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Intraventricular Hemorrhage Induces Deposition of Proteoglycans in Premature Rabbits, but Their *In Vivo* Degradation with Chondroitinase Does Not Restore Myelination, Ventricle Size and Neurological Recovery

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Abstract

Intraventricular hemorrhage (IVH) results in white matter injury and hydrocephalus in premature infants. Chondroitin sulfate proteoglycans (CSPGs)—neurocan, brevican, versican, aggrecan and phosphacan—are unregulated in the extracellular matrix after brain injury, and their degradation enhances plasticity of the brain. Therefore, we hypothesized that CSPG levels were elevated in the forebrain of premature infants with IVH and that *in vivo* degradation of CSPGs would enhance maturation of oligodendrocyte, augment myelination, promote neurological recovery, and minimize hydrocephalus. We found that levels of neurocan, brevican, aggrecan, phosphacan, and versican were elevated, whereas NG2 expression was reduced in premature rabbit pups and human infants with IVH compared to controls. Intracerebroventricular chondroitinase ABC (ChABC) reduced the expression of neurocan, brevican, versican and aggrecan, but not NG2. However, ChABC treatment did not enhance maturation of oligodendrocytes, myelination, or neurological recovery in the pups with IVH. Moreover, ChABC did not reduce gliosis or ventriculomegaly. Our results demonstrate that IVH induces distinct changes in the components of CSPGs, and that reversing these changes by *in vivo* ChABC treatment neither promotes clinical recovery, myelination, nor reduces ventriculomegaly in preterm rabbit pups.

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Keywords

Chondroitin sulfate proteoglycans; neurocan; brevican; versican; aggrecan; phosphacan; oligodendrocyte; myelination; chondroitinase ABC

INTRODUCTION

Intraventricular hemorrhage (IVH) remains a major cause of both morbidity and mortality in premature infants. IVH results in post-hemorrhagic hydrocephalus and reduced myelination of the white matter. The development of hydrocephalus is attributed to the obstruction of CSF flow by blood clots and impaired CSF absorption in the cerebral ventricles and subarachnoid space; and the hypomyelination is ascribed to degeneration and arrested maturation of oligodendroglial progenitors in the periventricular white matter (Dummula, et al., 2011). Chondroitin sulfate proteoglycans (CSPGs) are major component of extracellular matrix (ECM) in the brain and are upregulated after brain injury around the site of lesion (Morgenstern, et al., 2002). Elevated CSPGs in the ECM reduce migration and formation of process-outgrowth of oligodendrocyte (OL) progenitors, and impede movement of extracellular fluid in the brain (Siebert and Osterhout, 2011, Sykova, et al., 2001). Therefore, we specifically asked whether CSPG levels were elevated in the forebrain of premature infants with IVH, and determined whether degradation of CSPG would restore myelination and minimize hydrocephalus in these infants.

CSPG consists of a core protein that is covalently linked to glycosaminoglycan (GAG) side chains. The CSPGs include lecticans, phosphacan and NG2; and the family of lecticans comprise aggrecan, versican, neurocan and brevican. CSPGs are elevated in animal models of cerebral hypoxia-ischemia, surgically injured cerebral cortex and spinal cord, and inflammatory brain disease (Li, et al., 2008, Matsui, et al., 2005). CSPG restricts plasticity of the brain after injury; and degradation of CSPGs by Chondroitinase ABC (ChABC) treatment enhances plasticity of the brain structures. Indeed, ChABC promotes sprouting of axons in the spinal cord and nigrostriatal tract injury, stabilizes synaptic contacts, and enhances outgrowth of purkinje cell neurites in the cerebellum (Bradbury, et al., 2002, Hunanyan, et al. 2010, Moon, et al., 2001). ChABC treatment removes the GAG chain and thus, allows the CSPG core protein to be cleared by plasmin proteolytic system and other endogenous proteases (Bukhari, et al., 2011, Tsirka, et al., 1997).

