Neurocan contributes to the molecular heterogeneity of the perinodal ECM*

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Summary. Neurocan is a central nervous tissue-specific chondroitin sulfate proteoglycan of the lectican family. Mainly expressed during modeling and remodeling stages of this tissue, it is thought to play an important role via binding to various extracellular matrix and cellular components. In adults, neurocan expression is associated with the perineuronal net structures. This study shows the neurocan immunolocalization at the node of Ranvier in mouse central nervous tissues. The N-terminal fragment of neurocan (Ncan130) was the predominant form detected in the optic nerve. The expression of neurocan in the white matter of brain tissue and nerve tracts revealed differential expression profiles compared with those of versican V2 and brevican, other perinodal extracellular matrix molecules. Double immunolabeling for neurocan and a nodal marker, Bral1, or a paranodal marker, caspr, demonstrated that neurocan was localized at the node of Ranvier. Neurocan expression was found at many—not all—nodal regions, and neurocan-positive nodes outnumbered brevican-positive nodes. The nodal localization of neurocan was diminished in Bral1-deficient mice. Taken together, these findings indicate that neurocan contributes to the molecular heterogeneity of the perinodal matrix, and its nodal expression is dependent on Bral1.

Introduction

The myelin sheath at the nodes of Ranvier is interrupted, and a few micrometers of the axonal membrane are directly exposed to the extracellular fluid, thereby enabling nerve impulse propagation in the myelinated nerves. However, the molecular details and physiological activity of the perinodal extracellular matrix (ECM) were unclear until recently. Hyaluronan is enriched at the extranodal matrix, thus leading to the accumulation of hyaluronan-associated ECM molecules (Oohashi and Bekku, 2007). In the adult central nervous system, versican V2 and Bral1 (also called Hapln2) are the predominant components of the perinodal ECM (Schmalfeldt et al., 2000; Oohashi et al., 2002). Brevican, another member of the lectican family, localizes at the large diameter node, and determines the co-localization of tenascin-R and phosphacan (Bekku et al., 2009). In versican V2 or Bral1-deficient mice, the perinodal accumulation of ECM molecules is largely impaired, without any effect on sodium channel clustering or paranodal structures (Dours-Zimmermann et al., 2009; Bekku et al., 2010). As a result of this impaired ECM accumulation, the conduction velocity of the optic nerve was reduced in Bral1-deficient mice. The diffusion of substances in the extracellular space (ECS) is defined by Fick’s diffusion equations (Nicholson and Phillips, 1981) incorporating three diffusion parameters: extracellular volume fraction (α), tortuosity (λ), and non-specific uptake k’ (Sykova and Nicholson, 2008). Analyses of the ECS diffusion parameters in Bral1-deficient mice using

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a real-time iontophoretic method and diffusion-weighted magnetic resonance imaging (MRI) revealed a facilitated diffusion in the white matter of the mutant mice (Bekku et al., 2010). The extranodal matrix assemblies were therefore hypothesized to be a potential diffusion barrier that modulates the accumulation of ions during salutatory conduction (Bekku et al., 2010).

Neurocan is a member of the chondroitin sulfate proteoglycans (CSPG) lectican family that includes aggrecan, versican, brevican, and neurocan (Bandtlow and Zimmermann, 2000). Among the lecticans, neurocan is the predominant lectican in the extracellular environment during the developmental stage (Rauch et al., 1991; Hirakawa et al., 2000). Neurocan is thought to play roles in brain development and tissue remodeling processes through interactions with other extracellular or cell surface molecules (Matsui and Oohira, 2007). The protein is subjected to proteolytic cleavage, giving rise to N- and C-terminal fragments (Ncan150, Ncan130, and Ncan90) (Rauch et al., 1991; Oohira et al., 1994; Meyer-Puttlitz et al., 1995). In adults, neurocan is expressed in neurons (Engel et al., 1996; Miyata et al., 2005) and associated with the perineuronal net structures that constitute a highly condensed matrix surrounding the cell bodies and proximal dendrites of some classes of neurons (Celio et al., 1998; Matsui et al., 1998). Neurocan is expressed by neurons in these situations (Engel et al., 1996; Miyata et al., 2005). There is emerging evidence that CSPGs, including neurocan, are involved in the control of neuronal plasticity (Pizzorusso et al., 2002; Rhodes and Fawcett, 2004). Supporting this hypothesis is the fact that neurocan is upregulated in the injured brain and cytokine-treated astrocytes (Asher et al., 2000). CSPGs contribute to the inhibition of axonal regeneration and restrict plasticity.

