● RESEARCH ARTICLE

Modification of tenascin-R expression following unilateral labyrinthectomy in rats indicates its possible role in neural plasticity of the vestibular neural circuit

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Abstract

We have previously found that unilateral labyrinthectomy is accompanied by modification of hyaluronan and chondroitin sulfate proteoglycan staining in the lateral vestibular nucleus of rats and the time course of subsequent reorganization of extracellular matrix assembly correlates to the restoration of impaired vestibular function. The tenascin-R has repelling effect on pathfinding during axonal growth/regrowth, and thus inhibits neural circuit repair. By using immunohistochemical method, we studied the modification of tenascin-R expression in the superior, medial, lateral, and descending vestibular nuclei of the rat following unilateral labyrinthectomy. On postoperative day 1, tenascin-R reaction in the perineuronal nets disappeared on the side of labyrinthectomy in the superior, lateral, medial, and rostral part of the descending vestibular nuclei. On survival day 3, the staining intensity of tenascin-R reaction in perineuronal nets recovered on the operated side of the medial vestibular nucleus, whereas it was restored by the time of postoperative day 7 in the superior, lateral and rostral part of the descending vestibular nuclei. The staining intensity of tenascin-R reaction remained unchanged in the caudal part of the descending vestibular nucleus bilaterally. Regional differences in the modification of tenascin-R expression presented here may be associated with different roles of individual vestibular nuclei in the compensatory processes. The decreased expression of the tenascin-R may suggest the extracellular facilitation of plastic modifications in the vestibular neural circuit after lesion of the labyrinthine receptors.

Key Words: *nerve regeneration; extracellular matrix; brainstem; vestibular system; vestibular lesion; vestibular compensation; perineuronal net; neural plasticity; neural regeneration*

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Introduction

The vestibular system, or the system of balance, provides information about the motion, equilibrium, and spatial orientation of the body. Lesions of the vestibular system result in postural and visual deficits accompanied by dizziness, vertigo, and changes in cardiorespiratory and gastrointestinal functions. Signs of the disorder are classified as static and dynamic symptoms and many, but not all are regained spontaneously in the process of vestibular compensation (Dieringer, 1995; Vidal et al., 1998; Hitier et al., 2010). The vestibular compensation incorporates modifications in a number of processes, like changes in discharge properties of bilateral vestibular neurons (Dieringer, 1995, 2003; Vibert et al., 1999b; Straka et al., 2005; Dutia, 2010), in the efficacy of synaptic inputs from the existing non-labyrinthine pathways to the deafferented

vestibular neurons and in remodeling of synaptic connections through axonal sprouting and synaptogenesis (Dieringer, 1995, 2003; Vibert et al., 1999a, b, 2000; Beraneck et al., 2004; Straka et al., 2005). Based on previous results suggesting the modifications of extracellular matrix components in other parts of the nervous system after injuries, we suppose that these interrelated events of vestibular compensation have influence on the molecular assembly of the extracellular matrix also in the vestibular nuclear complex.

In the central nervous system, the major form of extracellular matrix, the perineuronal net, emerges in condensed form around the perikarya, proximal dendrites and axon initial segment (Carulli et al., 2006; Bruckner et al., 2008; Dityatev et al., 2010; Frischknecht and Seidenbecher, 2012; Lendvai et al., 2012; Blosa et al., 2013). Principal molecular constituents

of perineuronal net are the hyaluronan, chondroitin sulfate proteoglycan lecticans, tenascin-R and various link proteins (Celio et al., 1998; Zimmermann and Dours-Zimmermann, 2008; Kwok et al., 2011). The constituents of perineuronal net is activity dependent and its molecular assembly, as the parts of the synaptic machinery (Dityatev and Rusakov, 2011), can modify the synaptic transmission (Bukalo et al., 2007; Dityatev et al., 2010).

