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Traumatic Brain Injury Results in Disparate Regions of Chondroitin Sulfate Proteoglycan Expression That Are Temporally Limited

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Abstract

Axonal injury is a major hallmark of traumatic brain injury (TBI), and it seems likely that therapies directed toward enhancing axon repair could potentially improve functional outcomes. One potential target is chondroitin sulfate proteoglycans (CSPGs), which are major axon growth inhibitory molecules that are generally, but not always, up-regulated after central nervous system injury. The current study was designed to determine temporal changes in cerebral cortical mRNA or protein expression levels of CSPGs and to determine their regional localization and cellular association by using immunohistochemistry in a controlled cortical impact model of TBI. The results showed significant increases in versican mRNA at 4 and 14 days after TBI but no change in neurocan, aggrecan, or phosphacan. Semiquantitative Western blot (WB) analysis of cortical CSPG protein expression revealed a significant ipsilateral decrease of all CSPGs at 1 day after TBI. Lower CSPG protein levels were sustained until at least 14 days, after which the levels began to normalize. Immunohistochemistry data confirm previous reports of regional *increases* in CSPG proteins after CNS injury, seen primarily within the developing glial scar after TBI, but also corroborate the WB data by revealing wide areas of pericontusional tissue that are deficient in both extracellular and perineuronal net-associated CSPGs. Given the evidence that CSPGs are largely inhibitory to axonal growth, we interpret these data to indicate a potential for regional spontaneous plasticity after TBI. If this were the case, the gradual normalization of CSPG proteins over time postinjury would suggest that this may be temporally as well as regionally limited.

Keywords

chondroitin sulfate proteoglycans; cortex; immunohistochemistry; mRNA; RT-PCR; traumatic brain injury; controlled cortical impact injury; perineuronal nets

Traumatic brain injury (TBI) is associated with significant neurological deficits that in most cases do not completely resolve with time. Because axonal injury produced by shearing impact

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forces is a major hallmark of TBI (Adams et al., 1989), it seems likely that therapies directed toward enhancing axon repair and connectivity have the potential to improve functional outcomes after TBI. However, there is a large amount of literature that describes a significant axon growth inhibitory environment after central nervous system (CNS) lesions (Asher et al., 2000, 2002; Levine et al., 2001; Chen et al., 2003; Dobbertin et al., 2003; Sandvig et al., 2004; Silver and Miller, 2004; Dubreuil et al., 2006; Marklund et al., 2006; Thompson et al., 2006). This environment consists of increases in axon growth inhibitory extracellular matrix and myelin-associated molecules (growth-IMs) as well as increases in reactive astrocytes, microglia, and oligodendrocytes, all of which are associated with glial scar formation and can contribute to prevention of axon plasticity or synapse formation (Fawcett and Asher, 1999). Unfortunately, there has been little research to determine how the brain responds to TBI in terms of the glial scar and the local brain environment for growth inhibition. By addressing this gap in our knowledge, it should be possible to determine whether axon sprouting could underlie some spontaneous recovery of function after CNS injury, as some studies suggest (Castro-Alamancos and Borrell, 1995; Christman et al., 1997; Hulsebosch et al., 1998; Emery et al., 2000), and then proceed to determine whether this might be enhanced through therapeutic measures.

The major growth-IMs so far identified consist of myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, chondroitin sulfate proteoglycans (CSPGs), and NOGO, and these growth-IMs are generally, but not always, up-regulated after CNS injury (Fawcett and Asher, 1999; Sandvig et al., 2004; Silver and Miller, 2004). The CSPGs are extracellular molecules composed of secreted and transmembrane forms. They have a central protein core attached to glycosaminoglycan side chains containing repeating dissacharide units. The members of the CSPG family, versican, neurocan, phosphacan, brevican, neurocan, and NG2, differ in the protein core and the number and length of sulfation of the side chains (Morgenstern et al., 2002), which confers binding properties and hence function (Properzi et al., 2003). CSPGs undergo proteolytic cleavage to create additional forms (Morgenstern et al., 2002), and most are normally expressed by astrocytes, although there is now good evidence that immature oligodendrocytes either produce and/or associate with neurocan, versican, and NG2 (Ong and Levine, 1999; Asher et al., 2000, 2002) and that neurons produce at least some species of aggrecan, brevican, and neurocan (Oohira et al., 1994; Lander et al., 1998; Seidenbecher et al., 2002).

The CSPGs are present in normal brain, either within the extracellular space or aggregated as perineuronal nets that encase or surround neurons (Lander et al., 1998). They have been shown to be up-regulated after spinal cord injury (Lemons et al., 1999; Jones et al., 2003; Tang et al., 2003), stroke (Carmichael et al., 2005), hypoxic-ischemic injury (Aya-ay et al., 2005), or nigrostriatal lesion (Moon et al., 2002), and they are major inhibitory components of the resulting glial scar (Fawcett and Asher, 1999). A CSPG receptor site has not been identified, but Rho GTPase, which is increased after TBI (Dubreuil et al., 2006), appears to play a role (Jain et al., 2004) as does the epidermal growth factor receptor (Koprivica et al., 2005). The idea that all CSPGs are largely up-regulated after CNS injury and that they are all inhibitory to axon outgrowth is an oversimplification. Individual CSPGs have differential and even opposing effects on outgrowth, depending on the specific neuronal population and the developmental state of the neuron (Maeda and Noda, 1996). Previous reports suggest that the CSPG response to injury is quite heterogenous and may vary as a function of injury model or clinical disease state, temporal factors, or the specific species of growth-IMs (Levine, 1994; Jaworski et al., 1999; Asher et al., 2000, 2002; Lemons et al., 2001; Garwood et al., 2003; Dobbertin et al., 2003; Okamoto et al., 2003; Miyata et al., 2004).

Although on the face of it one might anticipate similar pathophysiology after TBI and other CNS injuries such as stroke, the initial insult very clearly differs between these two injuries,

especially with regard to the mechanism of primary axonal damage (Bramlett and Dietrich, 2004). Differences might therefore be expected in the temporal and/or spatial expression of molecules that affect axonal repair as a function of injury model. We reasoned that a study designed to investigate CSPGs associated with the glial scar after TBI would be an important first step for future efforts to design therapies that might enhance axon repair and functional outcome following TBI. The current study was designed to determine temporal changes in mRNA and protein expression levels of the CSPGs aggrecan, phosphacan, versican, and neurocan and to determine their regional localization and cellular association by using immunohistochemistry, in a controlled cortical impact (CCI) model of mild-to-moderate TBI.

MATERIALS AND METHODS

Experimental Groups

Separate groups of adult male Sprague Dawley rats (250–275 g) were used in experiments to determine CSPG mRNA, protein expression, or regional and cell-specific CSPG proteins. Initial pilot Western blot data suggested a peak response between 1 and 7 days after injury, so we opted to acquire the first mRNA data time point between these times and at weekly intervals thereafter. Rats were randomly assigned to postinjury day groups 4, 14, and 21 (n = 12, 12, and 6/time point, respectively, for groups with CCI or sham injury) for analysis of cerebral cortical CSPG mRNA by PCR. For analysis of CSPG protein by Western blots, rats were randomly assigned to postinjury day groups 1, 7, and 14 (n = 8/time point in groups with CCI, sham injury, and for naïve control groups). Ten rats were used for immunohistochemistry (postinjury day 7; n = 5 in CCI and sham injury groups). All procedures were approved by the UCLA Chancellor's Committee for Animal Research.

