Chondroitin sulfate expression around spinal motoneurons during postnatal development in rats

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ABSTRACT

Perineuronal nets are extracellular matrix structures that surround neuronal cell bodies and their proximal dendrites in the central nervous system. Chondroitin sulfate proteoglycans, which contain chondroitin sulfates (CSs) are major components of perineuronal nets. CSs are considered to have inhibitory roles in neural plasticity, although the effects differ according to their sulfation pattern. In the present study, we investigated the expression of the CS subtypes CS-A and CS-C surrounding spinal motoneurons in different postnatal periods to explore the potential influence of altered CS sulfation patterns on spinal development. CS-A-positive structures were observed around motoneurons in the cervical, thoracic, and lumbar segments as early as postnatal day (P) 5. Most motoneurons were covered with CS-A-positive structures during the first 2 postnatal weeks. The percentage of motoneurons covered with CS-A-positive structures decreased after P20, becoming lower than 70% in the cervical, and lumb segments after P35. CS-C-positive structures were occasionally observed around motoneurons during the first 2 postnatal weeks. The percentage of motoneurons covered with CS-C-positive structures increased after P20, becoming significantly higher after P25 than before P20. The expression pattern of Wisteria Floribunda agglutinin-positive structures around motoneurons was similar to that of the CS-C-positive structures.

The present findings revealed that CS-A and CS-C are differentially expressed in the extracellular matrix surrounding motoneurons. The altered sulfation pattern with increased CS-C expression is associated with the maturation of perineuronal nets and might lead to changes in the motoneuron plasticity.

1. Introduction

Perineuronal nets (PNNs) are unique lattice-like extracellular matrix (ECM) structures that surround cell bodies and proximal dendrites of some neurons in the central nervous system. PNNs are formed relatively late in the developmental process as mature synaptic connections are established. PNNs are implicated to have a major role in synaptic stabilization and maturation, i.e., restricting the formation of new connections with advancing axons (Berardi et al., 2004; Karetko and Skangiel-Kramska, 2009; Dzyubenko et al., 2016). The formation of PNNs is considered to coincide with the closure of the critical period for plasticity (Wang and Fawcett, 2012).

A major component of PNNs is chondroitin sulfate proteoglycans (CSPGs), among which the sulfated glycosaminoglycan (GAG) side chain is considered to contribute to neural plasticity and development (Caterson, 2012; Dyck and Karimi-Abdolrezaee, 2015; Soleman et al., 2013). There are 5 sulfation patterns in the repeating disaccharide unit of GAG, i.e., chondroitin sulfate (CS)-O, CS-A, CS-C, CS-D, and CS-E. In the spinal cord, CS-A and CS-C, also termed chondroitin-4-sulfate and chondroitin-6-sulfate, respectively, are expressed in the PNNs (Mikami and Kitagawa, 2013). CSs are considered to have inhibitory roles in neural plasticity, although the effects differ according to their sulfation pattern (Properzi et al., 2005; Wang et al., 2008; Butterfield et al., 2010; Lin et al., 2011; Swarup et al., 2013). In previous studies, PNNs were usually visualized by immunohistochemistry using antibodies against CSPG core proteins, tenascin-R or CS-56, a pan marker for CSs, as well as by staining with the plant lectin Wisteria Floribunda agglutinin (WFA; Irvine and Kwok, 2018; Miyata et al., 2012; Sigal et al., 2019). Few studies, however, have examined the expression of CS by classifying the subtypes, such as CS-A and CS-C.