Although the role of extracellular matrix in the lesion-induced plasticity and regeneration was confirmed in various parts of the central nervous system (Dityatev and Schachner, 2003; Moon et al., 2003; Galtrey and Fawcett, 2007; Lin et al., 2009; Dityatev et al., 2010; Alilain et al., 2011; Dityatev and Rusakov, 2011; Michaluk et al., 2011), similar works on the vestibular system were published only from our lab (Halasi et al., 2007; Deak et al. 2012). We observed that unilateral labyrinthectomy is accompanied by the radical decrease of hyaluronan and chondroitin sulfate proteoglycans expression in the lateral vestibular nucleus during the compensatory period. The reorganization of extracellular matrix assembly in the perineuronal net and neuropil correlated with the time course of recovery from the postural deficits and reappearance of normal resting discharge of vestibular neurons (Dieringer, 1995; Curthoys, 1996; Vidal et al., 1998; Curthoys and Halmagyi, 1999; Darlington and Smith, 2000), suggesting the involvement of hyaluronan and the lecticans in the process of vestibular plasticity (Deak et al., 2012). Here, we extend this study to the tenascin-R as the third major contributor of the perineuronal net integrity, forming the ternary network with the hyaluronan and lecticans (Koppe et al., 1997; Bruckner et al., 2000; Pesheva and Probstmeier, 2000; Dityatev and Schachner, 2003; Carulli et al., 2006; Deepa et al., 2006; Zimmermann and Dours-Zimmermann, 2008; Kwok et al., 2010; Wang and Fawcett, 2012). Other studies on various species indicated that tenascin-R is an important modulator of neural plasticity and repair processes in various parts of the central nervous system (Apostolova et al., 2006; Anlar and Gunel-Ozcan, 2012). Based on these findings, we hypothesized that the tenascin-R expression in the superior, medial, lateral, and descending vestibular nuclei varied following unilateral vestibular lesion and subsequent compensation.

Materials and Methods
Animals and surgical procedures
The experiments were carried out in accordance with European Community guidelines and state regulations and with the approval of the University Animal Care Committee (DEMÁB, 11/2011/DE MAB). All efforts were made to minimize animal discomfort and reduce the number of animals used. Adult female Wistar rats, aged 12–14 weeks old, weighing 250–300 g, were used in the experiments ($n = 12$). The animals were anesthetized using an intramuscular injection of 2% xylazine (10 mg/kg, CP Pharma Handels GmbH, Germany) and 10% ketamine (100 mg/kg, CP Pharma Handels GmbH). The surgical procedure was performed under operating microscope (Leica, Wild M3C). A 1.5 cm-long skin

incision was made behind the left external acoustic meatus; the cervical muscles, posterior belly of the digastric muscle, the stylohyoid and their nerves were spared. The ventral wall of the tympanic bulla was carefully opened and the labyrinth containing the vestibular sensory organs was approached by breaking the promontory. The left vestibular sensory organs were mechanically destroyed with special care taken on keeping the stapedial artery and facial nerve intact. After a period of 1, 3, 7 or 14 days of survival, the rats (three animals at each time point) were re-anesthetized with intraperitoneal administration of 10% urethane (1.3 mg/100 g, Reanal, Budapest, Hungary) and perfused transcardially with physiological saline.

Tissue processing and immunohistochemical staining
The brainstem was removed and immersed into Sainte-Marie's fixative (99% absolute ethanol and 1% glacial acetic acid) for 1 day at 4°C. The specimens were embedded in paraffin and transverse sections of 8 μm thickness were made. The tenascin-R was detected by incubating the samples in polyclonal goat anti-tenascin-R antibody (R&D Systems, Minneapolis, MN, USA; AF 3867) diluted in 1% bovine serum albumin + 3% normal rabbit serum overnight at 4°C. The primary antibody incubation was followed by repeated rinse steps in PBS, and then biotinylated rabbit-anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Visualization of labeling was performed by incubating the samples with ExtrAvidin Peroxidase complex (Sigma-Aldrich) diluted in PBS for 1 hour at room temperature, followed by 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Sigma-Aldrich) with H_2O_2. After dehydration, sections were coverslipped with DPX mounting medium (Sigma-Aldrich). Specificity of tenascin-R antibody was assessed previously in our lab and the details were already published (Gaál et al., 2014; Rácz et al., 2014). Images were recorded by using Nikon Eclipse E800 (Nikon Corporation, Tokyo, Japan) conventional light microscope and processed by Photoshop CS4 v11.0 (Adobe Systems Inc., San Jose, CA, USA) with minimal adjustments of contrast and background.