This study reports for the first time the immunolocalization of neurocan at many—but not all—nodes of Ranvier in the mouse brain. The results may therefore provide clues for understanding the diversity of the molecular assembly of the perinodal matrix, which may be related to the creation of an ion diffusion barrier.

**Materials and Methods**

**Animals**

Adult C57BL/6J mice (Shimizu Laboratory Supplies, Kyoto) and Bral1-deficient mice (Bekku et al., 2010) were used for this study. All animal experiments and animal care were carried out in accordance with the guidelines of the Animal Care and Experimentation Committee of Okayama University. All efforts were made to minimize both the discomfort and the number of animals used.

**Primary antibodies**

The rabbit polyclonal antibodies against Bral1, brevican (Ab1058), and versican- a, have been described previously (Oohashi et al., 2002; Bekku et al., 2003). We used a rabbit anti-caspr polyclonal antibody ab34151: Abcam, Cambridge, MA, USA; 1 μg/ml for immunohistochemistry) and a sheep anti-mouse/rat neurocan polyclonal antibody (AF5800: R&D systems, Minneapolis, MN, USA; 8 μg/ml for immunohistochemistry and 2 μg/ml for immunoblotting) for our studies.

**Western blot analysis**

For immunoblotting, tissue samples from the adult mouse brain or optic nerves were homogenized on ice in a 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 10 mM CHAPS, and protease inhibitor cocktail (Sigma-Aldrich). After centrifugation at 1,000 × g, treatment of the supernatant with chondroitinase ABC (Ch’ase ABC; Seikagaku Kogyo, Tokyo) was performed as described previously (Bekku et al., 2003, 2009). The protein concentrations of the extracts were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hecules, CA, USA) using bovine serum albumin as a standard. Proteins were separated on 2–15% gradient gels (COSMO BIO, Tokyo) and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). Primary antibodies were visualized by HRP-conjugated goat anti-rabbit (1:1000; MP Biomedicals, Aurora, OH, USA) or HRP-conjugated donkey anti-sheep IgG (1:1000; R&D systems) and enhanced with the ECL plus detection system (GE Healthcare, Buckinghamshire, UK).

**Immunohistochemistry**

The mice were deeply anesthetized with diethyl ether and transcardially perfused with 50mM phosphate-buffered saline (PBS; pH 7.4) followed by 4% PFA in PBS. Thereafter, the brains and optic nerves were removed and cryoprotected with 30% sucrose in PBS for 18 h at 4°C. Next, the samples were embedded in OCT compound (Sakura Finetek Japan, Tokyo) and completely frozen in hexane, which was cooled with liquid nitrogen. Cryosections (5 μm) were cut on a cryomicrotome (Leica CM1900; Leica, Wetzlar, Germany). They were pretreated with Ch’ase ABC (0.05 U/ml; Seikagaku
Neurocan expression at the node of Ranvier

of these punctuate signals for neurocan, brevican, and Bral1 (Fig. 2) and for versican (data not shown) differed and was observed in the following order: versican V2 = Bral1 > neurocan > brevican. Simultaneous immunostaining with an anti-neurocan antibody in the perineuronal net-fashion was observed in the deep cerebellar nuclei on the same section (Fig. 2A). Similar punctate patterns for neurocan were also detected in the white matter regions of the corpus callosum and internal capsule and in the optic nerve and facial nerve tract with the concomitant staining of the perineuronal nets in adjacent gray matter, such as the superior olive and the zona incerta (Fig. 3).

Kogyo) for 30 min at 37°C. Immunohistchemistry was performed as previously described (Oohashi et al., 2002; Bekku et al., 2009). The bound antibodies were visualized with biotinylated rabbit anti-sheep IgG (1:500; Jackson Laboratories, West Grove, PA, USA) or biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) and a streptavidin-HRP complex ( Vectastain ABC elite kit, Vector). HRP activity was detected with 0.025% diaminobenzidine and 0.03% H2O2 in PBS. For the immunofluorescence detection, Alexa Fluor 488 chicken anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 555 donkey anti-goat IgG (1:500; Invitrogen) were used. The images were captured using either an Olympus BX50 light microscope (Olympus, Tokyo) equipped with a Pro150ES CCD-camera (Pixera, Los Gatos, CA, USA) or a laser-scanning microscope LSM510 (Carl Zeiss, Oberkochen, Germany). All images were processed using Adobe Photoshop CS4.