A previous study using immunohistochemistry for CSPG core proteins showed that the formation of PNNs in the rat spinal cord begins in the second postnatal week (Gaitrey et al., 2008). In rats, the corticospinal tract (CST) develops from late embryonic days to the first 2 weeks...
after birth. The CST axons reach the white matter at the cervical level at birth (postnatal day 0 \([P0]\)), the thoracic level at P3-P5, and the lumbar level at P7, and then enter the gray matter 2–3 days later to complete functional synapse formation after P16 (Donatelle, 1977; Schreyer and Jones, 1982; Gribnau et al., 1986). PNN formation, therefore, is coincident with the formation of synaptic connections between CST axons and spinal neurons. Although disaccharide units with different sulfation patterns might also have important roles in synaptic formation in the spinal cord (Dyck and Karimi-Abdolrezaee, 2015), the expression patterns of CS-A and CS-C in PNNs during the postnatal period are not well elucidated.

In the present study, therefore, to explore the potential significance of the differences in CS sulfation patterns in the PNNs during postnatal development, we investigated the percentage of motoneurons that were surrounded by CS-A- and CS-C-positive structures, as well as WFA-positive structures in various spinal segments.

2. Results

The postnatal development of WFA-, CS-A, and CS-C–positive structures surrounding motoneurons in the cervical (C7), thoracic (T8), and lumbar (L5) segments of rat spinal cord was analyzed in this study. The spinal regions that were analyzed are shown in Fig. 1. Higher magnification images are shown in Figs. 2–4.

2.1. WFA-positive PNNs

In the cervical spinal cord, choline acetyltransferase (ChAT)-positive neurons with WFA-positive structures were rarely observed at P5 (0.45 ± 0.45%), but gradually increased up to P15 (P10, 2.94 ± 2.94%; P15, 13.81 ± 7.34%). At P20 (53.51 ± 5.85%), the percentage of ChAT-positive neurons with WFA-positive structures was significantly higher than that at P5, P10, and P15 (\(P < 0.01\)), and it was maintained between 50% and 80% from P25 to P40 (Fig. 2M; P25, 71.35 ± 5.77%; P30, 69.19 ± 6.46%; P35, 65.67 ± 4.99%; P40, 79.93 ± 1.82%). The significant differences observed between groups are shown in Fig. 2M.

In the thoracic spinal cord, no WFA-positive structures were observed from P5 to P15. Over 40% of the ChAT-positive neurons, however, were covered with WFA-positive structures at P20 (43.44 ± 4.98%). From P25 to P40, the percentage of ChAT-positive neurons covered with WFA-positive structures was between 40% and 70% (P25, 58.52 ± 3.97%; P30, 57.17 ± 6.16%; P35, 56.24 ± 3.86%; P40, 62.72 ± 2.37%). The significant differences observed between groups are shown in Fig. 2N.

In the lumbar spinal cord, WFA-positive structures were completely absent at P5, and then the number gradually increased until P15 (P10, 5.15 ± 3.13%; P15, 3.50 ± 2.12). Thereafter, nearly 30% of ChAT-positive neurons were covered with WFA-positive structures at P20 (31.27 ± 8.90%), and the percentage of ChAT-positive neurons with WFA-positive structures increased from P25 to P40 (P25, 37.49 ± 3.80%; P30, 54.76 ± 2.06%; P35, 53.93 ± 5.04%; P40, 68.23 ± 2.84%).

Fig. 1. (A–C) Images of \(W. \) Floribunda agglutinin (WFA) histochemistry (green) and immunohistochemistry for choline acetyltransferase (ChAT, red) in the ventral horn of cervical (A), thoracic (B), and lumbar (C) spinal segments at P30. (D–F) Images of double-labeling immunohistochemistry for CS-A (green) and ChAT (red) in cervical (D), thoracic (E), and lumbar (F) spinal segments at P30. (G–I) Images of double-labeling immunohistochemistry for CS-C (green) and ChAT (red) in cervical (G), thoracic (H), and lumbar (I) spinal segments at P30. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The significant differences observed between groups are shown in Fig. 2 O.

