The vestibular dysfunction or balance disorder may have a significant impact on everyday life activities. Unilateral labyrinthectomy (UL), which destroys the unilateral vestibular receptors, evokes serious deficits in the posture, balance, and eye movements of the animal indicating functional asymmetry in the vestibular system. After a shorter or longer period of time, a more or less complete vestibular function returns spontaneously via a complicated series of processes called vestibular compensation, therefore the UL is an excellent model for studying the deafferentation-induced plasticity of the central nervous system (CNS). Over the last few decades, the role of extracellular matrix (ECM) molecules was demonstrated in the plasticity of various parts of CNS. Previously we have shown that the UL and subsequent compensation is accompanied by modification in the expression of chondroitin sulfate proteoglycan (CSPG) or lectican (major lectican molecules: aggrecan, brevican, neurocan, versican), tenascin-R (TN-R) and hyaluronic acid (HA) molecules in the brainstem vestibular nuclei. The most prominent changes were detected in the perineuronal nets (PNNs), which is the condensation of ECM around the neuronal cell bodies and dendrites. In the PNNs, the lecticans, along with the TN-R, the HA and the link proteins (e.g. HAPLN1) form a highly organized structure and interact with the neuronal membrane. One of the important functions of the PNN is to restrict the neural plasticity thereby it supports the synaptic stability. In this project we studied the potential role of individual ECM molecules in the plasticity of vestibular system. In order to observe the possible changes, knowledge on the molecular organization and distribution of the ECM in the structures of vestibular neural circuit is required. The ECM molecules were detected by using histochemical (HA probe; Wisteria floribunda agglutinin /WFA/ general marker of lecticans) and immunohistochemical methods (aggrecan, brevican, neurocan, versican, TN -R, and HAPLN1).

I) Expression of brevican in the superior vestibular nucleus following labyrinthectomy

Since the brevican is one of the major CSPGs in the CNS, we examined the changes of its expression in the superior vestibular nucleus (SVN) of rat following UL and during the compensation. Among the four vestibular nuclei, the SVN is the only one which exclusively mediates the vestibulo-ocular reflexes but it is not responsible for vestibulo-spinal and vestibulo-autonomic reflexes. We have found that *the percentage of brevican positive PNN-bearing neurons was practically unchanged after UL. In contrast, the optical densities of brevican reactions in the PNNs were significantly smaller on both sides with respect to the control on the postoperative days 1, 3, 7.* After a transitory decrease on the first postoperative

day, there was an elevation in the density of PNNs on day 3. As the vestibular symptoms show continuous improvement parallel to the restoration of ECM assembly in the PNNs, we expected further elevation in the optical density on the subsequent postoperative days. Contrary to this expectation, the optical density of brevican reaction showed a more pronounced statistically significant decrease on day 7 when the value was near to half of the control level bilaterally in the PNNs of SVN. This time point is crucial time point during the vestibular compensation as the static symptoms almost disappear by postoperative day 7 but the vestibulo-ocular reflexes are not still working properly. Several studies showed that CSPGs limit the CNS plasticity thus the reduction of brevican in the PNNs of superior vestibular neurons may promote the axonal growth to the neurons of SVN by suspending the non-permissive property of brevican in the restoration of PNNs assembly. On postoperative day 14, the optical density returned to the control values on both sides parallel to the partial restoration of disorders. It may indicate, on the basis of found on the other parts of CNS, that the brevican is involved in the stabilization of newly formed synaptic connections and seals the synaptic cleft to prevent the transmitter spillover. Furthermore, the brevican controls both the cellular and synaptic forms of plasticity by regulating the localization of potassium channels and AMPA receptors, respectively. The role of brevican was suggested in the high-speed synaptic transmission at the calyx of Held in the medial nucleus of the trapezoid body. This auditory structure of the brainstem has common embryonic origin with the vestibular nuclei, and it seems that its very rapid synaptic transmission is also essential in the vestibular system. Since the caliciform synapses are also found in the SVN we suppose that the elevated brevican expression at the postoperative day 14 is associated with its similar role in the SVN. Other role of brevican was observed in the spatial coupling of pre- and postsynaptic elements, as an important precondition for the ultrafast synaptic transmission required to the very quick response to the head and body displacement.