Surgery and CCI Injury

A previously characterized CCI injury model (Dixon et al., 1991; Sutton et al., 1993; Chen et al., 2003) was used to create a unilateral contusion injury to the left hemisphere. Animals were anesthetized with isoflurane [3% in 100% O_2 (1.5–2.0 liter/min flow rate) for induction; 1.5–2% for maintenance], and, when under a surgical level of anesthesia, they received an application of ophthalmic ointment to both eyes. During all surgical procedures, body temperature was maintained at 37°C ± 0.5°C with a thermostatically controlled heating pad (Harvard Apparatus, Edenbridge, KY). After shaving and sterilizing the scalp, the anesthetized animal was placed into a stereotaxic frame; a midline incision was made over the skull; and the skin, fascia, and temporal muscles were reflected bilaterally. By using a microscope and intermittent saline-cooled perfusion of the skull area, a 6-mm diameter craniotomy was made over the left sensorimotor cortex, centered at Bregma and 3.5 mm lateral to midline. An electronically controlled pneumatic piston (Hydraulics Control, Inc, Emeryville, CA), mounted on a stereotaxic micromanipulator (Kopf Instruments, Tujumga, CA) and angled at 28° to the vertical to allow the rounded 4 mm diameter tip to make contact perpendicular to the surface of the brain, was used to create the CCI. The injury (15 psi; 2 mm dural compression) was induced by depression and rapid contraction of the pneumatic piston. Hemostat material (Surgicel; Johnson & Johnson) was placed over the injury site before the bone flap was replaced and sealed in placed with cyanoacrylate glue, used sparingly to prevent excess from running onto the hemostat. Sham injury control rats were subjected to all surgical procedures, but did not experience induction of the CCI. Scalp incisions were sutured closed, and Bupivacaine (0.05–0.07 mg/kg, s.c.) was infiltrated into this wound margin. After recovery from anesthesia, rats were maintained in a warm recovery cage for ~30 min before being returned to home cages. Additional naïve control groups were used in Western blot experiments, and these animals did not experience any surgical anesthesia or surgical procedures.

RT-PCR and PCR

On postsurgery day 4, 14, or 21, rats were briefly anesthetized with isofluorane and decapitated, and the brain was quickly removed and placed on ice for dissection. Brains were cut in half longitudinally, and the brainstem and subcortical structures up to the level of the corpus callosum were blunt dissected away from each cortical hemisphere. The cortical mantles were then flattened between two glass slides to visualize the injury site or the homotypic area in the contralesional cortex. Tissue blocks, ~2 mm^3 in size, were dissected from 1) the grossly injured contusion site (herein referred to as "core"), 2) the interface between normal-appearing tissue and the injury core region (herein referred to as "border"), and 3) the homotypic contralateral cortex (see Fig. 1D). Similarly sized tissue blocks were dissected from the same regions in sham injury rats. Blocks were placed in RNA Later (Qiagen, Valencia, CA) at 4°C. Total RNA was isolated by using QUAzol lysis reagent (Qiagen) and purified by RNeasy Mini Kit spin columns (Qiagen).

The relative expression of mRNA for genes encoding neurocan and versican was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR), using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the internal control after validating its use in this trauma model (see Results). The aggrecan primers are specific for the hyaluronan binding regions used previously in the rat brain (Asher et al., 1995). The versican primers recognize part of the α-domain of the glycosaminoglycan attachment region that is present in the V2 isoform that is up-regulated after injury (Asher et al., 2002). The phosphacan primers used recognize the secreted form rather than the tyrosine phosphatase long and short forms (McKeon et al., 1999). The neurocan primers recognize the full-length and proteolytic fragments that are expressed after cortical injury (McKeon et al., 1999).

TaqMan EZ RT-PCR Core Reagents (Roche, Branchburg, NJ) were used for the reactions. A validation experiment was performed for each gene of interest and its control in order to determine the conditions for optimal concentration of primers and probes and to determine the efficiency of the PCR for each primer using serial dilutions of cDNA. The threshold cycle (C_T; the PCR cycle number at which gene amplification crosses from a linear to an exponential rate) was recorded for each gene by a real-time PCR machine (iCycler; Bio-Rad, Hercules, CA). C_T was used to calculate the -fold change in gene expression relative to the contralateral hemisphere of sham control rats, correcting for differences in the efficiency of gene amplification using published methods (Pfaffl, 2001). PCR products were confirmed to be the predicted size by gel electrophoresis.

We were not able to obtain a phosphacan probe of sufficient quality and specificity for our RT-PCR system. Aggrecan levels, though detectable at 4 and 14 days after injury using RT-PCR, were low and highly variable at 21 days. Therefore, phosphacan and aggrecan mRNA levels were determined by PCR rather than RT-PCR. PCR was performed with the following thermocycler parameters: 4 min at 95°C, 30 cycles of 1 min at 94°C, 30 sec at 58°C, 30 sec at 72°C, and finally 5 min at 72°C. The number of cycles was initially varied to determine the optimum over the sample range and to confirm the absence of template saturation. The reaction product was run on 2% agarose gel and then imaged using a Fluoromax System (Bio-Rad). Gel bands corresponding to each target gene and GAPDH internal control mRNA were quantified by integrated optical density (Rasband, 2008) and normalized to contralateral sham injury values.

RT-PCR (neurocan, versican) and regular PCR (aggrecan, phosphacan) were performed with the following forward (F) and reverse (R) primers and probes (P), with the indicated gene accession number (AN No.): neurocan [F = 5′-ACCTGGTAACCCTGGAAGTGA-3′, R = 5′-AGCGAAGGTCAACGCATAGC-3′, P = 5′-CGCCCGATAATGGAACACGACGCC-3′; AN No. NM_031653]; aggrecan [F = 5′-TGAGTGTGAGCATCCCTCAACCAT-3′, R = 5′

ATGCTGTTCACTCGAACCTGTCCT-3′; AN No. NM_022190]; versican [F = 5′-GCCATCGACAGCCTTCACAG-3′, R = 5′-CTCACTGGGCTCCTTCTCAAAG-3′, P = 5′-CCACACCCTTGAGGCCACCTTCGC-3′; AN No. AF_062402]; phosphacan [F = 5′-GAATTCTGGTCCACCAGCAG-3′, R = 5′-GGTTTATACTGCCCTCTTTAGG-3′; AN No. U04998].