Elevated CSPGs affect proliferation of neural cells and maturation of OL (Gu, et al., 2009). In culture experiments, elevated CSPGs inhibit migration, formation of process outgrowth, and differentiation of OL progenitor cells, thereby contributing to myelination failure (Siebert and Osterhout, 2011, Silver and Miller, 2004). Maturation of OL progenitors into mature OL requires both extension of processes to ensheath axons and expression of myelin proteins. CSPGs, including neurocan and phosphacan, strongly inhibit OL process outgrowth (Siebert and Osterhout, 2011). In addition, CSPG forms a significant barrier to the migration of OL progenitors. Consistent to these findings, ChABC treatment degrades CSPGs and promotes both migration as well as differentiation of OL in culture experiments and animal models of spinal cord injuries (Siebert, et al. 2011).

CSPGs are elevated in cerebrospinal fluid of preterm infants with post-hemorrhagic hydrocephalus, suggesting that CSPGs might be released into the CSF by the periventricular neural cells (Chow, et al., 2005). Importantly, accumulation of CSPGs in the ECM might reduce the flow of extracellular fluid and contribute to hydrocephalus (Del Bigio and Enno, 2008, Massicotte, et al., 2000, Shoesmith, et al., 2000). Despite this, CSPGs have neither been evaluated in the cerebral cortex of brain injury models of hypomyelination nor in a

paradigm of post-hemorrhagic hydrocephalus. Therefore, we hypothesized that CSPGs were elevated in the forebrain of premature newborns with IVH, and that degradation of CSPGs by ChABC treatment might enhance maturation of OL, myelination, and clinical recovery in premature infants with IVH. We also postulated that ChABC treatment would minimize hydrocephalus. To test our hypotheses, we used our animal model, in which rabbit pups (E29, term=32d) with glycerol-induced IVH exhibit arrested OL maturation, hypomyelination and hydrocephalus at 2-week age (Chua, 2008). We found that CSPGs, including brevican, neurocan, aggrecan, phosphacan, and versican were elevated, whereas NG2 levels were reduced in pups with IVH compared to controls. More importantly, degradation of CSPGs by *in vivo* ChABC treatment neither promoted myelination, clinical recovery, nor reduced gliosis and ventriculomegaly.

MATERIAL AND METHODS

Animal Experiments

The Institutional Animal Care and Use Committee of New York Medical College approved all experimental protocols. Timed-pregnant New Zealand rabbits were obtained from Charles River Laboratories, Inc. (Wilmington, Mass., USA). Cesarean-section was performed to deliver the pups prematurely at E29 (full-term=32 days). We kept them in an infant incubator that was pre-warmed to a temperature of 35°C. The pups were gavage-fed 2–4 ml (100ml/kg) of puppy formula (Esbilac, Petag, IL) twice a day and then feeds were advanced to 125, 150, 200, 250 and 280 ml/kg at postnatal days 3, 5, 7, 10 and 14 respectively. The rabbit pups of either sex were administered 50% glycerol (6.5 gm/kg) intraperitoneally at 2–3 h of age to induce IVH. Head ultrasound was performed at 6 h of age to assess the presence and severity of IVH using an Acuson Sequoia C256 (Siemens) ultrasound machine. Severity of IVH was diagnosed by measuring ventricle volume (length, breadth & depth in coronal & sagittal views) on head ultrasound at 6 h age. Pups were stratified based on ventricular volume into moderate (30–150 mm³) and severe (151–250mm³) IVH. Ventricular volume <30 mm³ indicate microscopic or no IVH. The pups with moderate and severe IVH were assigned to treatment and control group in such a manner that the severity of IVH was balanced between the comparison groups. To determine the effect of IVH on the expression of CSPGs, we compared pups with IVH and glycerol-treated control pups without IVH.

In vivo ChABC treatment

The rabbit pups with IVH were sequentially treated with either 20 µl ChABC (50 U ml⁻¹, Catalog # C3667, Sigma, St Louis, USA) or vehicle at days 2, 4 and 6. ChABC or vehicle (saline) was administered intracerebroventricularly. Briefly, the pups were mounted on rabbit pup restrainer after anesthetizing them with ketamine and xylazine. A needle of 27 gauge with 50 µl Hamilton syringe was mounted on a micromanipulator to inject the ChABC into the ventricle. We used the following coordinates from bregma: 1 mm anterior, 4 mm lateral, and 3 mm deep. The dose of ChABC was calculated based on its previous use in rats to treat spinal cord and cerebral injury (Bradbury, et al., 2002, Bruckner, et al., 1998, Harris, et al.) and our initial experiments showing reduction in CSPG core-protein by 95% on ChABC treatment (Supplemental Fig. 1). The severity of IVH, measured by ultrasound, was similar between the comparison groups—vehicle treated pups with IVH and ChABC-treated pups with IVH (ventricular volume 0.170±0.02 vs. 0.174±0.03 cm³).