When we examined the modification of staining intensities in the strong, medium and weak categories of PNNs the highest decrease was observed in the most strongly stained PNNs bilaterally during the first postoperative week. In the medium category, parallel to this reduction, the percentage of PNNs was elevated at day 1 and 3 and returned to the control level at postoperative day 7 at both sides. In the weakly stained group, the changes in the percentage of PNNs showed minor differences at the day 1 and 3 on the ipsi- and contralateral sides. At the postoperative day 14, the intensity of weak category returned to the control level on both sides, whereas the percentage of the strongly stained PNNs was smaller than the control value at the non-operated side. Since the SVN contains morphologically and

functionally different neuron populations, we may suppose that the different intensities in brevican reaction in the PNNs and the different time courses in their modification after the UL is associated with different properties of neurons. The bilateral changes in the brevican expression support the role of commissural vestibular fibers in the restoration of vestibular function after UL (Magyar et al. Neural Regeneration and Research, 2021 In press). Similar experiment was done for the aggrecan; data analysis is progress.

II) Expression of ECM in the vestibular nuclear complex-related structures of mesencephalon

In order to follow the changes of ECM in the vestibular nuclear complex-related brainstem structures after labyrinthectomy, we studied the expression and distribution pattern of ECM molecules in the red nucleus and the pararubral area of mesencephalon.

i) The **red nucleus** consists of a rostral parvicellular (pRN) and a caudal magnocellular division (mRN). Although these regions are conventionally distinguished, none of the cell types show exclusive position in any region of the nucleus. Rather, the two divisions are defined by their different afferent and efferent connections: the mRN part receives direct vestibular pathways from all four vestibular nuclei, whereas the input to the pRN area arrives from the motor cortex, the cerebellum and the thalamus. We have detected the condensed forms of ECM, i.e., the PNNs, the nodal ECM and axonal coats as well as the diffuse network of neuropil in both parts of the nucleus. *Different staining pattern of the ECM reactions between the two subdivisions* were clearly distinguished *only with the aggrecan, neurocan, and HAPLN1* reactions as being much stronger aggrecan immunoreactivity in the mRN part compared to the pRN region, whereas the anti-neurocan and anti-HAPLN1 antibodies labeled mostly the pRN division. On the other hand, unevenly stained irregular territories were shown in both subdivisions, reflecting the somatotopic organization of the afferent and efferent connections. We could detect PNNs by using reactions against each ECM reaction studied and observed that the *expression pattern was related with the cell size independently from the neuronal position* within the two subdivisions. Thus, the PNNs were absent or faintly stained around the small, mostly inhibitory neurons, whereas the large and medium sized excitatory cells were surrounded by a pericellular coat. Analysis of the staining intensity of PNN revealed that among the lectican reactions, the aggrecan showed the most intense staining in the PNNs of the large rubral neurons indicating an aggrecan-based condensation of the ECM. The most conspicuous difference in the ECM staining pattern between the parvi- and

magnocellular parts was observed with neurocan and HAPLN1 reactions showing much less intense staining in the magnocellular part. This expression pattern might be related to the projection of the magnocellular division: it is the only output of red nucleus to the spinal cord. Since the activity of rubrospinal neurons is continuously modified by direct and indirect cerebellar signals, the less dense ECM assembly may contribute to the high level of synaptic plasticity to ensure the requited precise movements during the reach-to-grasp behavior. Differential expression and intensity of the ECM molecules in the PNNs of neurons with various sizes supports the widely accepted view that the red nucleus comprises more different populations of neurons. Since the parvalbumin, characteristic marker of the small GABAergic cells of the RN, shows unequal distribution within the RN, we have studied the *colocalization of the ECM molecules with the parvalbumin*. We have found that the large cells, in both parts of the RN, showed weak staining with parvalbumin antibody, whereas the ECM reactions was very strong around these neurons. In contrast, the small cells of RN showed very intense parvalbumin positivity surrounded by weakly stained PNN, or the PNNs were not recognizable. Similar staining patterns were shown by using aggrecan and brevican reactions. This staining pattern was observed throughout the red nucleus (Rácz É, et al., Neuroscience 2016, Rácz et al., 2017; Szarvas et al.; 2018).

