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Alterations in chondroitin sulfate proteoglycan expression occur both at and far from the site of spinal contusion injury

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Abstract

Chondroitin sulfate proteoglycans (CSPGs) present an inhibitory barrier to axonal growth and plasticity after trauma to the central nervous system. These extracellular and membrane bound molecules are altered after spinal cord injuries, but the magnitude, time course, and patterns of expression following contusion injury have not been fully described. Western blots and immunohistochemistry were combined to assess the expression of four classically inhibitory CSPGs, aggrecan, neurocan, brevican and NG2, at the lesion site and in distal segments of cervical and thoracic spinal cord at 3, 7, 14 and 28 days following a severe mid-thoracic spinal contusion. Total neurocan and the full-length (250 kDa) isoform were strongly upregulated both at the lesion epicenter and in cervical and lumbar segments. In contrast, aggrecan and brevican were sharply reduced at the injury site and were unchanged in distal segments. Total NG2 protein was unchanged across the injury site, while NG2+ profiles were distributed throughout the lesion site by 14 days post-injury (dpi). Far from the lesion, NG2 expression was increased at lumbar, but not cervical spinal cord levels. To determine if the robust increase in neurocan at the distal spinal cord levels corresponded to regions of increased astrogliosis, neurocan and GFAP immunoreactivity were measured in gray and white matter regions of the spinal enlargements. GFAP antibodies revealed a transient increase in reactive astrocyte staining in cervical and lumbar cord, peaking at 14 dpi. In contrast, neurocan immunoreactivity was specifically elevated in the cervical dorsal columns and in the lumbar ventral horn and remained high through 28 dpi. The long lasting increase of neurocan in gray matter regions at distal levels of the spinal cord may contribute to the restriction of plasticity in the chronic phase after SCI. Thus, therapies targeted at altering this CSPG both at and far from the lesion site may represent a reasonable addition to combined strategies to improve recovery after SCI.

Keywords

Neurocan; Aggrecan; NG2; CSPG; Perineuronal net; Spinal cord injury; Plasticity

Introduction

Spinal cord injury (SCI) results in the loss of mobility and sensation from segments below the injury level. Damage to ascending and descending tracts and nerve roots can also lead to secondary health consequences including autonomic dysfunction, chronic pain, and complications due to loss of bone, muscle and epidermal integrity (Chiodo et al., 2007; Ho et al., 2007). To date, the only approved treatment for SCI in North America is the use of methylprednisolone as an acute neuroprotective agent (Bracken et al., 1990), but even this drug is not currently employed as a standard of care due to the uncertainties of the benefit to risk ratio (Hugenholtz, 2003). Future treatments will most likely include regenerative bridges or transplantation strategies directed at the site of injury as well as pharmacological and rehabilitation therapies to maximize neural plasticity of circuits that are spared from direct injury (rev. in Jakeman et al., 2011; Kwon et al., 2010). A number of preclinical and clinical rehabilitation therapies have shown promise in both these areas (Courtine et al., 2009; Garcia-Alias et al., 2009; Nooijen et al., 2009; Wessels et al., 2010). However, difficulties encountered in replication of modest treatment effects across animal models (*e.g.* Sharp et al., 2010) and limited translation of the most promising findings to clinical practice suggest that the injured spinal cord contains mechanisms that are refractory to both cellular and activity based repair. To date, however, the factors that prevent more robust recovery are still poorly understood.

The extracellular matrix (ECM) of the central nervous system contributes to the stabilization and regulation of synaptic activity and plasticity in development and adulthood (rev. in Dityatev et al., 2010; Kwok et al., 2008). Chondroitin sulfate proteoglycans (CSPGs) are a major group of anionic glycoproteins that form the scaffold of the neural ECM. Changes in CSPG expression and composition occur at the end of periods of developmental plasticity (Galtrey et al., 2008; Hockfield et al., 1990; Matthews et al., 2002), implicating these molecules in an essential role in regulating activity dependent changes in synaptic function. Indeed, CSPG containing networks called perineuronal nets surround highly active neurons and are associated with increased synaptic stability and reduced plasticity. CSPGs and glycosaminoglycans also inhibit axon growth *in vivo* and *in vitro* (Barritt et al., 2006; Houle et al., 2006; Snow et al., 1990; Zuo et al., 1998). One of the major groups of CSPGs which inhibit axonal extension is the hyaluronan-binding CSPGs of the lectican family, including aggrecan, neurocan and brevican (Friedlander et al., 1994; Lemons et al., 2003; Yamada et al., 1997), and the membrane-bound CSPG, NG2 (Dou and Levine, 1994). The lecticans are produced by neurons and glial cells and also contribute to formation of perineuronal nets (Galtrey et al., 2008; Matthews et al., 2002), while NG2 is found on the surface of oligodendrocyte progenitor cells and expressed by a variety of cell types following injury (Jones et al., 2002; McTigue et al., 2006).

Partial transection and knife-cut injuries damage the spinal meninges and permit invasion of peripherally derived cells into the lesion site. These injuries and cellular interactions induce a well-characterized pattern of changes in expression of CSPGs at the lesion borders. Changes include an upregulation of neurocan, brevican and NG2 (Jones et al., 2003a; Massey et al., 2008; McKeon et al., 1999; Tang et al., 2003), which contribute to the chemical barrier to axonal extension at the site of injury (Fitch and Silver, 2008; McKeon et al., 1995). In contrast, much less is known about the changes in CSPG expression following contusion injuries, which leave the spinal meninges intact, and can represent approximately

half of observed clinical neuropathology cases (Bunge et al., 1993; Norenberg et al., 2004). Prior studies have shown that both neurocan and NG2 protein levels are slightly increased (Iaci et al., 2007) in segments surrounding the lesion site in a rodent compression injury, and that CSPG glycosaminoglycan staining (Lemons et al., 1999) and NG2 immunoreactivity (McTigue et al., 2006) are increased after contusion injury. However, a full comparison of protein levels and distribution of these important CSPGs has not been established in the contusion models. Brook and his colleagues (Buss et al., 2007, 2009) have also shown altered expression of NG2 and phosphacan in post-mortem SCI specimens, indicating that these molecules may be important in clinical pathology. As mechanistic studies begin to reveal the signaling pathways evoked by CSPG activation (Coles et al., 2011; Duan and Giger, 2010; Monnier et al., 2003), it is increasingly important to expand current knowledge of the evolving composition and distribution of CSPGs at the contusion injury border. These studies are also important in order to design and interpret efforts to enhance integration of cellular transplants or bridging grafts (Fouad et al., 2005; Houle et al., 2006; Karimi-Abdolrezaee et al., 2010).