Human subjects

The Institutional Review Board of New York Medical College, Valhalla, NY approved the use of autopsy materials from premature infants for this study. The study materials included forebrain tissue samples taken from the premature infants with and without IVH of 23–26

weeks of gestational age and 7–14 days of postnatal age (Table 1). Samples were obtained less than 24 h postmortem. We excluded premature infants with meningitis, hypoxic-ischemic encephalopathy, culture proven sepsis, major congenital anomalies and chromosomal defects. We included 4–5 infants in each group--IVH and no IVH. The wall of the cerebral hemisphere in premature infants consists of ventricular zone (VZ), subventricular zone (SVZ), intermediate zone, cortical plate, and marginal zone as described by the Boulder Committee (Bystron, et al., 2008). In the present study, we described intermediate-zone embryonic white matter synonymously with white matter and cortex for the cortical plate.

Rabbit tissue collection and processing

We processed the tissues as we previously described (Ballabh, et al., 2007). The brain slices were immersion-fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 0.1 M, pH 7.4) overnight and then were cryoprotected by immersing into 20% sucrose in 0.1 M PBS buffer for 24 hours followed by 30 % sucrose for the next 24 hours. Tissues were frozen after embedding into optimum cutting temperature compound (Sakura, Japan). Frozen coronal blocks were cut on a cryostat into 12 μ m thick coronal sections.

Human tissue collection and processing

We processed the tissues as in our prior studies (Ballabh, et al., 2007). Approximately 2–3 mm thick coronal slices were taken at the level of thalamostriate groove from the frontal lobe. The coronal blocks from the frontal lobe included the cortex, white matter, and germinal matrix. The samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 12–18 h and were then cryoprotected by immersing into a 20% sucrose solution in PBS. The tissues were frozen after embedding them into optimum cutting temperature compound (Sakura, Japan). Frozen coronal blocks were cut into 12 μ m sections. For Western blot analyses, pieces of tissues were directly harvested from the cortex, white matter and germinal matrix and frozen immediately on dry ice.

Immunohistochemistry

Immunostaining was performed as described previously (Ballabh, et al., 2007). The primary antibodies used in experiments included mouse monoclonal brevican (Novus biologicals, catalog # H00063827), mouse monoclonal RTP β (catalog # 610179, BD biosciences), mouse monoclonal aggrecan (Catalog # mab 5284, Millipore), rabbit polyclonal NG2 (Catalog # ab5320, Millipore), sheep neurocan (Catalog # AF5800, R & D), goat polyclonal versican (catalog # AF3054, R&D), goat polyclonal Ki67 (Catalog # 275R-14, Cell Marque, Rocklin, CA), goat polyclonal Olig 2 (Catalog # AF-2418, R & D, Minneapolis, MN, USA), mouse monoclonal GFAP (catalog # G6171, St Louis, MO, USA), rat monoclonal myelin basic protein (catalog # AB7439, Abcam, Cambridge, MA, USA), mouse monoclonal NG2 (catalog # 5384, Millipore, Billerica, MA), mouse monoclonal APC (Catalog # OP80, EMD Chemicals, Gibbstown, NJ). The secondary antibodies used were Cy-3 conjugate donkey anti-mouse, cy-3 conjugate donkey anti-goat and FITC conjugate donkey anti-rat (Jackson ImmunoResearch, West Grove, PA, USA). Briefly, we hydrated the fixed sections in 0.01M PBS and incubated with the primary antibodies diluted in PBS at 4°C overnight. After washing in PBS, the sections were incubated with secondary antibody diluted in 1% normal goat serum in PBS at room temperature for 60–90 minutes. Finally, after washes in PBS, sections were mounted with Slow Fade Light Antifade reagent (Molecular Probes, Invitrogen, CA, USA) and were visualized under a Confocal microscope (Nikon Instruments, Japan). Stereology was performed using Fluorescent microscope (Axioskop 2 plus, Carl Zeiss Inc) with motorized specimen stage for automated sampling (ASI, Eugene, OR, USA), CCD color video camera (Microfire; Optronics, Goleta, CA) and stereology software (Stereologer, SRC, Baltimore, MD).