2.2. CS-A in the ECM surrounding spinal motoneurons.

In the cervical spinal cord (C7), CS-A immunoreactivity was observed around all ChAT-positive neurons at P5, and the percentage of ChAT-positive neurons covered with CS-A-positive structures was decreased at P10 (90.96 ± 2.20%), P15 (74.16 ± 2.34%), P20 (76.05 ± 6.04%), P25 (96.11 ± 2.39%), P30 (74.11 ± 3.59%), P35 (46.24 ± 5.24%), and P40 (66.51 ± 4.71%). The significant differences from P40 are shown in Fig. 3 M. In each segment, there was a transient increase at P25.

CS-A immunoreactivity was also examined using a different monoclonal antibody (Clone LY111) in the cervical segment sections. Positive structures surrounding ChAT-positive neurons were observed at different postnatal days (P10, 84.05 ± 3.32%; P20, 73.26 ± 2.36%; P35, 70.95 ± 3.37%; P40, 77.28 ± 3.40), as shown in Suppl. 1.

Fig. 2. (A-L) Higher magnification images of WFA histochemistry (green) and immunohistochemistry for ChAT (red) in cervical (A-D), thoracic (E-H), and lumbar (I-L) spinal segments at P10 (A, E, and I), P20 (B, F, and J), P30 (C, G, and K), and P40 (D, H, and L). Arrowheads indicate WFA-positive perineuronal nets around ChAT-immunoreactive motoneurons. Scale bars = 20 μm. (M-O) Percentage of motoneurons with WFA-positive perineuronal nets in the cervical (M), thoracic (N), and lumbar (O) spinal cord. Blue bars indicate the percentage of motoneurons with greater than 80% WFA-positive surrounding structures, and red bars indicate between 20% and 80% WFA-positive surrounding structures. Data are expressed as the mean ± SEM (>80% and >20%). Statistical analyses of percentage of motoneurons with WFA-positive structures were performed using one-way ANOVAs, followed by Tukey-Kramer test. Significant differences from P5, P10, and P15 are expressed as ††: P < 0.01. Other significant differences are expressed as *: P < 0.05 and **: P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.3. CS-C in the ECM surrounding spinal motoneurons

Contrary to CS-A–positive surrounding structures, in the cervical spinal cord (C7), there were few ChAT-positive neurons covered with CS-C–positive structures at P5 (7.68 ± 3.56%), P10 (5.33 ± 0.10%), and P15 (14.77 ± 5.65%). The percentage of ChAT-positive neurons covered with CS-C–positive structures at P20 (28.53 ± 5.67%) was significantly higher than that at P10 (P < 0.01) and P5 (P < 0.05). The CS-C–positive surrounding structures were significantly increased at P25 compared with P5, P10, P15, or P20 (P < 0.01), whereas the percentages of ChAT-positive neurons covered with CS-C–positive structures after P30 were almost unchanged compared with that at P25 (P25, 76.06 ± 3.11%; P30, 78.03 ± 0.55%; P35, 70.38 ± 3.32%; P40, 80.30 ± 1.64%). The significant differences observed between groups are shown in Fig. 4M.

In the thoracic spinal cord (T8), the CS-C–positive surrounding structures were poorly formed at P5 (3.89 ± 0.86%), P10 (0 ± 0%), and P15 (6.46 ± 1.81%). Interestingly, unlike in the C7 segment, no CS-C–positive surrounding structures were observed at P10. After P20, the expression patterns of CS-C–positive surrounding structures in the T8 segment (P25, 79.38 ± 2.37%; P30, 76.30 ± 5.71%; P35, 81.67 ± 3.83%; P40, 86.28 ± 2.50%) tended to be similar to those in C7. The significant differences between groups are shown in Fig. 4N.

In the lumbar spinal cord (L5), the expression patterns of the CS-C–positive surrounding structures were analogous to those in the T8 segment (P5, 0.90 ± 0.78%; P10, 0 ± 0%; P15, 6.51 ± 1.21%; P20, 26.77 ± 4.31%; P25, 68.18 ± 2.41%; P30, 69.08 ± 2.30%; P35, 62.31 ± 2.02%; P40, 71.83 ± 1.61%). In addition, no ChAT-positive neurons covered with CS-C–positive structures were observed in the L5 segment at P10. The significant differences between groups are shown in Fig. 4O.