Western Blots

On postsurgery day 1, 7, or 14, rats were briefly anesthetized with isofluorane and decapitated, and the brain was quickly removed and placed on ice for dissection. Pilot studies indicated there was insufficient protein to detect CSPGs reliably in small core or border tissue samples. Therefore, larger tissue blocks of ~3–4 mm^3 were taken from the injury zone containing both the injury core and the border region in the left cortex and from the homotypic cortex contralateral to TBI and from a similar region in the left/ipsilateral cortex of sham injury and naïve rats (see Fig. 2E). Tissue was harvested as described above for PCR and was homogenized in lysis buffer containing Tris-HCl (0.05 M, pH 7.5), NaCl (0.15 M), and protease inhibitors (Complete; Roche) and centrifuged at 13,000 rpm for 30 min at 4°C. Protein concentration was measured with the RC-DC Protein Assay Kit (Bio-Rad). Protein (30 μg) from each brain region was incubated with chondroitinase-ABC (1 μ/ml; Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris (pH 8.0) containing 0.3 M sodium acetate for 3 hr at 37°C before being mixed with 2× Laemmli's buffer and boiled for 5 min (DTT was excluded for versican blots). Proteins were resolved on a 3–8% Tris-actetate gel (Bio-Rad) overnight running at 8 mA at 4°C. Sets of high- and low-molecular-weight standards [Precision Plus Standards 10–250 Kd (Bio-Rad) and HiMark Protein Standards 31–460 kD (Invitrogen, Carlsbad, CA)] were run on the two outside lanes, and samples from each group were evenly dispersed over the 26-well gel. Proteins were electrotransferred to polyvinylidene diflurodide membrane (Bio-Rad). Blots were stained for total protein with Sypro Ruby (Bio-Rad) and imaged under ultraviolet light (Fluoromax System; Bio-Rad) for visual confirmation of equal lane loading. After washing in a solution of Tris-buffered saline and Tween-20 (TTBS) and blocking in 5% milk for 1 hr, membranes were probed overnight with the primary antibodies versican 2C5 (1:500; Developmental Studies Hybridoma Bank (DHSB, Iowa City, IA), aggrecan cat316 (1:800; Chemicon, Temecula, CA), phosphacan 3F8 (1:1,000; DHSB), and neurocan 1F6 (1:500; DHSB). Membranes were washed in TTBS for 3 × 10 min and then incubated with a HRP-conjugated secondary antibody, hostspecific for the primary antibody, in 5% milk (1% BSA only for versican) for 60 min (e.g., goat anti-mouse HRP; 1:10,000; Dako, Carpinteria, CA). After washing, antigen detection was achieved by using an enhanced chemiluminescent substrate kit (ECL Plus; Amersham, Arlington Heights, IL), and images of the membranes were acquired with a camera system sensitive to chemiluminescence (Fluoromax System; Bio-Rad). After imaging, all gel membranes were tested for nonspecific staining by stripping the antibody-chemiluminescent complex in stripping agent (Bio-Rad) and reexposing the membrane to secondary antibody and antigen visualization. Specific protein bands were determined by reference to the molecular weight standards and were quantified by integrated optical density measurements (Rasband, 2008). All gels were run with naïve control samples, which were used to normalize the data for all samples from each gel in order to control for minor changes in experimental conditions. All gels were run in duplicate, and the results were averaged.

Immunohistochemistry

After an overdose of sodium pentobarbital (100 mg/kg, i.p.) on day 7 postsurgery, rats with CCI or sham injury were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) at approximately mean arterial blood pressure, followed by 4% paraformaldehyde in PBS. After cryoprotection in 20% sucrose in PBS until the brain sank, the brain was frozen, and coronal sections were cut at 50 μm. Standard free-floating immunohistochemistry was

performed. In brief, antigen retrieval was carried out with citrate buffer (1 M, pH 6.0, incubation overnight at 4°C), followed by two rounds of 5 min heating to 95°C and 5 min cooling. After Tris-buffered saline (TBS; 0.2 M) washes, sections were blocked in streptavidin and then biotin solution (15 min each according to the manufacturer's instructions; Vector Laboratories, Burlingame, CA) and in 10% primary-antibody-host-species-specific serum solution and 5% BSA in TBS (20 min). Sections were then incubated in one mouse-host primary monoclonal antibody at room temperature overnight [dilutions: phosphacan 3F8 antibody, 1:250; neurocan 1F6 antibody, 1:500; versican 12C5 antibody, 1:3,000 (DHSB), aggrecan CAT316 antibody, 1:10,000 (Chemicon)]. After rinsing in TBS, sections were incubated in a rat-adsorbed anti-mouse biotinylated secondary antibody for 2 hr (1:500 dilution in 1% NGS; Dako). Antigens were visualized with streptavidin-conjugated fluorescent Alexa-488 and -555 probes (Molecular Probes, Eugene, OR; 1.25 μg/ml in TBS for 60 min). Double labeling was then achieved by using another biotin blocking step followed by mouse-on-mouse blocking reagent (Dako) and a serum blocking step as before, all of which facilitated the use of additional mouse-host primary antibodies on the same preparation [overnight incubation at room temperature in mouse monclonal anti-RIP, 1:1,000 (DSHB); anti-NeuN, 1:5,000 (Invitrogen); or polyclonal rabbit anti-GFAP, 1:5,000 (Dako)]. Antigens were visualized as described above but using a different-colored fluorophore. Sections were mounted on slides and coverslipped with antibleaching medium containing DAPI (Vectashield; Vector Laboratories). Sections were imaged by confocal microscopy (LSM Pascal System; Zeiss) in single-channel acquisition mode to prevent cross-talk and with fluorophore-appropriate laser excitation and filter sets configured for optimal emission. Montage data were acquired with a 12-μm optical section thickness at ×40 magnification over four to eight fields of view in automatic mode. Z-axis data were acquired at ×63 at 1-μm optical thickness. Optimal primary and secondary antibody concentrations were obtained by using standard dilutions as single labels, and test sections were run without primary antibody to confirm the specificity of the labeling for each secondary antibody isotype. Double-label test sections were run exhaustively without one of the primary antibodies to confirm the specificity of antibody binding, especially when using two mouse primary antibodies.

Statistical Analysis

Group numerical data are expressed as mean ± SEM. Data were tested for a statistical difference using group, region, and time parameters by univariate or multivariate analysis, followed by post hoc comparisons with Tukey's HSD test using R (Hornik, 2008). Differences were considered significant at $P < 0.05$. The Western blot data showed some effect of craniotomy alone on ipsilateral protein expression, although the cortex contralateral to TBI was unaffected and was similar to that of naïve controls. We therefore ran our post hoc Western analyses testing for significant differences between contralateral cortex values in TBI rats and ipsilateral values in sham and CCI injured groups.

RESULTS

CSPG mRNA Levels Are Either Increased or Remain Unaffected by Injury

We first investigated the effect of unilateral CCI injury on CSPG mRNA levels, because the analysis of protein levels can be affected by significant posttranslational modification (Domowicz et al., 1995). GAPDH was used here as the denominator for comparison of gene expression levels. Although GAPDH is not changed after brain ischemia (Harrison et al., 2000), variable changes in GAPDH were reported at 24 hr after experimental TBI in the mouse (Thal et al., 2008). We found no group differences in GAPDH C_T values using RT-PCR, validating its use in the current study (Fig 1C).

Versican mRNA determined by RT-PCR was significantly increased (2.6-fold, $P < 0.05$) above sham contralateral levels in the injury core region by 4 days after CCI, and expression of this CSPG remained elevated for 2 weeks before declining to sham values at 3 weeks postinjury ($P < 0.05$; Fig. 1A). Versican mRNA was unaffected by surgical/injury procedures in all other brain regions examined in both the TBI and the sham injury groups.