Semiquantitative assessment of histochemical and immunohistochemical reactions
For the semiquantitative assessment of tenascin-R reaction, pictures from identical cross sectional levels of each individual vestibular nucleus of three animals were captured using the same magnification, contrast, and brightness. On the images, the staining intensity of tenascin-R reaction was evaluated on the same computer screen by using four-grade scaling: –: no staining, +: weak staining, ++: moderate staining, +++: strong staining (**Table 1**). The subjective grading was performed by two authors (BG and IW) and checked by the other (CM), independently. The optical density measurement would fail to detect the clear-cut distinction between the diffuse ECM and its condensed forms (Carulli et al., 2006, 2007; Costa et al., 2007; Galtrey et al., 2008; Gati et al., 2010; Lendvai et al., 2012; Rácz et al., 2013) and this method

was successfully applied in the inferior olive (Kecskes et al., 2014).

As the exact quantification of our data was difficult because there are no objective "distances" between the grades of staining intensity established by semiquantitative assessment, we considered these data as ordinal variables allowing application of nonparametric statistical methods to confirm our conclusions. Two different hypotheses were tested. The first statistical analysis was devoted to test the differences in staining intensities of perineuronal nets between the operated and unoperated sides in the vestibular nuclei of the same animal. The variables for this analysis were determined by calculating the median values of the intensity scales of tenascin-R provided by the three independent investigators. For the statistical analysis, the Wilcoxon signed rank test was applied.

In the second statistical analysis, we examined whether the staining intensity has been changed during the postoperative period in the individual vestibular nuclei by using Kruskal-Wallis analysis of variance. In those vestibular nuclei where changes were detected, the subsequent days were compared with Mann-Whitney U test. In each statistical analysis, $P < 0.001$ was considered statistically significant.

The statistical analysis was performed using SPSS 21.0 software (SPSS, Chicago, IL, USA).

Results

In the unoperated animals, as shown in our previous work (Racz et al., 2014), the tenascin-R immunoreactivity was intense in the perineuronal nets of each vestibular nucleus (**Figure 1A, C, E, G, I, K, M, O** and **Figure 2A, C, E, G, I, K, M, O; Table 1**), except for the caudal part of the descending vestibular nucleus which showed weaker staining in the pericellular area (**Figure 2Q, S, U, W**). The neuropil showed a diffuse, reticular appearance presenting strongly stained areas in the superior, medial and lateral nuclei, as well as in the rostral parts of the descending vestibular nucleus, whereas the staining intensity was weaker in the caudal part of the descending vestibular nucleus.

In the superior vestibular nucleus, the tenascin-R staining of perineuronal nets completely disappeared on the side of labyrinthectomy on the first postoperative day, whereas the pericellular staining did not change on the intact side (**Figure 1A, B; Table 1**). In the neuropil, the intensity of reaction was similar to that of the control animals, bilaterally. On survival day 3, the tenascin-R staining pattern was similar to day 1 with the exception of the lighter staining of ipsilateral neuropil (**Figure 1C, D**). On survival days 7 and 14, perineuronal nets were recognizable at both sides, showing minor decrease of staining in the perineuronal nets of the operated side (**Figure 1E–H; Table 1**). The staining intensity of neuropil was stronger on the unoperated side compared to postoperative day 3 (**Figure 1E–H**).

In the magnocellular part of the medial vestibular nucleus, the staining pattern of perineuronal nets and neuropil showed similar appearance to that of superior vestibular nucleus bilaterally on postoperative day 1 (**Figure 1I, J**). On

postoperative day 3, the staining of perineuronal nets no longer showed recognizable difference on the operated side and it was also similar on the following survival days (**Figure 1K–P; Table 1**). Neuropil remained the same in intensity on both sides.