Quantification of oligodendrocytes and proliferation

In ChABC-treated and vehicle treated pups with IVH, we counted proliferating OL in sections double-stained with Olig2 and Ki67 antibodies. Olig2 (+) cells were quantified in corpus callosum and corona radiata. Maturation of OL was evaluated in sections double-labeled with O4 and APC antibodies. From each brain, 5 coronal sections (20 μ m thick) taken as every 3rd section at the level of midseptal nucleus were used for immunolabeling. Counting was performed under confocal microscope using 60x lens (Nikon Instruments, Japan) by a blinded investigator in an unbiased fashion and random basis. We counted objects in ~25 images (5 images x 5 coronal sections) per brain region for each pup (n = 5–6 pups each group) for every parameter.

Stereological assessment of myelin and astrocytes in the white matter

Unbiased stereology methods, with assistance from a computerized software system (*Stereologer*, Stereology Resource Center, Chester, MD), were employed to quantify a range of parameters. Briefly, coronal sections of 30 μ m thickness were cut on a cryostat with a section sampling interval of 90 μ m to achieve at least 6 sections at the level of mid-septal nucleus. The sections were double-stained with myelin basic protein (MBP) antibody and DAPI (nuclear stain) and quantified as follows. The reference spaces (corona radiata, corpus callosum) were first outlined on the section under 5x objective. The volume of the outlined area (reference space) was quantified using a point counting probe (frame 25 μ m x 25 μ m; guard zone 2 μ m, inter-frame interval = 300 μ m). The total volume fraction (load) of myelin stained by antibodies to MBP through a defined reference space was quantified using the object area fraction probe under 60x oil lens. For the area fraction probe (frame 25 μ m x 25 μ m; guard zone 2 μ m, interframe interval 400 μ m), the user clicked on the grid points that touched myelin fibers in sections labeled with MBP. The area fraction of myelination was quantified as the ratio of product of the area per point and number of points hitting reference area [$a(\text{point}) \cdot \Sigma P_{\text{ref}}$] over the product of the area per point and number of points hitting the sampled area [$a(\text{point}) \cdot \Sigma P_{\text{samp}}$], as previously reported (Mouton, et al., 2009). Sampling continued until the coefficient of error (CE) was < 0.10. To assess gliosis, we performed: a) astrocyte count (GFAP labeled) using the optical disector method (frame 25 μ m x 25 μ m; guard zone 2 μ m, interframe interval 280 μ m); and b) total volume fraction of astrocyte cell body and glial fibers (Mouton, et al., 2009). Volume fraction of astrocytes was quantified in similar manner as for myelin.

Western blot analyses

We homogenized the frozen brain tissue in sample buffer (3% SDS, 10% glycerol, 62.5mMol TRIS-HCL, and 100 mM DTT) using a mechanical homogenizer and boiled the samples immediately for 5 minutes. We next determined protein concentration in the samples using BCA Protein Assay Kit (Pierce cat # 23227, Thermo Scientific, IL, USA) and used dilutions of BSA as the standard. On these lysates, Western blot analyses for myelin basic protein (MBP), myelin associated glycoprotein (MAG), GFAP, and decorin was performed. However, for Western blot analyses of brevican, neurcan, phosphacan, versican, aggrecan and NG2, we prepared ChABC-digested lysates. Briefly, rabbit forebrain slices of postnatal days 3, 7 and 14, were homogenized in lysis buffer (0.1 M Tris-HCl, pH 8.0), 10 mM DTT, 2% Lithium dodecyl sulfate and 5% Glycerol) and homogenate was centrifuged at 12,000 rpm for 30 min at 4°C. The protein concentration in the supernatant was determined using BCA Protein Assay Kit (Pierce cat # 23227, Thermo Scientific, IL, USA), similar to those of undigested lysates. 100 μ g Protein from each sample was incubated with ChABC (2U/ml; Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris (pH 8.0) with 0.3 M sodium acetate and 10% triton-x 100 for 2 hr at 37°C. After incubation, total protein was precipitated and re-dissolved in original lysis buffer with bromophenol blue. Total protein samples were separated by SDS-PAGE according to the previously described