Neurocan mRNA changes determined by RT-PCR were more variable, and, although they were raised ~2.5-fold above sham contralateral levels within core and border regions from CCI rats and in ipsilateral cortex of sham injury rats at 4 days, these increases were not significant (Fig. 1B). At later time points, sham ipsilateral values of neurocan normalized to sham contralateral values, while ipsilateral core and border values remained elevated in CCI groups, although these increases also did not reach statistical significance (Fig. 1B).

Aggrecan was expressed at much lower levels than versican and neurocan, so that at 21 days postsurgery we could not detect it reliably by RT-PCR. With PCR we determined that there was a trend toward minor reductions in aggrecan expression in core and border regions at 4 and 21 days after CCI, but these ipsilateral decreases in aggrecan mRNA levels were not significant (Supp. Info. Fig. 1A). The PCR analyses for phosphacan revealed no difference in mRNA levels within any region or between groups, as shown qualitatively (Supp. Info. Fig. 1B) or as determined by the integrated optical density measurements of the gel bands (data not shown).

CSPG Protein Levels Are Significantly Decreased After Injury

We next investigated the effect of unilateral CCI injury on CSPG protein expression at 1, 7, and 14 days. We were unable to obtain enough protein by using the small sampling procedure described for mRNA analysis of core and border regions. We therefore sampled tissue containing both of these regions for ipsilateral protein determinations.

Versican protein expression (400 kD) was detected using the 2C5 antibody,. which detects the V2 isoform present in the adult brain (Asher et al., 2002; Deepa et al., 2006). Significant decreases were detected in ipsilateral cortex at 1 day after either CCI or sham injury ($P < 0.05$; Fig. 2A). At later time points, versican protein levels gradually increased toward naïve control levels, although they were still reduced by ~20% in the ipsilateral cortex at 2 weeks after CCI.

Similar effects were found using the 1F6 antibody to neurocan, which detects intact neurocan at 275 kD (Fig. 2B; Asher et al., 2000). Reductions in the left/ipsilateral cortex were marked and significant for both TBI and sham injury groups at days 1 and 7 postsurgery, although CCI injury resulted in the greatest reductions ($P < 0.05$). TBI-induced reductions in neurocan levels were sustained to 2 weeks postinjury ($P < 0.05$), whereas the sham levels were normalized to control. No significant changes in the N-terminal 130-kD neurocan fragment were found (Fig. 2B; quantitative data not shown) which may indicate that only part of the neurocan protein undergoes significant proteolysis.

Western blots for aggrecan protein reproducibly resulted in several high-molecular-weight bands at ~170, 240, 270, and >460 kD, as reported by others (Deepa et al., 2006). We chose to analyze the >460 kD band corresponding to the soluble form of aggrecan because we anticipated that this might be more sensitive to the effects of trauma compared with the smaller, less soluble bands. Unlike the other CSPG species examined, there were no significant differences in aggrecan protein by day 1 after surgery in either group. By 1 week, the cortex ipsilateral to TBI contained up to 35% less aggrecan compared with contralateral cortex, but similar significant effects were noted in sham-ipsilateral cortex (Fig. 2C; $P < 0.05$). However, reductions in aggrecan protein levels in the injured cortex were sustained by up to 20% ($P < 0.05$) to 2 weeks postinjury, when the effect of craniotomy alone was no longer present.

Aggrecan levels in ipsilateral cortex increased significantly from 7 to 14 days ($P < 0.05$) after TBI, indicating either a loss of tissue containing low levels of aggrecan or an increase in production of the aggrecan protein.

We used the 3F8 antibody to detect phosphacan and resolve a band at ~400 kD (Fig. 2D), which corresponds to the core protein (Dobbertin et al., 2003;Deepa et al., 2006). Although there was a significant effect of craniotomy alone on phosphacan levels during the first week after surgery (35–50% reduction), TBI resulted in greater reductions in phosphacan within the ipsilateral cortex (50–75% reduction) that were sustained throughout the 2 week postinjury period compared with contralateral cortex ($P < 0.05$). No significant changes in any CSPG species were detected contralateral to the injury.

CSPG Protein Is Increased in Glial Scar Regions

We next used immunohistochemistry methods to confirm the Western blot findings, using the same primary antibodies employed for the immunoblot study. We chose to analyze tissue at 7 days after injury because our Western data indicated fairly robust changes in all four CSPGs at this time point. In addition, previous data from our laboratory has shown that this time overlaps with the peak of the glial scarring response in this TBI model (Chen et al., 2003).

In apparent contradiction to the Western blot data, there was substantial *up-regulation* of neurocan and versican in areas corresponding to the glial scar in the CCI group (Figs. 3A, 4A) and an increase in all CSPGs in cortical regions underlying the contusion site and proximal to the underlying white matter (Figs. 3A, 4A, 5A, 6A). CSPG protein up-regulation was markedly increased through expression within GFAP-labeled astrocytes, as illustrated for phosphacan (Fig. 5J-L) and the similar co-expression noted in regions of high GFAP expression for neurocan (Fig. 3A–D), versican (Fig. 4A–D), and aggrecan (Fig. 5A'–C'). Increased RIP^+ staining, indicative of a major oligodendrocyte response after injury, was primarily associated with neurocan and versican (Figs. 3J, 4J, respectively) but not with phosphacan (Fig. 5M–O) and only partially with aggrecan (Fig. 6I–N). Strong and diffuse extracellular expression was present in areas adjacent to the primary injury site in the case of phosphacan (Fig. 5A,A') but not for any of the other CSPGs examined.

CSPG Protein Is Decreased Within Large Areas Surrounding the Injury Site

In agreement with the Western blot data showing decreased protein expression after CCI, there were wide expanses of normal-appearing, DAPI-stained tissue immediately adjacent to the injury site that exhibited substantially decreased immunofluorescent staining for all of the CSPGs examined (Figs. 3A,I, 4A,I, 5A,A', 6A,A') compared with the contralateral hemisphere (Figs. 3E,M, 4E,M, 5D, 6E). Further evidence of this CSPG protein deficiency is the complete absence of the normal perineuronal net arrangement of the CSPGs (see Figs. 6A vs. 6E and, at higher power, Figs. 6R vs. 6O). The loss of these perineuronal nets and the decrease in CSPG immunofluorescence are not due to neuronal death, insofar as neuronal staining appears normal within regions adjacent to the injury site (Fig. 6O–Q).

DISCUSSION

The data show that experimental TBI results in increases in cortical mRNA for the CSPG versican and a trend for increasing neurocan mRNA within injury core regions at 4 or 14 days, but little or no change in mRNA for aggrecan and phosphacan. Adjacent border regions were less affected, and all regional CSPG message levels were not significantly different from normal by 21 days after TBI. Surprisingly, all species of ipsilateral CSPG protein expression examined by Western blot were significantly attenuated within 1 week after CCI injury and, although thereafter they began to normalize, by 14 days CSPG protein levels were still lower

than normal. In contradiction to this, a more detailed regional analysis by immunohistochemistry (IHC) at 7 days revealed substantial increases in CSPG protein associated with glial scar regions, as expected. However, in accordance with the Western data, the IHC data demonstrated much larger expanses of tissue containing reduced extracellular and perineuronal net CSPG protein expression in regions adjacent to the primary site of the injury.

