Székely G, Módis L: Distribution of tenascin-C and fibronectin in the nervous system of the frog. IBRO International Workshop, Budapest, 2004. Clinical Neurosci. 57: 1. 65.

9. Halasi G, Bácskai T, Matesz C: Connections between the superior vestibular nucleus and the oculomotorius and red of the rat: An electronmicroscopical study. 4th European Conference on Comparative Neurobiology: Evolutionary Development Biology of Brains, Congress Book of 4th ECCN. Oxford. 2004.

10. Matesz C, Módis L, Halasi G, Szigeti ZM, Felszeghy S, Bácskai T, Szekely G: Extracellular matrix molecules and their possible role in the regeneration of frog nervous system. Congress Book of 4th ECCN. Oxford. 2004.

11. Halasi G, Matesz C, Jancsik V: Expression of dystrophin-glycoprotein complex in the nervous system of the frog. Congress of Hungarian Neuroscience Society (MITT), Pécs, 2005. Clinical Neurosci. In press.

OTHER ABSTRACTS OF POSTER PRESENTATIONS:

1. Bácskai T, Halasi G, Matesz C: Synaptic connections on the hypoglossal nucleus of the frog. IBRO International Workshop, 2004. Budapest. Clinical Neurosci. 57:1. 4.
2. Bácskai T, Veress G, Halasi G, Matesz C: Involvement of the vestibular system in the prey catching behavior of the frog. Congress of Hungarian Neuroscience Society (MITT), 2005. Pécs. Clinical Neurosci. In press.
3. Rácz E, Bácskai T, Halasi G, Matesz C: Dye-coupled connections of the primary afferent vestibular fibers in the cerebellum of the frog. 1st International Conference on Basic and Clinical Immunogenomics, Budapest, 2004. Tissue Antigens 64: 317-441.
4. Rácz E, Kovács E, Bácskai T, Halasi G, Matesz C: Dye-coupled connections of the primary afferent fibers in the cerebellum of the frog Congress of Hungarian Neuroscience Society (MITT), 2005. Pécs. Clinical Neurosci. In press.