In addition to limiting axon growth and plasticity at the lesion site, there is also recent evidence that changes in expression of CSPGs at sites away from the lesion can contribute to the inhibition of collateral sprouting in denervated terminal fields. Massey et al. (2008) recently demonstrated that following a precise lesion of the low cervical dorsal columns, there were pronounced changes in CSPG expression in the denervated nucleus gracilis, which is the site of terminals of the injured ascending fibers. The alterations in CSPG expression in the target region were associated with inhibition of axon sprouting from adjacent uninjured dorsal column axons or growth of transplanted sensory neurons. Following a more severe contusion injury, Wallerian degeneration is extensive and long-lasting and leads to chronic degeneration of terminals in segments far from the lesion site.

The present study was done to characterize the time course of changes in both the expression and distribution of selected growth inhibitory CSPGs after a severe mid-thoracic contusion injury. Aggrecan, neurocan and brevican are developmentally regulated members of the lectican family and are strongly localized to perineuronal net structures in the adult CNS, which are affected by changes in neuronal activity and contribute to restricted plasticity (Carulli et al., 2010; Dityatev et al., 2007; McRae et al., 2007). In addition, NG2 is produced by and largely associated with proliferating and reactive oligodendrocyte progenitor cells, and is also expressed transiently by microglial cells in regions of neurodegeneration (Massey et al., 2008; Sandvig et al., 2004; Waselle et al., 2009). Selection of these four CSPGs provided a representative illustration of the major changes in ECM composition in both at and far from the lesion site and permitted direct comparison with the findings of Massey et al. (2008). Protein expression was measured both at the lesion site and in the distal spinal enlargements containing local circuitry for fore-limb and hindlimb function. To determine if the changes far from the injury were associated with denervation-induced astrogliosis, regional analysis of glial fibrillary acidic protein (GFAP) and neurocan immunoreactivity was performed upon tissue sections obtained from the cervical and lumbar enlargements. Combined Western blot and immunohistochemistry revealed changes in expression of the lecticans and NG2 both at and far from the site of injury. The lesion borders were characterized by a prolonged loss of aggrecan and transient downregulation of brevican, coupled with increases in expression of neurocan and changes in the distribution of NG2+ profiles. In addition to changes at the epicenter, neurocan and NG2 expression was increased far from the lesion site in lumbar and cervical spinal cord regions. The chronic changes in ECM composition both at and far from the site of a severe contusion site implicate the distal ECM as a viable target to improve the efficacy of combined therapies to restore function after SCI.

Methods

Animals and SCI

Adult female Sprague–Dawley rats (200–225 g, Harlan Laboratories) were housed in barrier cages in a temperature- and humidity-controlled room with *ad libitum* access to food and water. All experimental procedures were approved by the Ohio State University (OSU) Institutional Laboratory Animal Care and Use Committee and in accordance with the *NIH Guide to the Care and Use of Laboratory Animals*. Forty-eight rats were assigned to six groups. One group was designated for tissue harvest only (naïve; n=8). The rest were designated for laminectomy control (LAM, n=8), or survival times of 3, 7, 14, or 28 days post injury (dpi) (n=8/group). For surgery, the rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (80 mg/kg; Vedco, Inc.) and xylazine (20 mg/kg; Ben Venue Laboratories) and given prophylactic antibiotics (Gentamicin, 1 mg/kg, Vedco, Inc.). A dorsal laminectomy was performed at the T8 vertebral level (Ankeny et al., 2004; Jakeman et al., 1998). For injuries, the lateral processes of the T7–T9 vertebrae were secured using forceps clamps, and the Infinite Horizon (IH) Device was used to administer a severe (250 kdyn) contusion injury (Scheff et al., 2003). After impact, the overlying muscles were sutured and skin openings were closed. Sterile saline was administered (5 cm³, s.c.) and the rats were allowed to recover in warmed cages overnight. All rats were inspected daily. All injured rats received antibiotic (Gentamicin, 1 mg/kg, s.c.) and saline (2–5 cm³) for the first 5 days post injury (dpi). Bladders were manually expressed twice per day until a reflex bladder was restored, and one rodent Fruit Crunchies™ pellet (Bio-Serv) per rat was provided daily to maintain urine acidity. At the designated dpi, the rats were re-anesthetized with ketamine (120 mg/kg) and xylazine (15 mg/kg) and spinal cord tissues obtained for protein analysis (n=4/group) or immunohistochemistry (n=4/group) as described below. Tissues from naïve animals (n=4) and laminectomy (sham) animals (n=4) were collected and prepared in parallel with injured tissues. One laminectomy specimen was collected for each group at 3, 7, 14 and 28 days post-surgery.

Western blots

Rats were anesthetized, subjected to pneumothorax, and the spinal cords were rapidly dissected from the vertebral column. A 10 mm segment of the cervical enlargement, 6 mm spanning the SCI site and 10 mm of the lumbar enlargement were isolated as shown in Fig. 1A. The tissue blocks were weighed and stored at –70 °C. For Western blotting, the frozen tissue samples were thawed and homogenized in 40 mM Tris–HCl, pH 7.6, containing 40 mM sodium acetate and protease inhibitors (Complete, Roche Applied Science) (Massey et al., 2008). Aliquots of the homogenates at a final protein concentration of 2–3 mg/ml were treated with 0.3 U/ml chondroitinase ABC (chABC, Sigma-Aldrich) for 8 h at 37 °C. Samples were denatured and 10–15 µg total protein was electrophoresed on reducing 5–10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose (Bio-Rad) and transfer was confirmed using Amido Black. The membranes were incubated in primary antibodies overnight as follows: mouse monoclonal anti-aggrecan (Cat 301 raised against cat cortex CSPGs, MAB5284 Millipore; 1:400), mouse monoclonal anti-neurocan (clone 650.24 raised against embryonic rat brain proteoglycans, MAB5212 Millipore; 1:1500), rabbit anti-brevican (B61, raised against recombinant brevican amino acids 506–527; Viapiano et al., 2003; 1:10,000) and rabbit polyclonal-anti NG2 (AB5320 raised against purified rat NG2, Millipore; 1:2000). After washing and incubation for 2 h in the appropriate HRP conjugated secondary antibodies (Jackson ImmunoResearch; 1:200), the immunoblots were developed by chemiluminescence (Pierce ECL) using BX Film (Midwest Scientific). The films were scanned and the integrated optical density (O.D.) of each target protein was quantified using Gel-Pro Analyzer software (v3.1, Media Cybernetics). Blots were stripped and reprobed with antibodies against α -tubulin as a loading control (mouse anti β -tubulin; Abcam

1:2500). O.D. ratios for each protein and tissue region (O.D. target protein/O.D. β -tubulin) were determined and the value was normalized to the naïve or laminectomy control bands on the same gel to obtain a fold change for each specimen. In preliminary experiments, no differences in O.D. ratios were found between naïve and laminectomy samples from any time point, so laminectomy samples were used as controls on subsequent blots. Ratios for each band were expressed as mean $\pm$ SEM of 3–4 independent samples per group. The results were compared by one-way ANOVA followed by Dunnett's multiple comparison test to control using GraphPad Prism 5.0 (GraphPad Software, Inc.). All samples were measured in at least two separate loading experiments to ensure reproducibility.