Results

Specificity of the antibody against neurocan

The antibody against neurocan used in the current study was characterized by immunoblotting. The anti-neurocan antibody was raised against a recombinant mouse neurocan protein (aa 23-637) expressed by CHO cells according to the manufacturer’s instructions (AF5800: R&D systems). Neurocan-specific IgG was then purified by mouse neurocan affinity chromatography. On the immunoblot of the adult brain lysates after Ch’ase ABC treatment, the full-length neurocan band (Ncan full) as well as proteolytic fragments of neurocan (Ncan150, Ncan130, and Ncan90) were specifically detected (Fig. 1). The size and patterns of these bands were obviously different from those of brevican and versican V2: the absence of a cross-reacted band at around 35–45 kDa, which corresponds to the size detectable for link proteins (Crt11, Bral1, and Bral2), was noticed. Importantly, the N-terminal fragment of neurocan (Ncan130) was predominantly detected in the optic nerves of adult mice (Fig. 1, on, indicated by **).

Immunohistochemical detection of neurocan in the white matter

Immunoreactivity of the neurocan antibody was assessed in the white matter of the adult mouse cerebellum. The immunoreactivity was evident with a typical punctate fashion (Fig. 2A) as was demonstrated with anti-brevican and anti-Bral1 antibodies (Fig. 2B, 2C). The density of these punctuate signals for neurocan, brevican, and Bral1 (Fig. 2) and for versican (data not shown) differed and was observed in the following order: versican V2 = Bral1 > neurocan > brevican. Simultaneous immunostaining with an anti-neurocan antibody in the perineuronal net-fashion was observed in the deep cerebellar nuclei on the same section (Fig. 2A). Similar punctate patterns for neurocan were also detected in the white matter regions of the corpus callosum and internal capsule and in the optic nerve and facial nerve tract with the concomitant staining of the perineuronal nets in adjacent gray matter, such as the superior olive and the zona incerta (Fig. 3).

![Fig. 1. A Western blot analysis of an affinity-purified anti-neurocan antibody. Adult brain or optic nerve lysates (brain, 30 µg; optic nerve, 15 µg) treated with Ch’ase ABC were resolved in 2–15% gradient SDS-PAGE gels and immunoblotted with an affinity-purified anti-neurocan antibody, anti-brevican antibody, and anti-versican-a antibody. Notice the specific bands for the full length neurocan (Ncan full) and proteolytic fragments (Ncan190, Ncan150, and Ncan90). Also notice the absence of cross-reacted bands for link proteins at around 35–45 kDa (asterisk). Ncan: neurocan, Bcan: brevican, V2: versican V2, on: optic nerve.](image-url)
Fig. 2. Immunohistochemical staining of cerebellar sections from adult mice. Cerebellar sections were stained using antibodies against neurocan (A), brevican (B), or Bral1 (C). Notice the presence of the punctuate-immunoreactivity in the white matter with a concomitant perineuronal net type-immunoreactivity in the deep cerebellar nuclei (DC) with the anti-neurocan antibody. Ncan: neurocan, Bcan: brevican. Bar: 100 μm

Fig. 3. Immunohistochemical staining of neurocan in the white matter region and the nerve fiber tracts of the brain. The dotted lines indicate the border of the white matter region. Punctate patterns of neurocan-positive staining are observed in the corpus callosum (A, cc), the internal capsule (B, ic) and the zona incerta (B, ZI), the optic nerve (C, on), the facial nerve tract (D, 7n) and the superior olive (D, SO). Bar: 50 μm
Fig. 4. Immunofluorescent labeling of the optic nerve in wild-type mice (A, C–E) and Bral1-deficient mice (B, F–H) using antibodies to neurocan (magenta), Bral1 (green), and caspr (green). A neurocan signal is clearly shown between caspr-positive paranodes (A). Punctate signals of neurocan are accompanied with Bral1 (E, arrows). Notice the presence of some neurocan-negative nodes (arrow heads). Ncan: neurocan, Bcan: brevican. Bars: 10 μm
Neurocan is expressed at the node of Ranvier in the CNS