In the lateral vestibular nucleus, the staining of perineuronal nets and the neuropil was the same as in case of the superior and medial vestibular nuclei on postoperative day 1 (**Figure 2A, B**). On postoperative day 3, the staining intensity of perineuronal nets occurred slightly weaker on the operated side, but was considerably increasing after postoperative day 1 on the side of labyrinthectomy (**Figure 2C, D; Table 1**). The perineuronal nets showed the same intensity bilaterally on postoperative day 7 and the staining of ipsilateral pericellular areas was lighter compared to that on postoperative day 3 (**Figure 2E, F**). By postoperative day 14, the staining of pericellular area showed no bilateral differences (**Figure 2G, H; Table 1**). The staining of neuropil remained unchanged during the postoperative periods.

In the rostral part of the descending vestibular nucleus, the perineuronal nets were not recognizable bilaterally on postoperative day 1, and the staining intensity of neuropil was weaker compared to the control animals at both sides (**Figure 2I, J**). On postoperative day 3, the staining intensity of perineuronal nets and neuropil appeared bilaterally at the level of control animals (**Figure 2K, L; Table 1**). The same pattern of tenascin-R immunoreactivity was shown on postoperative day 7 (**Figure 2M, N**). On postoperative day 14, the staining intensity decreased bilaterally both in the perineuronal nets and neuropil (**Figure 2O, P**). In the caudal part of the descending vestibular nucleus, moderately stained pericellular areas, similar to that of control animals, were observed bilaterally during the postoperative periods (**Figure 2Q–X**).

Statistical analysis revealed that in the superior vestibular nucleus, the changes of staining intensity of tenascin-R reaction were non-significant between days 1 and 3 and days 7 and 14, whereas they were statistically significant between days 3 and 7 ($P < 0.001$). In the magnocellular part of the medial vestibular nucleus, the statistical analysis did not show any significant changes in the staining intensity of tenascin-R reaction during the postoperative period. In the lateral vestibular nucleus and the rostral part of the descending vestibular nucleus, the staining intensity was statistically significant between days 1 and 3 ($P < 0.001$) and no significant changes were detected between the other survival days (**Table 1**).