Tissue preparation for histology and immunohistochemistry (IHC)

Subjects designated for IHC were perfused with 0.1 M phosphate buffered saline (PBS) followed by 300 ml 4.0% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The spinal cords were exposed and spinal levels marked, and the entire cord was dissected from the vertebral column. Tissues were postfixed 2 h in 4% PFA, rinsed overnight in 0.2 M PB, and cryoprotected 2–3 days in 30% sucrose. After equilibration, the spinal cord tissues were cut as illustrated in Fig. 1A. In order to ensure inclusion of the entire lesion and perilesional areas in the resulting slide-mounted tissue sections, and to allow for illustration of the distribution of immunostaining in both transverse and longitudinal orientations, a 20 mm length of epicenter tissue was transected at the lesion epicenter creating two blocks of 10 mm epicenter tissue from each spinal cord. Blocks of 10 mm in length from cervical, epicenter, or lumbar spinal cord were embedded in Tissue TekTM (OCT) compound and rapidly frozen. Each block contained 1–2 injured and one laminectomy or one naïve specimen. Serial transverse sections of 20 μ m thickness were cut with a cryostat through the cervical or lumbar enlargement. At the lesion site, the 20 mm block was cut at the visual epicenter of the lesion, and transverse (n=3–6/group) or longitudinal sagittal sections (n=2–3/group) were prepared through the rostral or caudal epicenter blocks to illustrate CSPG distribution along different planes. All sections were thaw mounted onto Superfrost+ glass slides (Fisher Scientific), with equally spaced sections stored on 10 adjacent slide sets for selected staining. One set of sections from each block was stained with eriochrome cyanine (EC, Sigma-Aldrich) and counterstained with 0.1% cresyl violet to determine the distribution of neurons and myelin. The remaining sections were stored at –20 °C and used for subsequent IHC.

IHC

Adjacent series of transverse or longitudinal sections from blocks spanning the lesion epicenter regions were stained with antibodies as follows: mouse monoclonal anti-aggrecan (Cat 301, MAB5284 Millipore; 1:500), mouse monoclonal anti-neurocan (clone 650.24, MAB5212 Millipore; 1:300), rabbit anti-brevican (B61, Viapiano et al., 2003; 1:300) and rabbit polyclonal anti NG2 (C5067-71 raised against recombinant NG2 amino acids 1592–2222, US Biologicals; 1:100), rabbit anti-bovine GFAP (Z0334, Dako; 1:1000), and goat anti-ChAT (AB144P raised against human placental antigen, Millipore; 1:50). To illustrate the extent of the glial scar, one set of sections was stained with rabbit anti-glial fibrillary acidic protein (GFAP; Dako, 1:1000) and visualized in brightfield after indirect enzyme amplification. After blocking with 5% normal goat serum (NGS) and 3% BSA in 0.1% triton-x-100 PBS, sections were incubated in anti-GFAP overnight at 4 °C. The sections were blocked and incubated in biotinylated goat-anti-rabbit IgG (1:200), washed, and visualized using avidin biotin-HRP (ABC Elite, Vector Labs) developed in the presence of H₂O₂ using Vector SG as a chromagen, which yields a dark gray reaction product (White et al., 2010). Additional sets of equally spaced sections from the epicenter, cervical and lumbar enlargement blocks were incubated with primary antibodies raised against CSPG core proteins and selected counterstain markers and visualized by immunofluorescence using

Alexafluor-tagged secondary antibodies (Invitrogen). Staining markers included anti-neurocan and anti-GFAP for astrocytes, anti-neurocan and anti-NG2, anti-brevican counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label cell nuclei, and anti-aggrecan with anti-choline acetyltransferase (ChAT) to label cholinergic neurons. Sections stained with aggrecan antibodies were pretreated with 0.2 U/ml ChABC at 37 °C for 40 min prior to primary antibody incubation. All immunostaining was performed by staining all slides spanning the full time course of the study at the same time, with the same solutions, to ensure consistency. Controls for antibody specificity were included by substitution of normal serum in place of each of the primary or secondary antibodies. Selected regions for brightfield or widefield fluorescence illustrations were photographed using a Zeiss Axioscop with Sony 970 analog camera and appropriate filters and captured using the MCID Elite image analysis system (Imaging Research). For dual fluorescence, sections were viewed using Olympus Fluoview 1000 laser scanning confocal microscopes at the OSU Campus Microscopy and Imaging Facility. Selected images and calibrations were exported in .tif format and labeled plates were prepared using Photoshop CS. Brightness and contrast were optimized for illustrative plates only.

IHC analysis at cervical and lumbar levels

To identify the distribution of spared white matter and neuronal cell bodies, one set of sections was stained with eriochrome cyanine (EC) and cresyl violet (CV) to detect white matter and gray matter regions, respectively. Low power images of (EC/CV) stained sections were obtained using a 2.5× objective and used to prepare a map of the full length of each of the cervical and lumbar tissue blocks. From these maps, the range of sections spanning 2.5 mm at the C5 and L5 spinal levels for each specimen was determined based on gray and white matter distribution. A total of 4 sections spaced approximately 800 μm apart were identified for analysis of GFAP and neurocan staining in adjacent slides (n=4 per group). For each stain, the sections were viewed using a 20× objective and the camera gain and offset optimized to maximize the full range of contrast using a test section from a representative specimen. A full set of images was then obtained from 8 regions of each section. The regions of interest (dorsal column, DC; dorsal corticospinal tract, DCST; dorsal horn, DH; intermediate gray, IG; lateral white matter, LWM; medial dorsal columns, MDC; ventral horn, VH; and ventral white matter, VWM) were defined in advance and anatomical landmarks including the ventral sulcus, central canal, and dorsal fissure were used to align each image in the same manner. All images were collected under identical lighting conditions in the same viewing session by an investigator with no knowledge of the treatment code. For analysis of GFAP immunoreactivity, a threshold intensity setting that consistently distinguished astrocyte profiles from background was determined in uninjured regions. The area of positive stained processes (target area) was measured and expressed as a proportion of the area of the region of interest (scan area). The resulting proportional area (PA) across the full spinal level represents a volumetric proportional estimate of positive staining. For analysis of neurocan immunoreactivity, the diffuse extracellular staining pattern precluded identification of specific profiles or establishment of an intensity threshold. The same regions of interest were defined anatomically and the average fluorescence intensity was measured for each region using the MCID software. GFAP proportional area and neurocan intensity values were averaged across sections and compared using two-way ANOVA with repeated measures for region and time post-injury as the dependent variables. P values < 0.05 for main effects were considered significant and followed by Dunnett's multiple comparison test to compare each dpi with control values.