method (Ballabh, et al., 2007). Equal amounts of protein (10–20 mcg) were loaded into 4–15% gradient precast gel (Biorad, CA, USA). The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane by electro-transfer. The membranes were then incubated with primary antibodies. We detected target proteins with chemiluminescence ECL system (Amersham) by using secondary antibodies conjugated with horseradish peroxidase (Jackson immunoresearch, PA, USA). We next stripped the blots with stripping buffer (Pierce) and incubated with β actin primary antibody followed by secondary antibody and detection with chemiluminescence ECL system. As described previously (Ballabh, et al., 2007), the blots from each experiment were densitometrically analyzed using J-image. We quantified the major molecular species as indicated in the images. The optical density values were normalized by taking ratio the target protein and β actin.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was performed as described previously (Ballabh, et al., 2007). Briefly, total RNA were isolated from 1 mm thick slice taken at the level of mid-septal nucleus of the forebrain using Mini RNA isolation kit (Roche, NJ). RNA was reverse-transcribed using Superscript II RT (Invitrogen, CA). Real-time reverse transcriptase-PCR was used to analyze mRNA expression using the Stratagene MX3000, GmbH (Bernried, Germany). Quantification was performed using the efficiency-corrected $\Delta\Delta CT$ method. The sequences of primers used for both rabbits are as follows: brevicin: 5'-TCTGCCCGCTATGCTTTCTCCTTT-3' (sense), 5'-CCAGCCAGCATCACATTGCTCATA-3' (antisense); neurocan: 5'-TGAAGGACCCACTGTGAATCCCAT-3' (sense), 5'-GGTGGTGCTTTTCGGCTTCTTCAA-3' (antisense); phosphacan 5'-GCGGAGGAGTTTCAGAAATGGTGT-3' (sense), 5'-ATCGGTCCGCATCAAAGCAGTAGA-3' (antisense); aggrecan: 5'-ACAGTGCCTATCAGGACAAGGTCT-3' (sense), 5'-ATGGAACACGATGCCTTTCACCAC-3' (antisense); Versican: 5'-TGCCTACTTTGCCACCCAGTTACA-3' (sense), 5'-TTTGGGCCACAAGGACAGTAGTCT-3' (antisense); NG2: 5'-CGTGGTCTTCGCTTTCACCAACTT-3' (sense) 5'-CCTCACGGTCACGTTACCA-3' (antisense); GAPDH AACGAATTTGGCTACAGC (sense), AGGGTACTTTATTGATGGTACAT (antisense).

Neurobehavioral assessment

We performed neurobehavioral evaluation of pups at postnatal day 14 as in previously described scoring protocol (Chua, 2008, Georgiadis, 2008). The assessment was performed by two physicians, who were blinded to the group assignment. We tested cranial nerves by testing smell (aversive response to ethanol), sucking, and swallowing (formula delivered by a bottle with small nipple). We scored the responses on a scale of 0–3, 0 being the worst and 3 the best response. Motor evaluation consisted of tone (modified Ashworth's scale), motor activity, locomotion at 30° angle, righting reflex, and gait. Tone was examined by active flexion and extension of fore- and hind-legs (score 0–3). The righting reflex was evaluated by the ability and rapidity to turn prone when placed in supine position. Sensory evaluation included touch on face (touching face with cotton swab) and extremities as well as pain on limbs (mild pin prick). Grading of tone, gait, and locomotion at 30° angle are described in the footnote of Table 2. To assess coordination and muscle strength in fore- and hind-legs, we evaluated the ability of the pups to hold their position on a ramp pitched at a slope of 60°. The test was conducted on a rectangular surface (18 X 6 inch) kept at 60° inclination. We placed the pup at the upper end of the inclination and measured the latency to slip down the slope. We performed the visual cliff test to test the vision.