TBI Results in a Significant Glial Scar That Is Rich in CSPG Proteins

Although the reduction in CSPG protein demonstrated by Western blot data is somewhat surprising, it is not incongruent with the IHC results, in that large areas of low-intensity staining for all CSPGs were observed on all sections containing injury core and pericontusional brain tissue. During tissue sampling for Western blots, care was taken to avoid sampling of normal-appearing tissue outside the approximately 3–4 mm^3 volume of the injury. It is likely, therefore, that the Western blot data indicate the net effect of injury, a reduction in brain CSPGs. The IHC results show that, despite large regions of hypointense CSPG staining, there are regions of significant enhancement of CSPG staining in the developing glial scar around the injury site. These apparent differences in data do underscore the difficulty in assigning tissue outcome after TBI based solely on Western blot, which, though more quantitative than IHC, requires more careful regional analysis to determine the nature of changes in the tissue environment fully.

The occurrence of enhanced CSPGs in the glia scar is in agreement with previous findings of increased CSPG message or protein expression after spinal cord injury (Tang et al., 2003), stroke (Carmichael et al., 2005), stab wound (Asher et al., 2000, 2002), hypoxicischemic injury (Matsui et al., 2005), or kainic acidinduced seizures (Okamoto et al., 2003). This was most certainly the case for the CSPGs neurocan and aggrecan in the current study, which were highly expressed by astrocytes and oligodendrocytes at the contusion borders (Figs. 3, 6). These increases are also in good agreement with our previous data showing enhanced NG2 proteoglycan at the injury site at 4–7 days after TBI (Chen et al., 2003). At least for an astrocytic source of neurocan, the mechanism for increased CSPG expression is likely related to the cytokine transforming growth factor β1 (Smith and Strunz, 2005), which is greatly increased in core regions in the lesioned brain (Logan et al., 1992). The increased concentration of CSPGs and the densely packed glial scar will likely represent a significant barrier to axonal growth cones. Although we have shown that brain injury results in decreases in CSPG proteins in regions adjacent to the injury site, lack of full spontaneous functional recover after trauma might suggest that any axonal sprouting component to recover is hampered by those regions with significantly enhanced CSPG expression. Overcoming this impediment to growth inhibition represents a significant field of research in CNS injury today, and several groups have shown the functional benefits of reducing the CSPG or other growth-IM burden after CNS lesioning (Moon et al., 2001b), spinal cord injury (Bradbury et al., 2002; Barritt et al., 2006; Houle et al., 2006), and TBI (Thompson et al., 2006). Future work is needed to determine whether these therapies can improve axonal growth or impart substantial functional benefits in various models of TBI.

Injury Border Regions are Deficient in CSPG Protein

A novel and potentially important finding of this study is the measured decrease in CSPG protein expression in areas adjacent to the primary injury site. We had fully anticipated, based on our previous work in this model (Chen et al., 2003), that the current results would reflect a glial/inflammatory environment that is largely inhibitory for neuronal sprouting. Although there is some evidence of spontaneous axonal sprouting after brain injury (Christman et al., 1997; Hulsebosch et al., 1998; Emery et al., 2000), the persistent deficit in sensorimotor function observed in similar contusion models of TBI (Lindner et al., 1998) would be consistent

with our predicted overall growth inhibitory environment. However, the observed decrease in CSPG proteins in border regions after injury in the present study is not without precedent. For example, phosphacan protein is decreased after kainite-induced epilepsy (Matsui et al., 2002; Okamoto et al., 2003), within some astrogliotic lesions (McKeon et al., 1999), and after neonatal hypoxia ischemia (Matsui et al., 2005). In addition, phosphacan, versican, and brevican were all reduced at least within the first 2 weeks after spinal cord injury (Tang et al., 2003). Furthermore, mRNA levels of the receptor protein tyrosine phosphatase beta isoform of phosphacan are decreased for at least the first 2 days after CNS knife wound (Dobbertin et al., 2003).

There are numerous mechanisms by which decreased CSPG expression could occur, and, although we did not find significantly reduced message coding for these proteins (Fig. 1), translation into protein might well have been affected by the post-TBI environment. While there is only sparse evidence for reduced protein synthesis after TBI (Singleton et al., 2002), we have found previously that injury core blood flow levels in the CCI model (Sutton et al., 1994;Chen et al., 2004) are reduced to levels that affect protein synthesis after forebrain ischemia (Mies et al., 1991). In addition, the resolution of blood flow to normal values after injury that occurs in this model could account for a resumption of normal rates of protein synthesis and the gradual normalization of Western blot data demonstrated in this study (Fig. 2).

The extracellular environment after TBI is significantly enriched for proteases, for example, matrix metalloproteinases (MMP) 9 (Shigemori et al., 2006) and MMP3 (Kim et al., 2005), and many MMPs recognize a defined cleavage site in the central region of CSPG proteins that create N- and C-terminal protein fragments (Rauch, 2004). The present data showing an injury-induced reduction in intact neurocan at 275 kD would suggest that significant proteolytic cleavage occurs in this TBI model.

CSPG Reduction: An Environment for Brain Plasticity?

The overall decrease in CSPG protein burden around the injury site indicates that the environment is likely to be growth permissive. Literature evidence reviewed herein suggests that this would provide a potential window for plasticity after TBI. Decreases in phosphacan similar to those observed in this study have also been shown to coincide with brain regions undergoing synaptic remodeling in the rat (Miyata et al., 2004). Reductions in phosphacan and aggrecan have also been noted by IHC in a relatively plastic perilesional area after rat forebrain ischemia, although the corresponding increases in phosphacan mRNA in that model (Carmichael et al., 2005) would seem to suggest a failure in translation rather than, or in addition to, an increase in MMP or other protease activities after stroke. Endogenous, injury-induced CSPG cleavage by MMP action has been mimicked by pharmacological means to ablate CSPGs exogenously. The bacterial enzyme chondroitinase ABC applied after CNS injury or perturbation produces decreases in CSPGs similar to those observed within injury border regions after TBI. By using this CSPG ablation technique, improvements in axonal sprouting and functional outcome have been measured (Moon et al., 2001a; Bradbury et al., 2002; Pizzorusso et al., 2002; Tropea et al., 2003; Yick et al., 2003). The opposite effect of this treatment, an inhibition of MMPs after entorhinal cortex lesion, resulted in a paucity of hippocampal synaptic reorganization and failure to observe long-term potentiation, all of which normally develops in that injury model (Reeves et al., 2003). Finally, although we did not assess CSPGs at more chronic postinjury times, at least in the case of spinal cord injury the CSPG proteins increase at 1 month postinjury (Tang et al., 2003), which corresponds to the end of spontaneous sprouting in that model (Soares et al., 2007). The gradual normalization of CSPG proteins in this study (Fig. 2) does suggest a similar trend after experimental TBI, so that there may only be a short temporal window for growth permissiveness. It seems therefore

that the spontaneous decrease in CSPGs may represent a period for enhanced axonal sprouting after trauma, although further study is required to substantiate this supposition. If this were the case, then therapies directed to improve plasticity and axonal sprouting after TBI (Thompson et al., 2006; Smith et al., 2007) might benefit by timing the intervention with the nadir in CSPG expression.