Results

Progression of severe contusion injury pathology over time

The 250 kdyn spinal cord impact produced a severe contusion injury at the mid-thoracic spinal cord level as described previously (Rabchevsky et al., 2001; Scheff et al., 2003). White and gray matter areas were disrupted by a central region of necrosis and tissue damage as revealed with EC/CV staining (Figs. 1B–D). Transverse sections of the lesion epicenter illustrate the well-documented pattern of cellular infiltration and tissue loss over time. At 3 dpi, the lesion epicenter was enlarged and was occupied by cellular debris and infiltrating cells. Rostral and caudal extensions of the lesion were evident near the base of the dorsal columns. By 28 dpi, the irregularly shaped epicenter region was constricted. GFAP-positive astrocytes vacated the lesion center and established a distinct border at the lesion edge. Note that tissue blocks obtained for Western blots (6 mm) encompassed only a portion of the rostral and caudal borders. Some left-right asymmetry and rostrocaudal asymmetry of the lesion and spared tissue regions are typical of contusion injuries, so all regional analyses were obtained across the full width and length of the tissue blocks. This injury largely mimics a moderate range of closed contusion injuries in human clients, in terms of variation and secondary consequences (Metz et al., 2000).

CSPG expression at the lesion site

Previous studies have documented increased expression of chondroitin sulfate glycosaminoglycans (CSPGs) (Fitch and Silver, 1997; Lemons et al., 1999) and changes in expression of selected CSPG core proteins at the lesion site following knife-cut lesions (Jones et al., 2002; Massey et al., 2008) or contusion injuries (Iaci et al., 2007; Lemons et al., 1999). However, prior studies have not documented the corresponding distribution of these major inhibitory CSPGs after contusion injury. Aggrecan expression was evaluated using a monoclonal antibody that recognizes the Cat 301 antigen originally described on cortical neurons (Mize and Hockfield, 1989; Matthews et al., 2002). In intact spinal cord extracts, multiple bands were identified with sizes ranging predominantly from ~200 to 500 kDa, with smaller bands evident at ~140 and 75 kDa, reflecting a range of glycosylation and cleavage products (Lemons et al., 2001; Matthews et al., 2002). At 3 dpi, aggrecan was markedly reduced at the lesion epicenter and did not recover by 28 dpi (Figs. 2A,B). IHC revealed the prominent distribution of aggrecan in perineuronal net structures throughout normal gray matter (Fig. 3A). After injury, total aggrecan protein and specific labeling were eliminated both at the epicenter core and extending into the gray matter as far as 3–4 mm beyond the rostral and caudal borders (Figs. 2B, 3A,E).

In Western blots, the neurocan 5212 antibody recognized full length (~245–250 kDa) neurocan and a 150 kDa C-terminal proteolytic cleavage product (Asher et al., 2000). In the normal control thoracic cord, neurocan-150 was the predominant form, with very low levels of expression of the full length protein (Fig. 2A). Following contusion, there was a 4-fold increase in total amount of neurocan by 7 dpi, driven mostly by the pronounced elevation (40–60 fold) of the full-length form (Fig. 2B). The 150 kDa isoform was also increased by 2–3 fold and remained elevated through 28 dpi. In addition, an unidentified variant of ~180 kDa that was not present in intact spinal cord was identified in the epicenter tissues at all times post injury (Asher et al., 2000; Tang et al., 2003). In tissue sections from uninjured or laminectomy animals, the neurocan antibody stained neurons and the extracellular neuropil, including perineuronal net structures throughout gray matter, and also lightly stained glial cells in surrounding white matter (Fig. 3B). After injury, extracellular neurocan-like immunoreactivity was markedly increased, especially throughout spared white matter and in association with astrocytes at the glial border. In contrast to the loss of aggrecan immunoreactivity in adjacent spared tissues, the neurocan staining extended to the very edge

of the GFAP+ lesion borders. No neurocan immunoreactivity was found within the center of the lesion (Fig. 3E).

The polyclonal brevican antibody (B6) recognized the full length isoform (150 kDa) and C-terminal cleavage product (~100 kDa) in Western blots (Fig. 2A). Both isoforms were depleted from the lesion epicenter by 3 dpi, but partially recovered by 28 dpi. Immunostaining for brevican was distributed throughout both gray and white matter in control spinal cords. After injury, there was a distinct loss of brevican staining in the lesion, but brevican remained right up to the lesion border in both gray and white matter surrounding the lesion site. Expression in the spared white matter and the proximity of brevican to the lesion edge differed from that of aggrecan, which likely explains the recovery of protein expression in Western blots.

NG2 was detected as a single band of ~275 kDa in Western blots from control and injured spinal cord (Fig. 2A). A slight decrease in NG2 was detected at 3 dpi (Fig. 2B), but differences in levels of NG2 expression across time were not different by one way ANOVA. In control spinal cord, NG2 was associated with small cells, presumed to be oligodendrocyte progenitors, throughout the gray and white matter and also in some pericytes and meninges (Fig. 3D). After injury, there was an increase in the intensity of immunoreactivity in spared white matter and along the lesion border and a loss of NG2 within the lesion center. By 14 dpi, however, NG2 immunoreactivity extended into the lesion past the neurocan-positive borders, and at 28 dpi, NG2-positive profiles extended deep within the lesion site in association with elongated cells and fibrous processes (Figs. 3D,E) (McTigue et al., 2006).

Changes in CSPG expression in cervical and lumbar enlargements

After severe mid-thoracic contusion injury, there were chronic changes in expression of some of the CSPG core proteins in distal spinal cord segments (Fig. 4). Aggrecan was expressed in the enlargements at all time points, and the levels detected by Western blots were not significantly changed in either the cervical (Fig. 4A) or lumbar (Fig. 4B) segments, although there was a trend toward a decrease of this CSPG at the latest time point. Immunocytochemistry showed no change in the distribution of aggrecan in these segments. In contrast, both the 250 kDa and 150 kDa isoforms of neurocan were significantly increased far from the lesion site, resulting in a total increase in neurocan protein expression at both sites. In the cervical spinal cord, neurocan-250 was elevated by 15 fold from 3 to 7 dpi and the 150 kDa isoform was also increased at 7 dpi. Effects of injury on expression of neurocan in the lumbar spinal cord were even more striking; significant elevations in total neurocan and the uncleaved 250 kDa isoform were maintained throughout 28 dpi.