The WFA-staining combined with double-labeling immunohistochemistry for CS-C and ChAT showed that many ChAT-positive neurons were covered with both WFA-positive surrounding structures and CS-C–positive structures in the cervical segment at P30. The percentages of ChAT-positive neurons with both WFA-positive and CS-C–positive
surrounding structures was 56.54 ± 4.46%; with only WFA-positive surrounding structures, 24.50 ± 4.51%; with only CS-C-positive surrounding structures, 10.38 ± 1.19%; and without either WFA-positive or CS-C-positive surrounding structures, 8.57 ± 2.19% (Fig. 5).

3. Discussion

The present study revealed differential expression patterns of CS-A and CS-C in the ECM structures surrounding motoneurons in the spinal cord during postnatal development in rats. CS-A is highly expressed in structures surrounding the motoneurons in all spinal segments at early stages from P5 to P15, with expression decreasing after P35. Unlike CS-A, CS-C-positive surrounding structures were rarely observed until P15, but increased thereafter. Although the expression patterns seemed similar among the 3 spinal segments, CS-C-positive surrounding structures were observed earlier in the cervical segment than in the thoracic and lumbar segments from P5 to P15.

In the present study, we used the monoclonal antibody clone 2H6 to detect CS-A. Although this antibody effectively binds CS-A (Shimazaki et al., 2005), it recognizes multiple sequences containing CS-A units in CS chains (Deepa et al., 2007). Therefore, we compared another antibody to CS-A (LY111) in some sections and observed similar expression patterns of positive structures surrounding motoneurons. We used the monoclonal antibody clone 3B3 to detect CS-C. This antibody was raised against CS-C neoepitopes on CS-GAG chains that were pre-digested with either chondroitinase ABC or chondroitinase ACII, and efficiently recognizes CS-C stubs on the CS-GAG chains (Caterson, 2012). Therefore, before immunostaining using this antibody, we pretreated the tissue with chondroitinase ABC.

WFA lectin staining is a conventional marker for PNNs, and the staining intensity corresponds to the maturation of PNNs (Härtig et al., 1992; Slaker et al., 2016). The present study revealed that WFA-positive structures surrounding motoneurons rapidly increased after P15, and were preserved after P25, suggesting that the formation of PNNs around

**Fig. 4.** (A-L) Higher magnification images of double-labeling immunohistochemistry for CS-C (green) and ChAT (red) in cervical (A-D), thoracic (E-H), and lumbar (I-L) spinal segments at P10 (A, E, and I), P20 (B, F, and J), P30 (C, G, and K), and P40 (D, H, and L). Arrowheads indicate CS-C-positive structures around ChAT-immunoreactive motoneurons. Scale bars = 20 μm. (M–O) Percentage of motoneurons surrounded by CS-C-positive structures in the cervical (M), thoracic (N), and lumbar (O) spinal cord. Blue bars indicate the percentage of motoneurons with greater than 80% CS-C-positive surrounding structures surroundings, and red bars indicate 20%-80% CS-C-positive surrounding structures. Data are expressed as the mean ± SEM (>80% and >20%). Statistical analyses were performed using one-way ANOVAs, followed by Tukey-Kramer test. Significant differences from P5, P10, P15, and P20 are expressed as ††: P < 0.01. Other significant differences are expressed as *: P < 0.05 and **: P < 0.01.
motoneurons is most active between P15 and P25. These periods occur shortly after complementation of functional connections between CST axons and spinal neurons. Thus, the timing of PNN maturation appears to coincide with changes in the motoneuron plasticity, leading to closure of the critical period of synaptic formation with CST. The present study also revealed some differences in the expression patterns among the 3 spinal segments from P5 to P15. The WFA-positive surrounding structures in the cervical and lumbar segments gradually increased, but there were no WFA-positive surrounding structures in the thoracic segment. The CS-C expression pattern appears to be identical to the WFA staining pattern from P5 to P40. In fact, approximately 70% of motoneurons with WFA-positive surrounding structures were also positive for CS-C, and approximately 85% motoneurons with CS-C-positive surrounding structures were also positive for WFA. These findings suggest that CS-C expression coincides with the maturation of PNNs surrounding motoneurons. The CS-A expression pattern, on the other hand, was consistent with the CS-56 antibody pattern in a previous report (Wang et al., 2017), showing that CSPGs are highly expressed in the spinal cord at late embryonic and neonatal stages in mice. These findings might indicate that the CS components of PNNs change drastically from CS-A dominant to CS-C dominant in the spinal cord during postnatal development.