To determine the neurocan expression at the node of Ranvier, we conducted double immunolabeling in the optic nerve with the anti-Bral1 antibody as a nodal marker and with the anti-caspr antibody as a paranodal marker. The neurocan signal was always intercalated between the caspr signals (Fig. 4A), as was observed for Bral1 (Bekku et al., 2010). Double immunolabeling for neurocan and Bral1 showed that all of the neurocan-positive signals co-localized with Bral1. However, in some cases, Bral1-positive signals were not accompanied with neurocan (Fig. 4C–E, arrow heads). The ratio of neurocan-positive/Brail-positive spots in the total Bral-positive spots was 200/276 (72 %) (Fig. 4C–E). Double immunolabeling for neurocan and brevican revealed the neurocan-positive nodes to be more numerous than the brevican-positive nodes (Fig. 5). The neurocan signal was not detectable at the node of Ranvier in the Bral-deficient mice (Fig. 4B, F–H), suggesting that neurocan is stabilized by Bral1 at the node, as is the case for other nodal ECM molecules (Bekku et al., 2010).

Discussion

The current study has characterized an affinity purified anti-mouse/rat neurocan antibody (AF5800: R&D systems). Many of the antibodies being used for neurocan studies were produced in the mice as monoclonal antibodies (1D1, Rauch et al., 1991; 1G2, Oohira et al., 1994; 1F6, Meyer-Puttlitz et al., 1995) with limited availability of neurocan antibodies in other species (Milev et al., 1998; Zhou et al., 2001; Dours-Zimmermann et al., 2009). The immunoblotting was able to demonstrate the specific detection of neurocan—including full-length form as well as all of the processed forms (Fig. 1), so that all forms of neurocan were detectable in the mouse at the same time. Hence, the antibody characterized in the present study may be a valuable tool for investigation of the neurocan immunolocalization in mice.

The immunoblotting results of the tissue extracts from adult brain and optic nerves revealed the presence of different ratios of processed neurocan fragments. The optic nerve was used for immunoblotting, since it is located in a perineuronal nets-free environment and can be reliably dissected without contamination of the parenchymal extracellular matrix aggregates. In the optic nerve, the N-terminal fragment of neurocan (Ncan130) was predominantly detected while other forms were detected in trace amounts. This indicates that the difference may be due not only to the processing of core proteins, but also to the molecular associations of the C-terminal fragments (Ncan150).

The C-type lectin domain of neurocan has the lowest affinity to tenascin-R among the lecticans. It is therefore, unlikely that the C-terminus of neurocan is stabilized via

![Fig. 5. Double immunostaining for neurocan and brevican in the optic nerve. Notice the presence of brevican-positive nodes (arrows) and brevican-negative nodes (arrow heads) among the neurocan-positive nodes. Ncan: neurocan, Bcan: brevican. Bar: 10 μm](image)
tenascin-R, even though tenascin-R is present at the node.

The current study demonstrated that neurocan is expressed at the node of Ranvier and is widely distributed in the white matter of the cerebellum, optic nerve, facial nerve tract (Fig. 2, 3C, D), vestibuloocochlear nerve, trapezoid body, and spinal trigeminal tract (data not shown), where brevican immnoreactivity was also found (Bekku et al., 2009). In addition, neurocan immunoreactivity was also found in the corpus callosum and internal capsule (Fig. 2A, B), regardless of the co-localization of brevican (Bekku et al., 2009). It is likely that the nodal ECM is highly diverse and complex. While versican V2 localizes at all of the CNS nodes, and brevican localizes at the large-diameter nodes in the nerve tracts, neurocan seems to localize at the intermediate-type nerves (Bekku et al., 2009; Bekku et al., 2010). Further measurements of neurocan positive-axonal diameters may elucidate the differential expression of neurocan and other nodal ECM molecules at the node. However, such measurements are presently impossible due to the unavailability of an anti-sodium channel antibody which can be co-labeled with the anti-neurocan antibody used in the present study.

We recently reported the Bral1-dependent formation of perinodal ECM in the CNS (Bekku et al., 2010). The CNS nerve conduction is markedly decreased in Bral1-deficient mice, but there was no effect on the clustering or transition of ion channels at the nodes or in the tissue morphology around the nodes of Ranvier. This previous study suggested that Bral1-based ECM association creates a diffusion barrier around the nodes and provides an extranodal micromilieu as an 'extracellular ion pool' (Bekku et al., 2010). The current study demonstrated the presence of neurocan in many of the CNS nodes. Although the N-terminal processed form of neurocan (Ncan130) predominates at the node, the Ncan130 is predicted to contain two chondroitin sulfate chains. The presence of neurocan at the node may therefore positively influence the creation of an ion diffusion barrier.

In summary, the current results provide insight into the physiological roles of neurocan-associating ECM at the nodes of Ranvier in the CNS.

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