Discussion

Unilateral labyrinthectomy results in elimination of sensory inputs from the vestibular receptors and the subsequent deafferentation-induced plasticity contributes to the restoration of vestibular function. Our results demonstrated for the first time that unilateral labyrinthectomy and subsequent compensation is accompanied by the modification of tenascin-R staining pattern in the vestibular nuclei of the rat. The modification of tenascin-R expression showed regional differences in the vestibular nuclear complex which may be
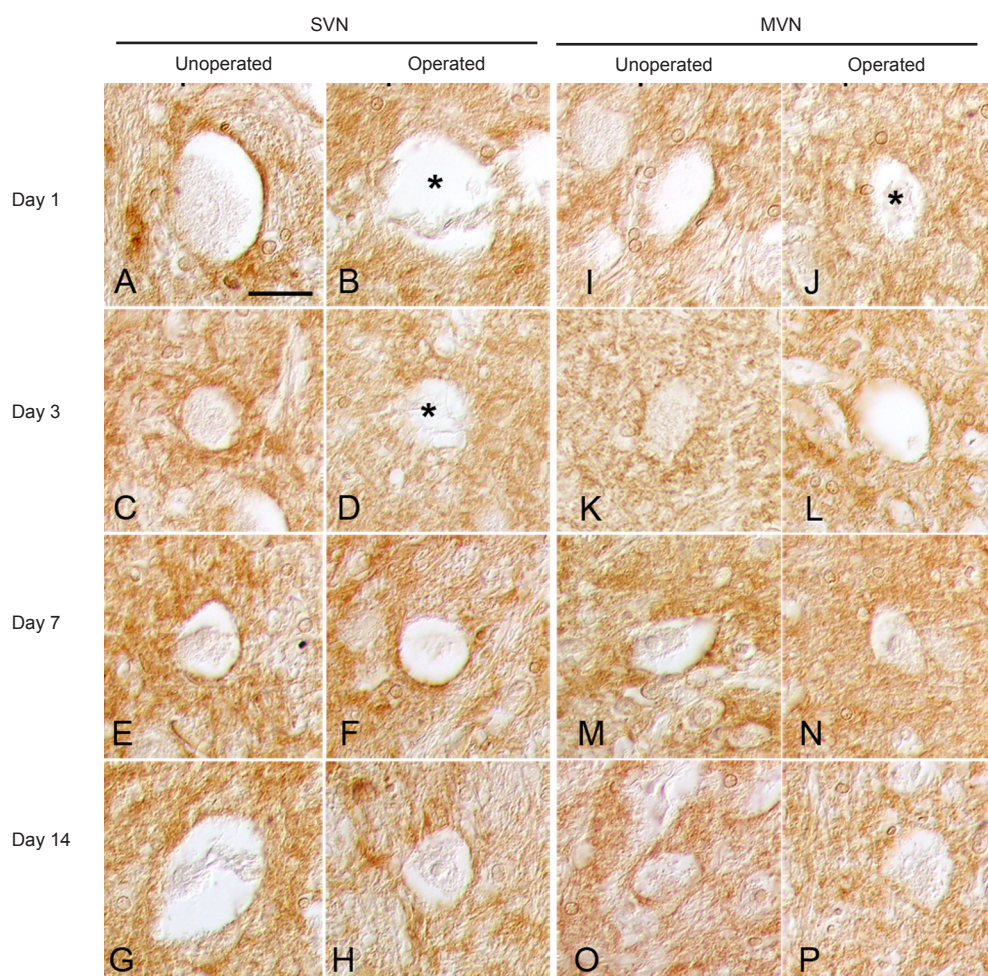
Figure 1 Distribution of tenascin-R staining intensity in the perineuronal nets of the superior (SVN) and medial vestibular nuclei (MVN) on the operated *versus* unoperated sides (A–P) on postoperative days 1, 3, 7 and 14.
In the SVN, decreased tenascin-R staining was seen in the perineuronal nets on the operated side on postoperative days 1 and 3 (B, D). In the MVN, weak tenascin-R staining in the perineuronal nets was only seen on postoperative day 1 on the operated side (J). Asterisks indicate decreased perineuronal net staining on the operated side. Scale bar: 20 μm.

associated with the morphological and functional heterogeneity of the individual vestibular nuclei and with their different roles in the compensatory processes.

The tenascin-R has versatile, sometimes opposite functions in the central nervous system depending on its location, the type of targeted cells, receptors, signaling pathways, the molecular composition of surrounding extracellular matrix as well as the embryonic and postembryonic periods of life (Pesheva and Probstmeier, 2000; Anlar and Gunel-Ozcan, 2012). Experimental studies showed that tenascin-R restricts functional recovery from spinal cord injury, and in agreement with this finding the tenascin-R-deficient mice recovered better than wild-type controls after spinal cord compression (Apostolova et al., 2006). Therefore, it appears reasonable that the temporary decrease in the tenascin-R expression presented in our study plays a role in the recovery from the vestibular disorder. In the lack of data on the role of tenascin-R in the vestibular system, we can merely state, at present, that the tenascin-R expression is changing after unilateral labyrinthectomy and during the subsequent com-

pensation. However, based on results of earlier experiments related to the role of tenascin-R in various parts of the central nervous system, we may suggest the following possible involvements of tenascin-R in the mechanisms of vestibular compensation (Dieringer, 1995; Dutia, 2010; Lacour and Tighilet, 2010).