Statistics and Analysis

Data are expressed as means and standard error of the mean (s.e.m.). To determine differences in the CSPG levels between pups with and without IVH at days 3, 7 and 14, 2-way analysis of variance was used. The independent factors in 2-way analysis of variance were postnatal age (d3 vs. d7 vs. 14) and presence of IVH (IVH vs. no IVH). To compare CSPG levels between human infants with and without IVH, in the cortex, white matter and germinal matrix, 2 way analyses of variance with repeated measures was used. The repeated measures were applied to the 3 white matter region—white matter, cortex and white matter. To assess the effect of ChABC at day 14 on MBP, MAG and GFAP, 3 group comparisons were done using one-way ANOVA. All post hoc comparisons to test for differences between means were done using Tukey multiple comparison test at the 0.05 significance level.

RESULTS

Brevican, neurocan, phosphacan, and aggrecan elevated in IVH

Validation of Western blot analyses for quantification of CSPGs—To identify and quantitate CSPGs in cerebral hemispheres, the tissues samples were denatured in lithium dodecyl sulfate and then treated with ChABC *in vitro* to remove GAG chain and generate core-proteins. The core-proteins readily transferred onto the PVDF membrane after ChABC digestion, unlike undigested proteins. The proteins on the membrane were quantified by staining procedures. A time course (0, 30, 60, 120 or 240 minutes) of *in vitro* digestion with ChABC showed that 2 hours of treatment was sufficient to recover about 95% or more of the CSPG core-protein (Supplemental Fig.1A, 1B; data shown for versican and neurocan). This demonstrated that our antibodies for CSPGs—neurocan, versican, aggrecan, phosphacan and NG2—detected core protein independent of the GAG chain in the Western blot analyses. Moreover, these antibodies detected CSPGs in undigested tissue sections in immunolabeling experiments indicating that the GAG chain does not interfere with the immunoreactivity.

Brevican—We quantified brevican levels in the forebrain of premature rabbit pups with IVH at postnatal days 3, 7 and 14 using Western blot analyses. We found that brevican [both isoforms 145 (kDa) and (75 kDa)] expression was higher in rabbit pups with IVH relative to pups without IVH (glycerol treated) at postnatal day 14 ($P < 0.01$), but not at days 3, 7 (Fig. 1A). Analyses of brevican expression as a functional of postnatal age revealed that brevican levels were higher at day 14 compared to days 3 and 7 among pups with IVH ($P = 0.002$, 0.036 respectively). However, brevican levels did not increase with advancing postnatal age in pups without IVH. Immunolabeling revealed that brevican was expressed primarily in the extracellular matrix. Brevican immunoreactivity was stronger in the periventricular regions of rabbit pups with IVH and weaker in controls without IVH (glycerol-treated) at postnatal day 14, but not at days 3 and 7 (Fig. 1B). However, brevican immunoreactivity was similar in the cortex and superficial white matter of pups with and without IVH at day 14, indicating that brevican was elevated only in brain regions adjacent to the hemorrhage. Double labeling of forebrain sections showed that immunoreactivity for brevican was strong on GFAP (+) astrocytes, weak on doublecortin (+) neurons and almost absent on Olig2 (+) cells of OL lineage in the periventricular region (Supplementary Fig. 2). Consistent with protein expression, brevican mRNA accumulation was higher in rabbit pups with IVH compared with controls without IVH at day 14 ($P < 0.05$), but not at days 1, 3 and 7 (Supplementary Fig. 3).

In agreement with the findings in rabbit pups, Western blot analyses of human brain samples showed that brevican [both isoforms 145 (kDa) and (75 kDa)] levels were higher in the germinal matrix of infants with IVH compared to infants without IVH ($P = 0.046$), but not in

the cortex or white matter (Fig. 1C). Similar to findings in rabbits, brevican immunoreactivity was more intense and abundant in the periventricular germinal matrix (SVZ and VZ) of premature human infants with IVH compared to controls without IVH (23–27 week gestational age, Fig. 1D). Brevican was expressed on a GFAP (+) astroglia, but not on doublecortin (+) neurons of the germinal matrix (Fig. 1E). Brevican immunoreactivity was almost absent on Olig2 (+) OL and Iba1 (+) microglia (data not shown). Together, IVH induced upregulation of brevican levels in the brain region around the lateral ventricles.