The functional significance of CS-A and CS-C is controversial. Both positive and negative effects on axonal outgrowth are observed with CS-A and CS-C (Properzi et al., 2005; Wang et al., 2008; Butterfield et al., 2010; Lin et al., 2011; Swarup et al., 2013). A previous study in goldfish showed that the descending axons regenerate to preferentially terminate on neurons not covered with CS-C-positive PNNs after spinal cord injury, suggesting that CS-C in the PNNs inhibits new contact formation between descending axons and spinal neurons (Takeda et al., 2018). The previous study in goldfish also showed that CS-A in the ECM or PNNs does not inhibit for regenerating axons after spinal cord injury (Takeda et al., 2017, 2018). The high CS-A expression in the ECM during the first postnatal 2 weeks, therefore, might be involved in the development of motoneuron connectivity. These results appear to be consistent with the present findings that expression of CS-C, but not CS-A might be associated with the maturation of PNNs, leading to closure of the critical period of plasticity. Further experimental studies are necessary to determine whether the altered CS sulfation pattern around motoneurons in the postnatal period is associated with the maturation of PNNs, leading to changes in motoneuron plasticity, and with the development of motor function for different spinal segments.

4. Experimental procedure

4.1. Materials

Wistar rats from P5 to P40 were purchased from Japan SLC (Hamamatsu, Japan) and used in the present study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Animal Research Center of Yokohama City University.

4.2. Tissue preparation

Both male and female rats at P5 (N = 3), P10 (N = 3), P15 (N = 3), P20 (N = 4), P25 (N = 4), P30 (N = 4), P35 (N = 4), and P40 (N = 4) were deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord was dissected and postfixed with 4% paraformaldehyde (PFA) overnight at 4 °C. The tissues were then cryoprotected in 25% sucrose for 2 days, embedded in OCT compound, and stored at −80 °C until sectioning. Sections of the 7th cervical (C7), 8th thoracic (T8), and 5th lumbar (L5) spinal cord were cut at 20 μm with a cryostat (CM3050 S, Leica, Nussloch, Germany), attached to slides, and stored at −20 °C.
4.3. Immunohistochemistry

The slides were dried for 1 h and washed 3 times in phosphate-buffered saline (PBS) for 5 min each. The sections were then incubated in PBS including 0.5% Tween 20 (PBST) for 30 min. For CS-C staining, the sections were pre-incubated with chondroitinase ABC (0.2 U/mL, Millipore Sigma, St. Louis, MO, USA) in 50 mM Tris-HCl (pH 7.5) containing 50 mM acetic acid at 37 °C for 1 h. After the blocking procedure with Block Ace (5%, UK-B80, DS Pharma Biomedical Co., Ltd), the sections were incubated in a moist chamber overnight at 4 °C with primary antibodies as follows: (1) goat polyclonal antibody against ChAT (1:100, AB144P, Merck KGaA, Darmstadt, Germany); (2) a mixture of goat polyclonal antibody against ChAT (1:100), and mouse monoclonal IgM antibody against CS-A (10 μg/mL, Clone 2H6, Cosmo Bio, Tokyo, Japan); and (3) a mixture of goat polyclonal antibody against ChAT and mouse monoclonal IgM antibody against CS-C (1:50, 3B3, Cosmo Bio). Some additional cervical segment sections at P10 (N = 3), P20 (N = 4), P35 (N = 4), and P40 (N = 4) were incubated with mouse monoclonal IgM antibody against CS-A (10 μg/mL, Clone LY111, Tokyo Chemical Industry, Tokyo, Japan), instead of mouse monoclonal antibody against CS-A (Clone 2H6).