First, the tenascin-R is known to activate the microglia cells which, in response, secrete cytokines and growth factors including brain-derived neurotrophic factor and nerve growth factor (Liao et al., 2005). In the deafferented vestibular nuclei of the labyrinthectomized rat, intense microglial reaction was detectable as early as day 1 after lesion and it persisted several weeks afterwards (Campos Torres et al., 1999). This microglial reaction constitutes one of the signals responsible for astroglial reaction observed in the vestibular nuclei during the 1-3 postoperative days (de Waele et al., 1996). As suggested by Campos Torres et al. (2005), growth factors as well as pro- and anti-inflammatory cytokines produced by activated astroglial cells could promote the survival of deafferented vestibular neurons and contribute to recovery of their resting
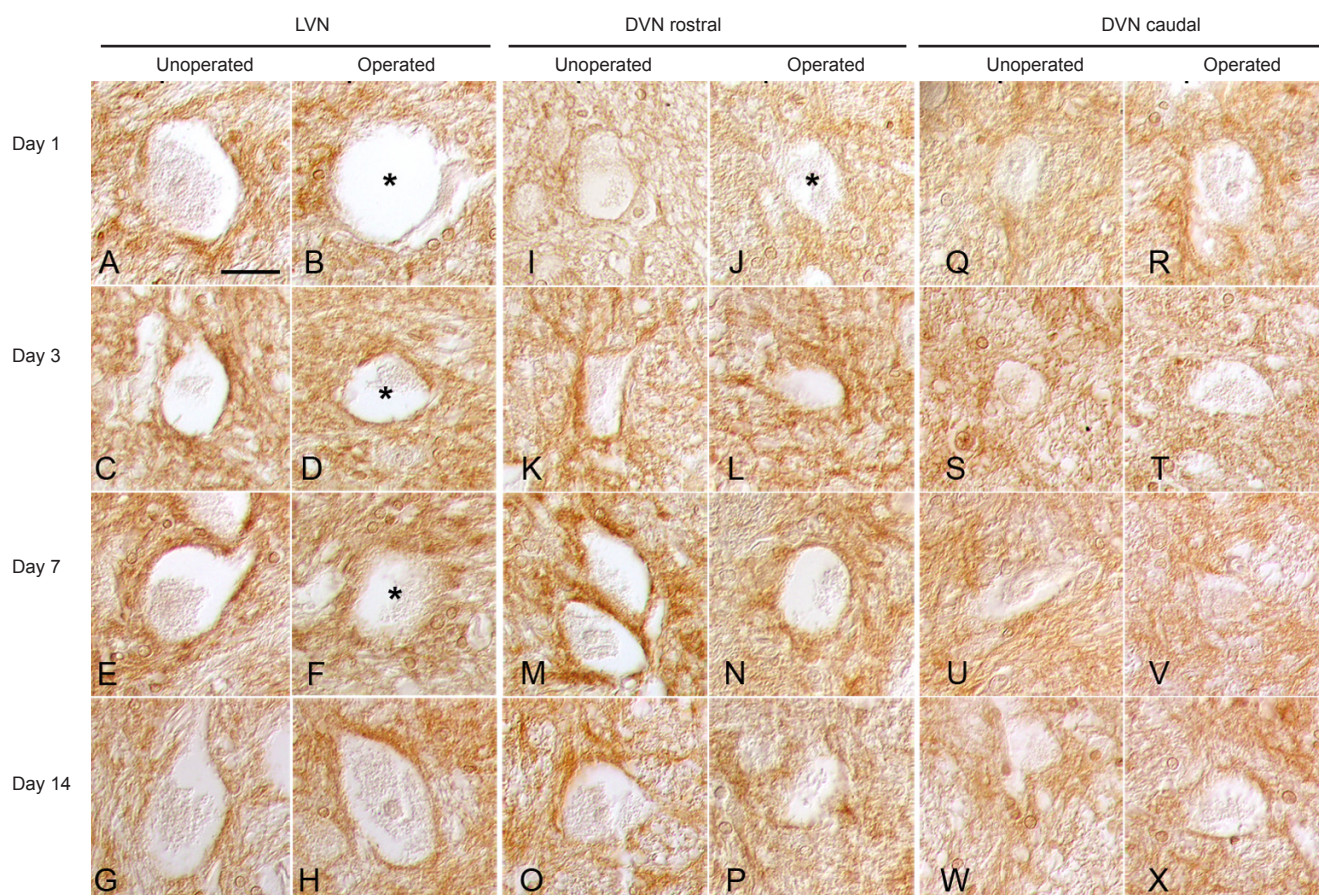
Figure 2 Distribution of tenascin-R staining intensity in the perineuronal nets of the lateral (LVN), rostral (DVN rostral) and caudal (DVN caudal) parts of the descending vestibular nucleus (DVN) on the operated *versus* unoperated sides (A–X) on postoperative days 1, 3, 7, and 14.
In the LVN, decreased tenascin-R staining was seen on the operated side on postoperative day 1 (B), and lesser difference was seen on postoperative days 3 and 7 (D, F). In the rostral part of DVN, weak tenascin-R staining in the perineuronal nets was only seen on postoperative day 1 on the operated side (J); in the caudal part of the DVN, no difference was observed throughout the postoperative period. Asterisks indicate decreased perineuronal net staining on the operated side. Scale bar: 20 μm.