Brevican showed little modulation in the distal spinal cord segments, while NG2 levels were altered modestly. In cervical spinal cord there was only a transient trend toward increased NG2 that did not reach significance, while in lumbar spinal cord samples, NG2 expression was increased compared with controls, at 3 dpi and again at 28 dpi.

Regional changes in GFAP and neurocan expression in distal segments

IHC analysis was used to examine the regional distribution of reactive astrocytes and increased expression of neurocan in the distal spinal cord. GFAP staining was measured by determining the proportional area (PA) of segmented images from selected regions of gray and white matter that exhibited clear and specific immunostaining above the background tissue level (Fig. 5A). In naïve specimens, the PA of GFAP staining was slightly higher in gray matter regions than in white matter regions, with the exception of the dorsal corticospinal tract (DSCT) located in the base of the dorsal columns. Values from cervical spinal cord were slightly higher than those in lumbar spinal cord. After injury, a significant

increase in GFAP reactivity was clearly evident in the medial dorsal columns of the cervical cord, corresponding to the location of the ascending dorsal column fibers undergoing Wallerian degeneration after mid-thoracic injury. In the lumbar white matter, there was a trend for a transient increase in GFAP staining, especially in the DSCT, but this did not reach significance by two-way ANOVA. Notably, GFAP immunostaining also revealed a robust, but transient increase in glial activation in the lumbar gray matter, peaking at 14 dpi where it reached significance in the intermediate gray, and returning to control levels by 28 dpi.

We hypothesized that neurocan expression would parallel the regional changes in GFAP immunoreactivity. Neurocan staining intensity was measured from selected regions that exhibited strong trends or significant changes in GFAP, including the medial dorsal columns (mdc), intermediate gray (ig) and ventral gray matter (vgm) in cervical spinal cord and the DCST (dcst), ig, and vgm in lumbar spinal cord (Fig. 5B). At cervical levels, neurocan immunostaining was unchanged in gray matter, but significantly increased in white matter by 14 dpi and maintained at 28 dpi. In the lumbar enlargement, there was a significant effect of time post-injury by two way ANOVA, with trends toward increased neurocan expression in all three regions. Neurocan expression was robustly increased in the vgm, which represents a primary target of the degenerating descending fiber tract terminals damaged by the injury. Notably, these changes in neurocan were greatest at the most chronic time point, which occurred well after the transient increase in GFAP immunoreactivity and corresponded to the changes seen in Western blot analyses (Fig. 4B). To illustrate the enhanced neurocan staining and transient GFAP increase, sections obtained from representative specimens closest to the mean for each group were stained with GFAP and Neurocan antibodies and photographed under identical laser intensity and collection conditions using the confocal microscope (Fig. 6). In cervical spinal cord, the greatest increase in both GFAP and neurocan immunoreactivity was evident in the medial dorsal columns (Fig. 6A). In lumbar spinal cord, neurocan immunoreactivity increased substantially with time and was found throughout the neuropil by 28 dpi (Fig. 6B), while changes in GFAP expression were more subtle, and appeared to peak at 14 dpi.

Discussion

A principal cause of clinical SCI is closed contusion due to fracture or rapid compression/distension of the surrounding bony vertebrae or vertebral discs. Cell rupture and hemorrhage and the immediate cascade of biochemical reactions lead to a central necrotic lesion within the parenchyma of the cord, while the meninges and a peripheral rim of tissue often remain intact (Bunge et al., 1993; Kakulas, 1999). A similar pathology is produced by contusion injuries or clip compression models in rodents, where inflammation and secondary injury events typically result in formation of large cystic cavities lined by a dynamic border of reactive glial cells (Bresnahan et al., 1991; Fleming et al., 2006; Metz et al., 2000; Noble and Wrathall, 1985; Norenberg et al., 2004; Poon et al., 2007).

Based on a large body of *in vitro* and *in vivo* studies, it is well recognized that the activated glial cells at the edges of a lesion in the brain or spinal cord contribute to inhibition by increasing expression of growth-inhibitory molecules, including CSPGs (Asher et al., 2000; McKeon et al., 1991; Properzi et al., 2005; Tang et al., 2003). Growing axons and migrating cells are stalled in the presence of CSPGs and their sulfated glycosaminoglycan side chains (Davies et al., 1999; Kim et al., 2006).

Surprisingly, however, much of the evidence of the expression and inhibitory role of CSPGs in animal models of SCI has been obtained from *in vitro* assays and studies of discrete knife-cut, aspiration, or hemisection lesions, where the lesion edge is clearly defined (Barritt et al.,

2006; Bradbury et al., 2002; Houle et al., 2006). In these injuries, the lesion edge is composed of a cellular interface between spinal cord neurons and the host glia and either grafted cells or peripherally derived cells that migrate into the lesion site in association with the cut meninges (Reier et al., 1989). In contrast, the patterns of expression of CSPGs after closed contusion injury are not well described.

The present study reveals important changes in CSPG expression at the injury site. Most striking was the dramatic and prolonged decrease in expression of aggrecan at the lesion site after contusion injury. Aggrecan is a CSPG predominantly found in the adult normal brain and spinal cord gray matter, in coincidence with synaptic maturation and the closing of the regional periods of synaptic plasticity (Matthews et al., 2002). In contrast with other CSPGs that have been described as having dual roles, promoting or limiting neural plasticity (rev. in Rauch, 2004), aggrecan is the prototypical inhibitory CSPG, with robust ability to inhibit axon growth (Chan et al., 2008; Lemons et al., 2003; Snow et al., 2001) and Schwann cell motility (Afshari et al., 2010). Aggrecan is expressed predominantly by neurons (Matthews et al., 2002) and is a major component of perineuronal nets (PNNs), which are associated with limiting synaptic plasticity (Carulli et al., 2010; Dityatev and Schachner, 2003; Giamanco et al., 2010; Hockfield et al., 1990). Furthermore, most *in vitro* models of CSPG mediated growth inhibition incorporate aggrecan because it is easily obtained from peripheral cartilage and is highly glycosylated and thus sensitive to chondroitinase treatment (Snow and Letourneau, 1992; Tom et al., 2004; Vahidi et al., 2008).