CSPGs exist both in the extracellular space and/or loosely bound to membranes and also very tightly bound together around cells in characteristic perineuronal net (PNN) arrangements. We observed a decrease in both extracellular CSPG and the association of CSPGs into PNN in tissue adjacent to the injury core. The dual loss of CSPG protein levels and organization, rather than CSPG levels alone, might suggest considerable proteolytic action, insofar as, at least in vitro, the digestion of CSPGs from tissue initially results in a decrease in extracellularly located species and much more vigorous digestion is required for removal of PNN-associated CSPGs (Deepa et al., 2006). The PNNs are complexes of CSPGs and other extracellular matrix molecules that surround mainly GABAergic neurons and have been implicated in stabilizing synaptic contacts while impeding novel ones (Celio et al., 1998). The loss of PNNs after TBI in this study and after ischemia (Carmichael et al., 2005) might therefore indicate a critical period for axonal sprouting. Furthermore, the loss of PNNs through exogenously applied enzymatic digestion reactivates ocular dominance plasticity in adult visual cortex (Pizzorusso et al., 2002) and, in culture, increases neuronal excitability without affecting neuronal number (Dityatev et al., 2007). Therefore, therapies directed toward enhancing PNN degradation after TBI might enhance cortical plasticity and ensuing brain function.

In summary, we have shown that experimental TBI results in disparate regional changes in CSPG expression, ranging from high mRNA and protein expression at the glial scar to considerable decreases in protein expression in adjacent border regions. Reduced extracellular and PNN-associated CSPGs indicative of a growth permissive environment suggest that there may be potential for regional spontaneous plasticity after TBI. The gradual normalization of CSPG proteins over time postinjury suggests that this could be temporally as well as regionally limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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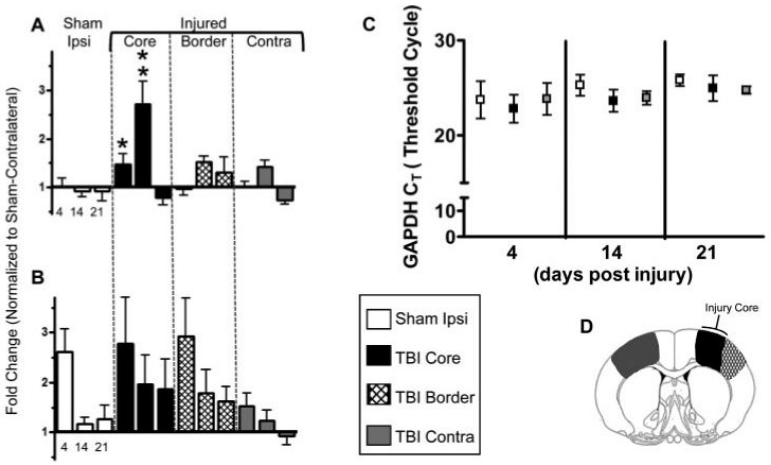

Fig. 1.

Semiquantitative mRNA values from cerebral cortex at 4, 14, and 21 days after injury obtained by real-time RT-PCR for (A) versican and neurocan (B). Data are plotted as -fold change from sham injury contralateral cortex for: sham ipsilateral (ipsi; open bars), TBI core (solid black bars), TBI border (hatched bars), and TBI contralateral (contra) cortex (solid gray bars). **A:** Versican expression was significantly increased in the TBI core at 4 and 14 days, but not at 21 days, and did not differ in any other regions compared with sham contralateral values or sham-ipsi. **B:** Neurocan expression was temporarily increased in sham-ipsi cortex at 4 days, and it remained increased in TBI core and border from 4 to 21 days, although this was variable and was not significantly different from sham contralateral values. For all CSPGs examined, there were no significant changes in the levels of contralateral CSPG mRNAs. $\star P < 0.05$, $\star\star P < 0.01$. **C:** RT-PCR threshold cycle number (C_T) for GAPDH expression in sham-ipsi, injured core, and contralateral regions (symbols color-coded according to the bar legend). There was no difference in GAPDH C_T cycle number between any of the groups studied (data plotted as means ± calculated 95% confidence interval). **D:** Diagram of the brain regions sampled for mRNA (color-coded to plotted bars).

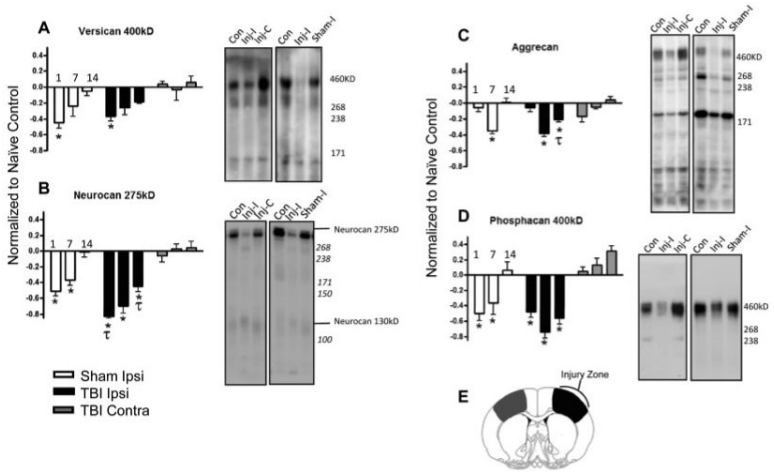

Fig. 2.

Semiquantitative cerebral cortical protein expression analysis of versican (**A**), neurocan (**B**), aggrecan (**C**), and phosphacan (**D**) and diagram of brain regions sampled for protein (**E**; color-coded to plotted bars) at 1, 7, and 14 days after sham injury, ipsilateral cortex (open bars, sham-I) or TBI ipsilateral (black bars, Inj-I) and contralateral cortex (gray bars, Inj-C). Values are normalized to naïve control (con) rat cortex. Craniotomy alone (sham injury) resulted in a significant reduction in all CSPGs at 1 and/or 7 days postsurgery, but this effect was completely normalized by 14 days. TBI also resulted in significant reductions in all CSPG species examined by 1 and/or 7 days. Although CSPG protein began to normalize by 14 days, levels of neurocan (B), aggrecan (C), and phosphacan (D) were still significantly reduced ipsilateral to TBI. There were no significant changes in the levels of CSPG proteins contralateral to the injury site. Each gel shown is representative of the day-7 time point. Thirty micrograms protein was run in each lane and high- and low-molecular-weight markers were run in the outermost lanes. ⋆Difference from TBI contralateral cortex, $P < 0.05$; ᵀdifference from sham-ipsi, $P < 0.05$, at the same time point.