ii) In the cytoarchitectonically homogeneous **pararubral area** (PRA), the characteristic *PNNs were only recognizable with WFA and aggrecan staining* around some of the medium sized neurons, whereas the small cells were rarely surrounded by a weakly stained PNN. The large neurons are not present in the pararubral area. The perikarya of PNN-ensheated neurons are excitatory projection neurons, whereas the majority PNN-free cells are interneurons with inhibitory function. With the other ECM reactions studied, no characteristic forms of PNN were identifiable, the HA and HAPLN1 reactions showed occasionally patch-like staining around the neuronal cell bodies. We found similar ECM expression pattern of PNN, with some differences, between the PRA and the pRN part of the red nucleus. Characteristic PNNs were observed with WFA and aggrecan staining in both areas, whereas the HA, brevican, neurocan, TN-R, and HAPLN1 reactions showed pericellular positivity only around the small and medium sized cells in the pRN. The similarity in the ECM staining pattern may be related to their common afferent connections from the somatosensory cortex and cerebellum and efferent projection to the rubrospinal neurons of red nucleus. The neurons of sensorimotor cortex and cerebellum excite the GABAergic neurons of PRA and pRN which inhibit the rubrospinal neurons in the magnocellular part of the red nucleus. In case of the cerebellum,

significant overlap with some differences was observed in the origin of projection fibers. Although the experiments revealed significant overlap in the termination areas, the functional differences were described about of the cerebellar nuclei indicate that the PRA is involved mostly in the visuomotor and vestibular activity. The visuomotor function of the PRA was suggested by its polysynaptic projections to orbicularis oculi muscle. Moreover, the pararubral nucleus receives substantial projection form the retina and by its indirect connection with the suprachiasmatic nucleus it plays a role in the circadian rhythm regulation (Szarvas et al., Neuroscience 2018).

iii) We studied the expression of ECM molecules in the **visual motor nuclei,** the major efferent targets of the vestibular nuclei. The PNN was present around the singly innervating (SI) motoneurons in each eye moving cranial nerve nucleus, the multiply innervating (MI) neurons and interneurons were free of PNN. Double imunohistochemical reactions revealed aggrecan and brevican molecules in the PNNs of cholinergic neurons, whereas the neurocan reaction was negative in the PNNs. With the help of optical density measurements, the most intense aggrecan reaction in the PNNs was detected in the trochlear nucleus followed by the abducens and then the oculomotor nucleus. The intensity differences are presumably due to the regional expression of different ECM molecules. Since the SI motoneurons have widespread afferent connections, which render possible the very quick and precise eye movements, the ECM deposition around the neurons helps to stabilize the established synaptic connections. Our results provide the basic information for future lesion experiments (Gaál et al., 2018).

iv) We have continued the studies on the expression of ECM **in the developing** mouse brainstem, with special emphasis to the **vestibular nuclei and related structures**. As suggested by many authors, the role of ECM in plastic responses of the adult nervous system is similar to those mechanisms which are used for regulation of different developmental stages during brain development. We found that HA, neurocan, versican, and TN-R positivity were detected as diffuse staining in the neuropil at early embryonic stage (E13.5). We could not find any aggrecan, WFA or HAPLN1 staining before birth. Several molecules were observed only postnatally, and the first appearance of the PNN was observed at P7. Postnatally, the HA, neurocan and TN-R reactions were detected throughout the brainstem including the vestibular nuclei, whereas WFA, aggrecan and HAPLN1 were restricted to the neuropil in some brainstem nuclei The PNNs were composed of WFA and aggrecan in the

vestibular nuclei and related parts of the reticular formation (Wéber at al. 2016, Birinyi et al 2018, Birinyi et al., 2018).