Table 1 Semiquantitative assessment of the staining intensity of tenascin-R in the perineuronal nets of individual vestibular nuclei on the operated *versus* unoperated sides on survival days 1, 3, 7, and 14 following unilateral labyrinthectomy

	SVN		MVN magno		LVN		DVN rostral	
	Unoperated	Operated	Unoperated	Operated	Unoperated	Operated	Unoperated	Operated
Day 1	+++	–	++	–	+++	–/+	+	–
Day 3	+++	–	++	+	+++	++	++	++
Day 7	+++	++	++	+	+++	+/++	+++	++
Day 14	++	++	++	+	++	++	++	+

The staining intensity was scored as –: no staining, +: weak staining, ++: moderate staining, +++: strong staining. Staining intensity of tenascin-R reaction in the SVN was statistically significant between days 3 and 7 ($P < 0.001$). In the LVN and the rostral part of DVN, the staining intensity was statistically significant between days 1 and 3 ($P < 0.001$). Statistical analysis detailed in the text. SVN: Superior vestibular nucleus; MVN magno: magnocellular part of medial vestibular nucleus; LVN: lateral vestibular nucleus; DVN rostral: rostral part of the descending vestibular nucleus.

discharge (Dieringer, 1995; Vibert et al., 1995; Li et al., 1999; Straka et al., 2005; Dutheil et al. 2013). To support these findings, Lacour and Tighilet (2010) confirmed bilateral up-regulation of brain-derived neurotrophic factors along with its TrkB receptor, both of which appeared as early as 1 day after unilateral labyrinthectomy and peaked at postoperative day 3 in the descending and lateral vestibular nucleus. As a result of glial reactions described above, the secreted growth factors facilitate the survival and recovery of deafferented vestibular neurons. The increased staining of tenascin-R in the neuropil from postoperative day 3 (sometimes from postoperative day 7) might be associated with the microglial activation thereby the tenascin-R may promote the plasticity of vestibular nucleus and contribute to the repair of vestibular disorders.

Second, the extracellular matrix in physiological conditions,

by stabilization of synapses, creates a barrier against the formation of new synaptic contacts and restricts the synaptic plasticity (Galtrey and Fawcett, 2007). In our present study, the common feature of the tenascin-R expression was the decrease or disappearance of tenascin-R immunoreactivity from the perineuronal nets in each vestibular nucleus. Decreased staining intensity of non-permissive tenascin-R may stimulate the formation of new synaptic contacts, as one of the possible mechanisms during the restoration of vestibular function (Dieringer, 1995; Li et al., 1999; de Waele et al., 2000; Lacour and Tighilet, 2010).