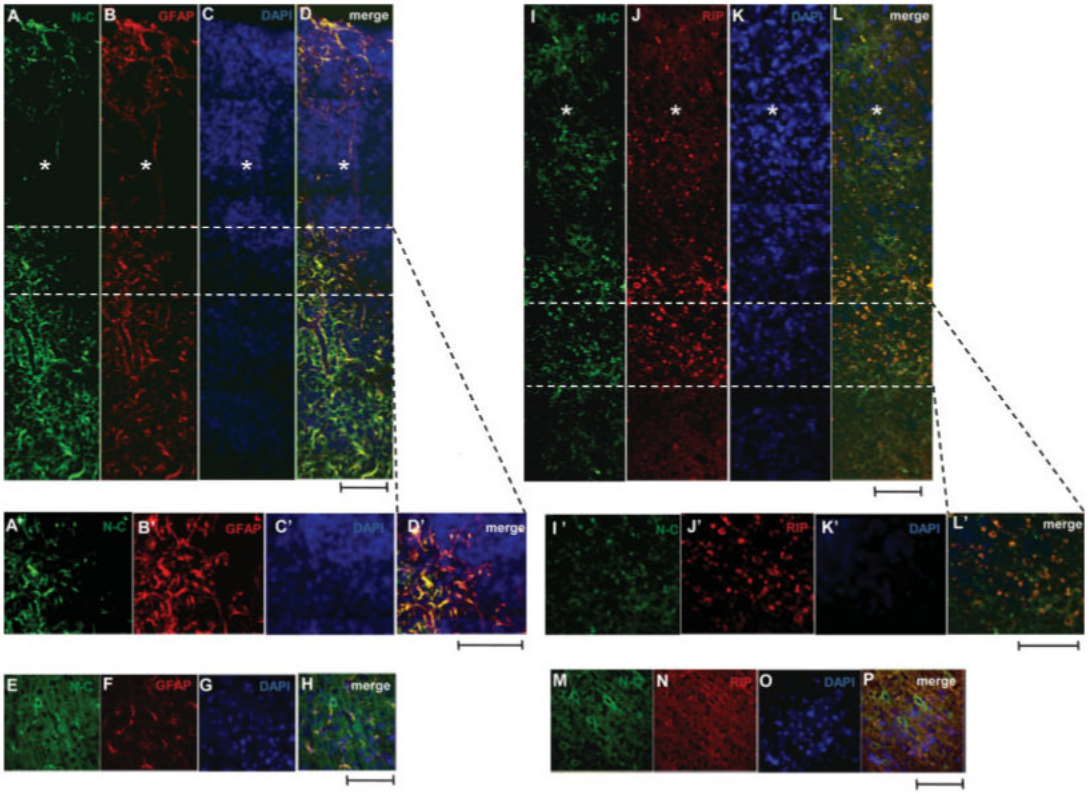

Fig. 3.

Neurocan (NC) immunohistochemistry. Confocal montages of the left/ipsilateral sensory-motor cerebral cortex from pia (top) toward the white matter (bottom) at 7 days after TBI (**A–D,A'–D',I–L,I'–L'**) compared with cortical layers IV–V of the contralateral homotypic cortex (**E–H,M–P**). After injury, NC protein (green, A,E) is highly expressed by $GFAP^+$ cells (red, B) in the glial scar surrounding the contusion (upper region of A–D), whereas it is low or absent in normal-appearing DAPI-stained regions closer to the contusion core (asterisk in A–D,A'–D') compared with contralateral regions (E–H). Many RIP^+ oligodendrocytes also coexpress NC after injury (I–L,I'–L') compared with contralateral regions (M,N,P). The normal perineuronal net "ring" arrangement of NC expression observed in contralateral tissue (M) was absent in the injured cortex (I), and it was only highly expressed in association with activated astrocytes and oligodendrocytes farther from the injury site in regions adjacent to the white matter (lower half of A–D,I–L). Scale bars = 100 μm.

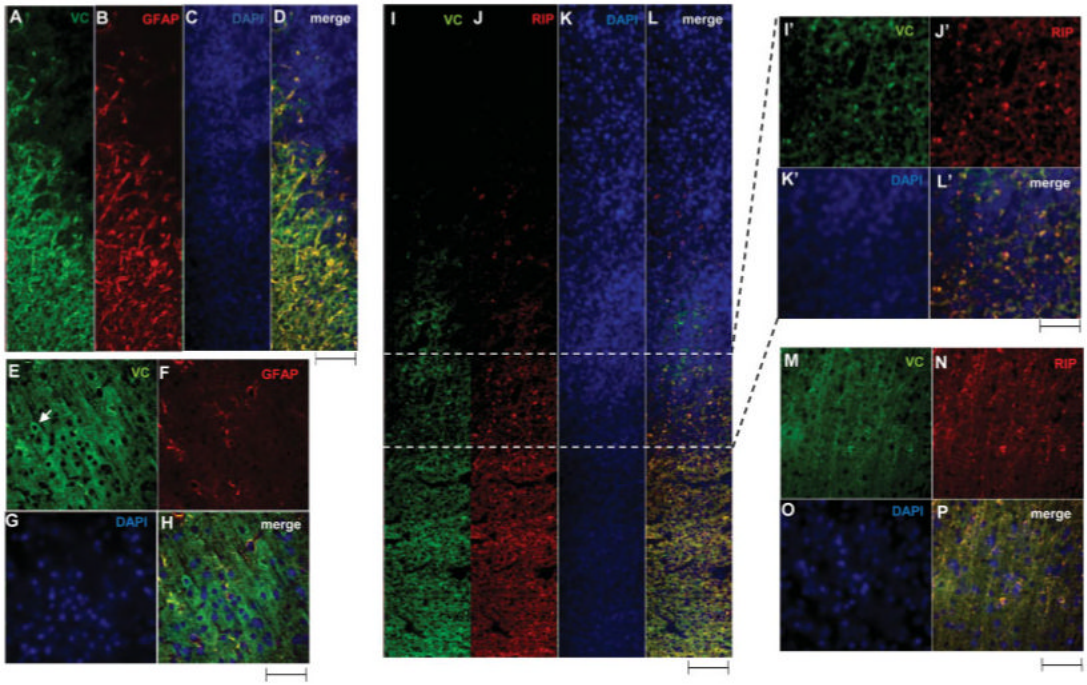

Fig. 4.

Versican (VC) immunohistochemistry. Confocal montages of the left/ipsilateral sensory-motor cerebral cortex from pia (top) toward the white matter (bottom) at 7 days after injury (**A–D,I–L,I'–L'**) compared with contralateral, homotypic layer IV–V cortex (**E–H,M–P**). After injury, VC protein is high in the glial scar on the edge of the contusion (A) compared with contralateral cortex (E) and is expressed by many $GFAP^+$ cells (B), presumed to be activated astrocytes. On the contralateral side, astrocytes remain either VC^- or are VC^+ only on glial end processes (E–H), and VC is more restricted to perineuronal nets (arrow, E) or the extracellular space. VC expression is reduced in regions near the contusion core (top of A,I and DAPI images C,K), being lower than even the contralateral cortex (E,M). Many RIP^+ cells and processes colabel for VC under the injured area (I–L) similar to the contralateral cortex (M–P). This occurs even in regions where RIP is enhanced (I–L,I'–L'). Scale bars = 100 μm.