Despite the inhibitory nature of aggrecan in these models, the loss of aggrecan staining at the lesion border and the lack of recovery by 4 weeks after injury indicate that aggrecan is not a major contributor to inhibition of axon growth or migration of grafted cells at the lesion edge. These findings extend the initial findings of Howland's group (Lemons et al., 2001), who showed decreased aggrecan expression in the spinal cord after a hemisection aspiration injury, and also confirm the time course of loss of aggrecan mRNA and protein from the spinal cord after a cervical dorsal column lesion (Massey et al., 2008). A similar loss of aggrecan and PNNs has been reported after cortical contusion injury (Harris et al., 2009), suggesting that focal sprouting may be enabled at the edge of the cortical lesion by the loss of aggrecan. However, recent studies with aggrecan knockout mice have shown that PNN structures still form in the absence of aggrecan, although the function of these PNNs has not been examined (Giamanco et al., 2010). Furthermore, the unchanged expression of this CSPG in cervical and lumbar spinal cord was initially surprising in light of prior findings following dorsal column lesions, where aggrecan expression was decreased at 3–7 days after denervation, corresponding to a transient loss of perineuronal nets around neurons in the target region (Massey et al., 2008). To date, however, there is no evidence of reorganization of PNNs in the distal regions of the spinal cord after contusion injury. These results suggest that a severe contusion injury induces only modest alteration in synaptic organization of neurons far from the lesion. Indeed, continued expression of basal aggrecan levels may contribute to resistance of the denervated lumbar and cervical spinal cord segments to reorganization by locomotor rehabilitation strategies alone (rev. in Jakeman et al., 2011).

Neurocan underwent the most striking change in processing after contusion. Neurocan is expressed early in brain development as an ~250-kDa polypeptide that can be cleaved by a yet-undetermined metalloprotease into 150- and 130-kDa products (Milev et al., 1998; Rauch et al., 2001). During postnatal development, the full-length form is reduced, and most neurocan is only detected as the cleavage products. This has raised speculation that the 250-kDa neurocan might contribute to early plasticity while the cleavage products are associated with consolidation of the inhibitory matrix. We observed both a significant increase in 150-kDa neurocan and a pronounced re-expression of the 250-kDa form at the lesion site. The

250-kDa form declined after 14 dpi, while the 150-kDa form remained elevated through 28 dpi. This time course of expression is similar to that described following knife-cut injuries by Massey et al. (2008) and by Tang et al. using a different antibody (1 F6) that recognizes a site on the 130-kDa product (Tang et al., 2003). In addition, an undefined product of 180-kDa was found in tissues from the lesion site at 3–28 dpi. A product of this size has been revealed in previous studies using the 5212 (Asher et al., 2000) and 1 F6 antibody (Tang et al., 2003) and may represent a poorly understood, but possible alternative processing event associated with injury. These complex, but reproducible, changes in neurocan expression suggest that the balance of proteases and inhibitors is an important component of the reorganization of the extracellular matrix after injury. Neurocan immunoreactivity was closely associated with astrocytes at the lesion border, consistent with evidence that astrocytes and O-2A glial progenitor cells produce neurocan in response to cytokine and growth factor activation (Asher et al., 2000; Smith and Strunz, 2005). Importantly, purified neurocan is inhibitory to axon growth *in vitro* (Asher et al., 2000). Thus, although developmental expression of the 250-kDa neurocan correlates with early axon growth, both the distribution and cellular localization of neurocan are after injury consistent with neurocan playing a major role in the inhibitory environment at the edge of the lesion.

In addition to effects around the lesion site, neurocan expression was also markedly increased far from the injury site in the cervical and lumbar enlargements. The full-length form of neurocan was upregulated within 3 dpi in both regions, while the 150-kDa form was increased by 7 dpi in cervical and 14 dpi in lumbar spinal cord. These results extend initial findings of Massey et al. (2008) in the cuneate nucleus following a dorsal column lesion. The analysis of regional intensity of neurocan staining demonstrated that neurocan is clearly upregulated by white matter astrocytes in spinal tracts undergoing prolonged Wallerian degeneration (Buss et al., 2004; Wang et al., 2009). However, not all of the increased neurocan expression was localized to these fiber tracts. Indeed, the gray matter of the lumbar and cervical spinal cord showed measurable increases in the intensity of neurocan immunoreactivity. Given the inhibitory effects of neurocan on axon growth *in vitro*, this chronic increase in neurocan in the distal spinal cord segments may contribute significantly to the limited plasticity and recovery of hindlimb function following contusion injury. Many years ago, Haas et al. (1999) showed a pronounced upregulation of neurocan mRNA and full length neurocan at the borders of the denervated molecular layer of the dentate gyrus after entorhinal cortex lesion. This was consistent with a guidance role of this CSPG to restrict axonal sprouting to appropriate layers of the dentate gyrus. Further studies will determine if extended expression of the developmentally expressed 250-kDa can extend plasticity in these regions, or whether alteration of the 150-kDa form would enhance sprouting.

Brevican and NG2 showed modest changes in expression after injury, but both of these CSPGs underwent changes in distribution around the injury epicenter that may reveal a bit about their possible contributions to the characteristics of the lesion borders. Brevican is a ubiquitous CSPG restricted to the CNS and expressed predominantly by astroglial cells but also by neurons and oligodendrocytes (Milev et al., 1998; Thon et al., 2000). This CSPG has been shown to inhibit axon growth (Yamada et al., 1997) and reduce axonal extension and cell motility (Snow et al., 2001) and it is another major component of PNNs. In the white matter, brevican is associated with nodes of Ranvier, where they may play an important role in assembly of the extracellular matrix and neuron-glial communication (Bekku et al., 2009). In contrast to the growth inhibitory nature of the full length protein, however, the cleavage products of brevican, which are generated by ADAMTS metalloproteases, can stimulate cell motility and explain in part the invasive ability of brevican-overexpressing glioma cells in the CNS (Hu et al., 2008; Viapiano et al., 2005). After contusion injury, brevican expression was decreased dramatically at the lesion epicenter, but unlike aggrecan, the distribution of staining loss was restricted to regions of neuronal death and cavitation, while staining in

both gray and white matter at the lesion edge was enhanced, and some recovery was seen by 28 dpi. Thus, the inhibitory characteristics of brevican at the lesion edge may contribute to inhibition of axon growth, while high expression in the peripheral white matter may contribute to changes in axonal conduction in spared fibers.