For WFA lectin staining, the sections were incubated for 2 h in Cy3-conjugated donkey anti-goat IgG, followed by incubation in fluorescein W. floribunda lectin for 2 h (diluted 1:100 in PBS, FL-1351, Vector Laboratories, Inc., Burlingame, CA, USA). For CS-A and CS-C, the sections were rinsed several times with PBST, and then incubated for 2 h at room temperature with a mixture of Alexa Fluor 488-conjugated donkey anti-mouse IgM (1:200, Jackson ImmunoResearch Laboratories, West Grove PA, USA) and Cy3-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch Laboratories) secondary antibodies. The slides were then rinsed in PBS and coverslipped with slow-fade reagent (SlowFade Gold antifade reagent, S36936, Invitrogen, Carlsbad, CA, USA).

Some cervical segment sections obtained at P30 (N = 4) were incubated overnight at 4 °C with a mixture of goat polyclonal antibody against ChAT (1:100, AB144P, Merck KGaA), and mouse monoclonal IgM antibody against CS-C (1:50, 3B3, Cosmo Bio), then incubated for 2 h at room temperature with a mixture of Cy3-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch Laboratories), Cy5-conjugated donkey anti-mouse IgM (1:200, Jackson ImmunoResearch Laboratories), and fluorescein W. floribunda lectin (diluted 1:100 in PBS, FL-1351, Vector Laboratories).

Antibody specificity was verified by incubation with 0.5% normal mouse serum (Jackson ImmunoResearch Laboratories) or 0.5% normal goat serum (Jackson ImmunoResearch Laboratories) instead of the primary antibodies.

4.4. Image acquisition and analysis

Sections including C7, T8, and L5 were digitally photographed using a Keyence BIORÉVO All-in-One Fluorescence Microscope (BZ9000, Keyence, Osaka, Japan) and transferred to Adobe Photoshop CS (Adobe, San Jose, CA, USA) to generate the figures. Contrast and brightness were adjusted with Adobe Photoshop Software. Three sections from each spinal segment were observed. ChAT-positive motoneurons in the lateral part of the ventral horn at C7 and L5, and in the ventral horn at T8 level were analyzed.

We analyzed WFA-, CS-A-, and CS-C-positive ECM structures surrounding ChAT-positive motoneurons. We identified ChAT-positive neurons for which 20% or more of the circumference was surrounded by CS-A-positive structures as positive for CS-A. We further divided ChAT-positive neurons into 3 groups: (1) those for which more than 80% of the circumference was surrounded by CS-A-positive structures, and (2) those for which 20% to 80% of the circumference was surrounded by CS-A-positive structures, and (3) those for which less than 20% of the circumference was surrounded by CS-A-positive structures. Similar criteria were applied to the CS-C-positive structures, and WFA-positive structures. We counted the number of ChAT-positive motoneurons for each group with the researchers blinded to group conditions. We then calculated the percentage of ChAT-positive neurons surrounded by WFA-positive, CS-A-positive, or CS-C-positive structures among all ChAT-positive neurons for each of the 3 groups. This process repeated in all rats. Statistical analyses were performed using SPSS. One-way ANOVAs followed by Tukey-Kramer test were used to analyze differences.

CRediT authorship contribution statement

Masahito Takiguchi: Methodology, Resources, Investigation, Validation, Formal analysis, Visualization, Writing - original draft. Sonoko Morinobu: Investigation, Formal analysis, Visualization. Kengo Funa-koshi: Conceptualization, Data curation, Writing - original draft, Funding acquisition.

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Appendix A. Supplementary data

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