II) Expression of ECM in the olfactory bulb

Since the impairment of smell sensation is frequently observed in the vestibular disorders, we have studied the ECM organization in the olfactory bulb (OB) in order to detect the possible changes in the labyrinthine lesion experiments in the future. The olfactory system has a high degree of plasticity throughout life, which is partially due to the renewal of olfactory epithelial cells and the continuous ingrowth of their axons into the OB.

Our results did not show positive reaction in the ***glomerular layer*** of the OB with versican antibody. In the other cases, the intensity of staining varied between reactions. The staining intensity was not uniform throughout the OB, darker and lighter *glomeruli* were recognizable without any regularity in their number and position. The other common characteristic of the ECM reactions was an inhomogeneous staining within the glomeruli. The irregular stained and unstained areas resemble the compartmentalization of either the olfactory nerve terminals or dendrites of periglomerular and mitral cells. The unequal distribution of ECM staining within the glomeruli may suggest that a given ECM molecule contribute differently to the synaptic plasticity in the two compartments. In the *periglomerular area* of the glomerular layer, the HA and brevican staining showed the strongest reaction, the staining was very weak with WFA, aggrecan, neurocan, but the reaction was almost negative with the versican, TN-R and HAPLN1 antibodies. The absence of versican and the very weak TN-R reaction may be related to the absence of Ranvier nodes in the glomeruli. The periglomerular area was stained with HA, neurocan and brevican reactions, thin PNNs were identifiable around periglomerular cells in only a few cases. Except the versican, all ECM molecules were detected in the ***external plexiform layer***. The staining intensity showed a largely homogeneous distribution which was strongest with the HA, aggrecan, neurocan and HAPLN1 with a weaker aggrecan immunoreactivity in the outer part of external plexiform layer. Interestingly, the WFA reaction was more intense in the deeper part of this layer. The beaded appearance of brevican staining and its colocalization with the neurofilament reaction may indicate large amount of brevican molecules at the node of Ranvier of projection neurons. Positive ECM reactions were shown with the ECM reactions in the ***internal plexifrom layer*** with moderate or strong staining intensities. The characteristic darkly stained bands with WFA and aggrecan reactions seemed to be arranged around the axons of mitral and tufted cells as reinforced by using neurofilament antibody. The ***granule***

cell layer showed the most intense staining with brevican reaction followed by the HA, neurocan, and TN-R reactions. The most characteristic staining pattern was the columnar organization of the versican positive dots representing the presence of this ECM molecule at the nodes of Ranvier.

In summary, one of the striking features of ECM staining pattern in the olfactory bulb is *that the ECM molecules were accumulated dominantly in the neuropil*. PNNs were present only in the periglomerular area with the HA and neurocan reactions and in the mitral cell layer with the WFA and aggrecan staining. *The PNNs exhibited thin or diffuse appearance,* robust forms were not recognizable. These results are in agreement with the life-long plasticity of olfactory system, as the PNNs limit the plasticity in adulthood by acting as a scaffold for molecules that can inhibit synapse formation, and limiting receptor motility at synapses. The other interesting point of our results is the comparison of the ECM expression in two compartments of the olfactory bulb which have major role in the plasticity. One of them is "glomerular map" in the glomeruli, whereas the other, the "intrabulbar map" is located in the internal plexiform layer. The expression of ECM molecules is very similar in the two "maps" except the very strong TN-R expression in the IPL, which is almost negative in the glomeruli. The possible explanation may be related to different periods in the modification of the neuronal circuits in the two maps. In collaboration, we found similar ECM expression pattern in mice and human OB (Hunyadi et al., Brain Structure and Function 2020).

Taken together results obtained during this OTKA grant revealed possible role of individual ECM molecules during the restoration of vestibular disorders. Our findings support the generally accepted view that the structural and molecular organization of the ECM shows regional variation in the CNS.

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