NG2 was the only CSPG examined that did not exhibit a significant change in total protein expression at the site of the contusion injury. In the intact central nervous system, NG2 is primarily expressed by oligodendrocyte progenitor cells scattered throughout the gray and white matter, with some low expression in pericytes (Nishiyama et al., 1996; Stallcup and Beasley, 1987). After injury, many of these cells are lost at the lesion center, but this occurs at the same time as increased proliferation and hypertrophy of NG2 cells around the injury site (McTigue et al., 2006). In addition to expression on progenitor cells, NG2 is also transiently upregulated in macrophages after SCI and is found in association with Schwann cells within the lesion (Jones et al., 2003b; McTigue et al., 2006). Although NG2 inhibits axon growth in *in vitro* assays, the close association of axons with NG2+ profiles after injury has favored the interpretation that a balance of inhibitory and permissive ECM molecules that are produced by cells within the contusion injury may facilitate axon growth. Indeed, mice lacking NG2 show no increase in capacity for regeneration after spinal cord injury (Hossain-Ibrahim et al., 2007). The present results from the lesion site conflict with the results of Iaci et al. (2007), who found increased NG2 expression in segments surrounding the site of a crush injury. The differences may be attributed to the more limited rostro-caudal extent of the crush injury and resultant cell death, so that increases in NG2 at the lesion border account for more of the total protein. Finally, the current study revealed a chronic increase in total NG2 expression in the lumbar enlargement after thoracic contusion injury. This response may reflect the reactivity of NG2 cells or microglia in response to chronic denervation or inflammation. However, immunocytochemistry demonstrated that NG2 was restricted to cells with morphology of oligodendrocyte progenitors (not shown). Furthermore, histological measurements of regional NG2 immunoreactivity from lumbar spinal cord segments did not reveal a significant evidence of increased numbers or size of NG2+ cells or changes in the distribution of staining compared with laminectomy controls. In contrast, Massey et al. observed a transient increase in NG2 immunoreactivity in the gracilis nucleus after denervation and this was localized to a short term upregulation of NG2 on microglial cells, but was resolved by 3 weeks post-injury. The differences between these findings are interesting as they may reflect effects of the lesion size and type or the distance from the lesion to the denervated neuropil. In the Massey study, the lesion was restricted to the dorsal columns at the C3 spinal level, and tissues were examined in the denervated nucleus gracilis. This is located approximately 9 mm rostral to the lesion and represents a compact terminal field for the vast majority of the cut axons. In contrast, the contusion injury is much larger, involving the full cross-sectional area at the lesion epicenter and extending as far as 10–12 mm in length. In turn, the spinal enlargements contain a widely distributed and extensively denervated target field of the injured axons and are located as far as 25–35 mm from the rostral and caudal ends of the lesion. Thus, the chronic (28 dpi) increase in expression of NG2 may reflect a long term widely distributed effect of injury on the reactivity of NG2 cells, whose functions as oligodendrocyte progenitors and potential synaptic interactions with neurons are a topic of intense and ongoing studies and controversy (Mangin and Gallo, 2011; Sakry et al., 2011).

This study provides important evidence of changes in CSPG expression both at and far from the site of a contusion lesion and indicates that strategies to enhance recovery of function need not be limited to application at the lesion site and should also be directed at distal segments. However, further work is essential to understand the extent to which these changes in core protein expression contribute specifically to inhibition or even to facilitation of axonal plasticity and resulting recovery of function. For example, the core proteins may

contribute directly to the inhibitory microenvironment. Indeed, NG2 has been shown to inhibit axon growth in the absence of glycosaminoglycan (GAG) side chains (Dou and Levine, 1994) and inhibition of NG2 expression can enhance outgrowth of neurons on isolated oligodendrocyte progenitor cells (Chen et al., 2002). Likewise, the aggrecan core is inhibitory to axon growth *in vivo* when stripped of its side chains (Lemons et al., 2003), and the neurocan core protein interacts directly with adhesion molecules in modulating axonal guidance, so it may also have some intrinsic growth inhibitory capacity (Retzler et al., 1996). However, it is now well understood that most of the inhibitory capacity of the accumulation of CSPGs after SCI can be attributed to the extensive increase in expression of the glycosylated side chains.

Treatment of the injured spinal cord with the enzyme chondroitinase ABC cleaves CSPG GAG side chains and increases sprouting near the lesion site (Bradbury et al., 2002). While this study clearly shows changes in neurocan processing in cervical and lumbar spinal cord, it is not yet known if GAG expression is altered in these distal spinal cord segments or if chondroitinase has effects on plasticity far from the site of injury. The mechanisms of chondroitinase function are multifaceted. When this enzyme cleaves GAG chains *in vivo*, it also degrades the hyaluronic acid backbone and disrupts the entire extra-cellular matrix, breaking perineuronal nets and releasing bound growth factors. Nevertheless, the direct role of the GAGs in inhibiting growth and plasticity have been demonstrated *in vitro* and *in vivo* by improved axon growth following administration of inhibitors of CSPG glycosylation, including knockdown of xylosyltransferase (Grimpe and Silver, 2004; Hurtado et al., 2008) and inhibition of GAG chain poly-merization (Laabs et al., 2007). In addition, the patterns of sulfation of CSPGs are also altered after SCI and may dictate the effects of these species on axonal extension. For example, a dramatic upregulation of chondroitin 6-sulfate and chondroitin 4,6 sulfate contributes to the growth inhibitory characteristics of CSPGs around the site of injury (Gilbert et al., 2005; Properzi et al., 2005). The enhanced sulfation of the CS-4,6 species is comparable to the inhibitory properties of the heavily glycosylated aggrecan (Gilbert et al., 2005), but recent work with sulfotransferase knockout mice suggests that increased CS-6 species may actually contribute to facilitating axon growth after injury (Lin et al., 2011). Thus, the present study provides a first step of many in understanding the role of chronic changes in the extracellular matrix surrounding the contusion injury and far from the site of injury. Successful therapeutic and rehabilitation strategies for treatment and care of long term SCI will have to incorporate knowledge of these changes in extracellular interactions in order to enhance plasticity and ultimately improve recovery of function.

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Fig. 1.

Experimental design and lesion extent. A. For Western blots, tissue blocks included 10 mm centered on the cervical or lumbar enlargement and 6 mm centered on the lesion epicenter (above diagram). For immunohistochemistry (IHC), spinal cords from PFA perfused rats were blocked with 10 mm centered on the cervical or lumbar enlargements or 20 mm centered on the epicenter. The epicenter IHC block was then severed at the lesion center and longitudinal or transverse cryostat sections were taken from the rostral and caudal ends. B. Eriochrome cyanine/cresyl violet (EC/CV) stained transverse sections from naïve thoracic spinal cord and the lesion epicenter of specimens obtained at 3, 7, 14, and 28 dpi, respectively. C, D. Midsagittal sections illustrating the extent of the spinal contusion site at 3 (C) and 28 dpi (D). In each pair, the top section was stained with EC/CV, and the bottom section with anti-GFAP. Arrow doublets (C) point to rostral and caudal lesion expansion in the dorsal column region. *(D) in GFAP-free lesion core; black arrows at GFAP+lesion border. Scale B=200 μ m; C,D=1.0 mm.