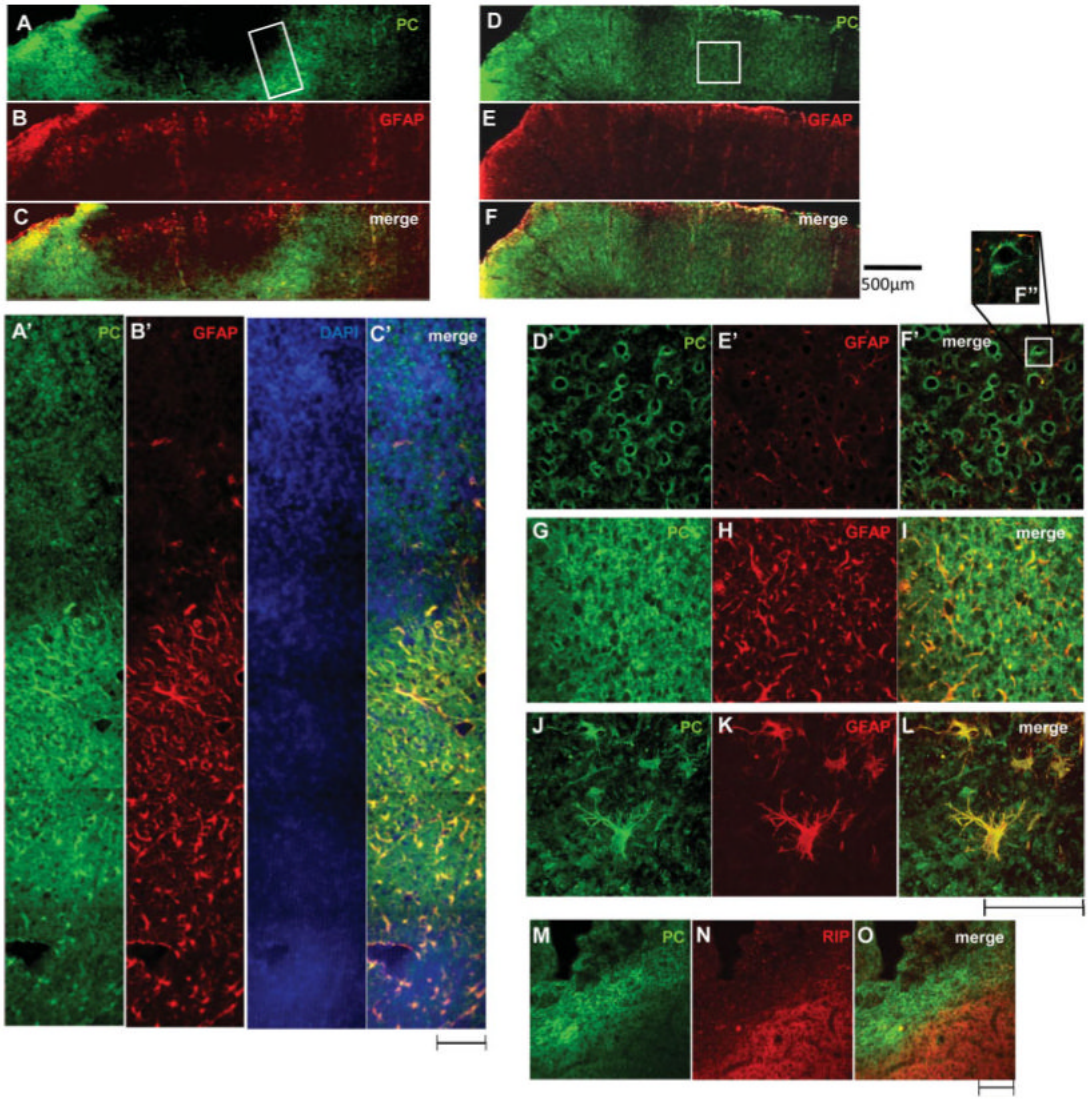

Fig. 5.

Phosphacan (PC) immunohistochemistry. Confocal montages of sensory-motor cerebral cortex at 7 days after TBI ipsilaterally (**A–C,A'–C',G–I,J–L,M–O**) compared with the homotypic, contralateral region (**D–F,D'–F'**). PC protein is low in the contusion center, where even scar-forming GFAP$^+$ astrocytes do not colabel (A–C). Farther from the injury site, PC is highly expressed and colabels with astrocytes (A–C,A'–C'). In the contralateral cortex (D–F) and at higher power (D'–F'), PC is highly expressed in perineuronal nets (merged F') and does not colabel with astrocytes. This organized structure is lost around the developing astrocytic scar after injury with high extracellular (G) and astrocyte expression (J–L), although PC is not associated with areas of oligodendroglial RIP$^+$ expression around the edge of the contusion (M–O). Scale bars = 100 μm, except where stated otherwise.

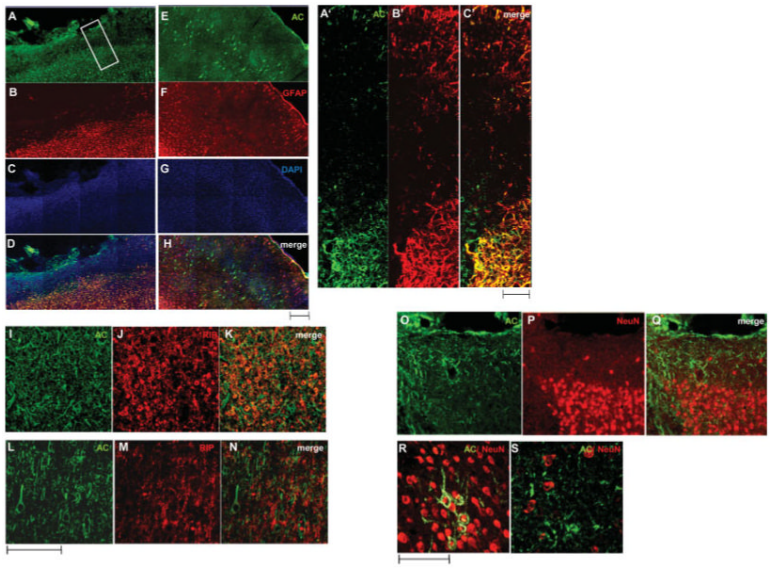

Fig. 6.

Aggrecan (AC) immunohistochemistry. Confocal montages of sensory-motor cerebral cortex at 7 days after TBI ipsilaterally (**A–D,A'–C'**) compared with the homotypic, contralateral region (**E–H**). Aggrecan is highly expressed in the developing glial scar under the contusion and is associated with astrocytes (A–D,A'–C') but remains low in bordering regions close to the contusion, within fully intact areas as indicated by DAPI staining (C). The perineuronal net arrangement present in contralateral regions (E and at high power **L–N,R,S**) as well as in sham rats (not shown) is lost ipsilateral to the injury (**O–Q**) and is replaced by a more random array of mainly AC^+ astrocytic glial processes and mature oligodendroglia processes (**I–K**). However, oligodendrocytes do not show a complete overlap with AC^+ cells. Scale bars = 100 μm.