The third possible involvement of tenascin-R in the vestibular compensation might be related to inhibitory commissural pathways existing between the bilateral vestibular nuclei (Holstein et al., 1999; Bergquist et al., 2008; Malinvaud et al., 2010). Unilateral labyrinthectomy results in severe imbalance in the GABAergic commissural system and it is regarded as a key cause of the static oculomotor and postural symptoms. Similarly, the asymmetry in spontaneous resting activity between the intact and deafferented vestibular neurons is due to the imbalance of GABAergic interaction between the ipsilateral and contralateral sides (Gliddon et al., 2004). The re-balancing of commissural inhibition occurs in parallel with the restoration of impaired resting activity and with the subsequent behavioral recovery during vestibular compensation (Gliddon et al., 2004; Straka et al., 2005; Tighilet et al., 2007; Bergquist et al., 2008). The tenascin-R regulates, *via* interactions of its HNK-1 (human natural killer cell) carbohydrate epitope, the $GABA_B$ receptor mediated perisomatic inhibition and thus influences synaptic transmission and plasticity in the hippocampus (Saghatelyan et al., 2000, 2001; Bukalo et al., 2001; Dityatev and Schachner, 2003; Brenneke et al., 2004). As the unilateral labyrinthectomy resulted in marked downregulation of the functional efficacy of $GABA_B$ receptors in the cells of the ipsilesional medial vestibular nucleus (Yamanaka et al., 2000; Horii et al., 2003) similar regulatory function of tenascin-R is possible in the vestibular system.

Fourth, the tenascin-R is detectable around the nodes of Ranvier predominantly at the large, myelinated axons (Apostolova et al., 2006; Bekku et al., 2009). Here, the tenascin-R is binding to the voltage-gated sodium channels and initiates the clustering of channels and then stabilizes the clusters after they have formed thereby it is regarded as a functional modulator of sodium channel beta subunits (Srinivasan et al., 1998; Xiao et al., 1999). In the tenascin-R-deficient mice, there was a significant decrease in conduction velocity of myelinated axons (Weber et al., 1999). As the vestibular nuclei have large caliber myelinated axons (Sotelo and Palay, 1970), similar function of the tenascin-R might be suggested here, as well.

Although the individual vestibular nuclei are different from each other in their morphological, physiological and biochemical characteristics associated with their different functions in balance, vestibulo-ocular reflexes, spatial cognition and automatic responses (Babalian and Vidal, 2000; Birinyi et al., 2001; Straka et al., 2005; Eugene et al., 2011;

McCall and Yates, 2011; Kodama et al., 2012; Racz et al., 2014) their specific role in the compensatory processes is not yet determined. Most of the experiments on vestibular lesion and subsequent compensation have been performed on the medial vestibular nucleus. This nucleus is engaged mostly in the postural reflexes, and restoration of which is known as the initial event during the vestibular compensation (Yamanaka et al., 2000; Beraneck et al., 2003; Beraneck and Idoux, 2012). The earliest re-establishment of tenascin-R reaction in the perineuronal nets around the neurons of medial vestibular nucleus might support these findings. The only part of the vestibular nuclear complex where the tenascin-R reaction remained almost unchanged following unilateral labyrinthectomy is the caudal part of descending vestibular nucleus. This subnucleus is involved in the rapid modulation of the cardiovascular, respiratory and digestive systems in response to locomotion and postural adjustments (Ruggiero et al., 1996; Matesz et al., 1997; Porter and Balaban, 1997; Holstein et al., 2011). In the light of our results, it is tempting to assume that the role of extracellular matrix is less important in the compensatory processes of impaired vestibulo-autonomic function.

Our results may provide new insights into the mechanisms of vestibular plasticity. The presented results contribute to our earlier findings on the spatially and temporally specific alterations of hyaluronan and chondroitin sulfate proteoglycans during vestibular compensation (Deak et al., 2012). The reduction of the immunostaining of tenascin-R also suggests the extracellular facilitation of plastic modifications of the vestibular circuit after lesion. The results may also assist in developing new therapeutic strategies for the treatment of symptoms of vestibular lesion.

Author contributions: *CM and BG conceived and designed this study, and wrote the paper; EÖJ, AD, AM and IW were responsible for data acquisition; EÖJ, AD, AM, IW, and BG analyzed the data; BG revised critically for the important intellectual content; CM supervised the study. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*

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