Fig. 2.

Changes in CSPG core protein expression at the site of a severe contusion injury. A. Western blots of aggrecan, neurocan, brevican and NG2 in normal and injured thoracic cord. B. Analysis of identified bands of aggrecan, neurocan, brevican and NG2 expression in control (CTRL) and injured rats (designated dpi or DPI). Aggrecan expression changed with time (one way ANOVA; $p < 0.001$) and decreased by 3 dpi, and remained absent through the remainder of the study. Total neurocan expression levels were increased 4 fold at 7 dpi and remain 3–4 fold elevated throughout 28 dpi. NG2 expression was not changed in the epicenter block. *Post-hoc* Dunnett's * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with control.

Fig. 3. IHC images of CSPG core proteins around the lesion epicenter after contusion injury. A. Aggrecan is expressed throughout the gray matter (dorsal horn, dh) and ventral horn (vh) in naïve spinal cord (a). The boxed area in (a) is enlarged in (c). Aggrecan is depleted throughout the epicenter (*) at 14 days after contusion injury (b). The boxed area in (b) is enlarged in (d) (ventrolateral white matter, vlwm). Scale a,b=200 μ m; c,d=50 μ m. B. (a) Neurocan (red) is expressed more highly in gray matter (dh, vh) than in white matter in naïve spinal cord. (b) Following injury, neurocan is upregulated throughout spared ventrolateral white matter regions (vlwm; 7 dpi). (c) Longitudinal section through the lesion border of another specimen at 7 dpi. The lesion (*) is at the top. (d) Scanning confocal micrograph from section at the lesion border at 7 dpi. Neurocan (red) is associated with hypertrophied astrocytes expressing high levels of GFAP (green). Scale a,b=200 μ m, c=50 μ m, d=10 μ m. C. (a) Uninjured spinal cord. Brevican immunoreactivity is distributed

throughout gray and white matter regions. (b) After injury, brevican staining intensity is increased throughout spared white and gray matter regions surrounding the lesion site (*) (14 dpi). (c) Enlargement of ventral horn from (a). (d) Enlargement of boxed area in (b) of ventrolateral white matter (vlwm) border and lesion epicenter (*) at 14 dpi. Scale a, b=200 μ m, c=20 μ m, d=50 μ m. D. (a) NG2+ cells are distributed throughout gray and white matter regions in uninjured spinal cord. By 7 dpi (b), NG2 immunoreactivity is observed in hypertrophic NG2+ cells and in extracellular fibrillary profiles and other cell types. At 14 dpi, NG2+ cells are found in high density at the lesion border and extend far beyond the neurocan positive border (c), and into the center of the lesion (d). Scale a,b=200 μ m, c=50 μ m, d=40 μ m. E. Photomontages of confocal images from adjacent dual-IHC stained sections of a specimen obtained at 14 dpi. The lesion epicenter is to the right of the panel (*) and the lesion border is outlined with a white line. Note that aggrecan staining is lost proximal to the lesion site (a), while neurocan and brevican (b,c) accumulate at the lesion border. NG2+ reactivity extends throughout the lesion center (d). Scale=400 μ m.

Fig. 4. CSPG expression at cervical (A) and lumbar (B) levels. Western blots (left) and analysis of identified isoforms (right). Aggrecan and both isoforms of brevican expression were unchanged in cervical and lumbar spinal cord after contusion. Total and 250-kDa were changed in the cervical enlargement over time (one way ANOVA $p < 0.01$ and $p < 0.02$ respectively) with significant increase in total neurocan at 3 and 7 dpi (1.56 and 2.63 fold increases, respectively), and again, a large increased expression of the 250 kDa isoform (15.79 and 12.78 fold increases) at 3 and 7 dpi, respectively. Changes in the 150 kDa isoform of neurocan were modest, with a 1.58 fold increase compared with controls at 7 dpi. In the lumbar spinal cord, total and 250 kDa neurocan increased by 3 and 10–13 fold, respectively at 7 and 14 dpi; and both species remained elevated compared with controls for as long as 28 dpi. Significant increases in NG2 expression were observed at lumbar spinal cord levels (one way ANOVA; $p < 0.01$) with a 2 fold increase ($p > 0.05$) at 3 dpi and 3 fold

increase ($p<0.001$) at 28 dpi. No differences in NG2 expression were observed at cervical spinal levels ($p=0.076$).

Fig. 5.

Regional expression of GFAP and neurocan in cervical and lumbar spinal cord. A. Distribution of GFAP+proportional area in white matter (top) and gray matter (bottom) regions. Top left, drawing of spinal cord enlargement with white matter regions of analysis; lateral dorsal columns (DC), medial dorsal columns (MDC), dorsal corticospinal tract (DCST), lateral white matter (LWM), and ventral white matter (VWM). Bottom left, gray matter regions; dorsal horn (DH), intermediate gray (IG), and ventral horn (VH). Graphs illustrate the proportional area measures across regions and time post-injury for cervical (center column) and lumbar (right column) enlargements. Two-way ANOVAs revealed a significant effect of region ($p < 0.001$) and interaction ($p < 0.05$) in both white and gray matter. Corrected *post-hoc* analyses showed increases in cervical MDC at 14 and 28 dpi and lumbar IG at 14 dpi (* $p < 0.05$). B. Neurocan staining intensity in selected regions, including the medial dorsal columns (mdc), dorsal corticospinal tract (dcst), intermediate gray (ig), ventral gray matter (vgm) and lateral white matter (lwm). Two-way ANOVA identified effects of dpi and interaction in cervical and lumbar cord, with an increase in neurocan in cervical mdc at 14 and 28 dpi. In lumbar spinal cord, there was a significant increase in vgm at 28 dpi. Scale bar=200 μ m.

Fig. 6. Distribution of GFAP and neurocan in cervical dorsal columns and lumbar ventral gray matter. A. Dual staining images of GFAP, neurocan, and merged confocal photomicrographs illustrate chronic increase in neurocan immunohistochemical staining intensity in the medial dorsal columns of the cervical spinal cord. B. Increased neurocan staining is seen throughout the parenchyma of the lumbar spinal cord ventral gray matter. Scale bar=50 μ m.