Neurocan—Western blot analyses revealed that neurocan [both neurocan 300 (kDa) and its proteolytic fragment (200 kDa)] levels were significantly higher in rabbit pups with IVH than controls without IVH at day 14 ($P=0.04$), but not at days 3 and 7. Neurocan levels did not change significantly with the advancing postnatal age in pups with or without IVH (Fig. 2A). Consistent with Western blot analyses, immunolabeling revealed that neurocan immunoreactivity was more abundant in the SVZ and the adjacent white matter of pups with IVH compared to controls without IVH at day 14, but not at earlier ages. Neurocan accumulated predominantly in the glial cells and extracellular matrix (Fig. 2B). Double immunolabeling demonstrated that neurocan was expressed extensively on GFAP (+) glial cells of the germinal matrix and weakly on doublecortin (+) neurons as well as on Olig2 (+) OL (Supplementary Fig. 2). Quantitative RT-PCR showed that neurocan mRNA expression was higher in pups with IVH than controls without IVH at day 7 ($P<0.05$), but not at days 1, 3 and 14 (Supplementary Fig. 3). Elevation in neurocan protein level at day 14 appears to follow an increase in gene transcription at day 7.

In human infants, Western blot analyses showed a trend towards higher level for neurocan in infants with IVH relative to controls without IVH, however the differences were not statistically significant (Fig 2C). Neurocan immuno-reactivity was more intense in the germinal matrix and adjacent white matter of IVH cases compared to controls without IVH (Fig. 2D). Similar to rabbits, neurocan was expressed in GFAP (+) glial cells and doublecortin (+) neurons of human infants (Fig. 2E), but not on Olig2 (+) OL (data not shown). Together, neurocan is expressed in the glial cells and extracellular matrix, and is elevated in the subventricular regions of both preterm infants and rabbit pups with IVH.

Phosphacan—Quantification of phosphacan levels by Western blot analyses showed that phosphacan [both isoforms 400 (kDa) and (250 kDa)] levels were significantly higher in pups with IVH compared to controls at day 14 ($P=0.004$), but not at days 3 and 7 (Fig. 3A). In addition, among pups with IVH, phosphacan levels were higher at day 14 relative to days 3 and 7 ($P=0.003$ and 0.007). Accordingly, immunolabeling revealed that phosphacan expression at day 14 was more extensive in the brain regions around the ventricle of pups with IVH compared to controls without IVH, but not in the cortex and superficial white matter (Fig. 3B). The phosphacan was expressed extensively in the extracellular matrix, doublecortin (+) neurons and GFAP (+) astrocytes of rabbit pups (Supplemental Fig. 2). In human infants, phosphacan immunoreactivity was also abundant on GFAP (+) astrocytes, but relatively scarce on doublecortin (+) neurons (Fig. 3D). Phosphacan was weak-to-absent on Olig2 (+) OL of both rabbits and humans (data on Olig2 not shown for humans). Quantification of phosphacan and aggrecan by Western blot analyses on human tissues could not performed satisfactorily with the commercially available antibodies. mRNA expression of phosphacan was comparable in the pups with and without IVH at all days (Supplemental Fig. 3), suggesting a non-transcriptional control of phosphacan levels in IVH.

Aggrecan—Aggrecan levels [both aggrecan isoforms 300–370 (kDa) and its proteolytic fragment (190 kDa)], measured by Western blot analyses, were significantly higher in rabbit pups with IVH compared to pups without IVH at day 14 ($P=0.024$), but not at days 3 and 7 (Fig. 4A). Immunostaining depicted that aggrecan was more abundantly expressed both in

the SVZ and adjacent white matter of rabbit pups and premature infants with IVH relative to controls without IVH (Fig. 4B and 4C). Aggrecan was expressed both on neural and glial cells as well as extracellular matrix of the forebrain. Aggrecan immunoreactivity was observed in GFAP (+) astrocytes, doublecortin (+) neurons and Olig2 (+) OL of the rabbit pups (Supplementary Fig. 4 and Fig. 4). In human infants, it was expressed abundantly on GFAP (+) glial cells, somewhat on doublecortin positive neurons, and Olig2 (+) cells but not on Iba1 (+) microglial cells (Fig. 4D; data on Olig2 and Iba1 not shown). Gene expression assay by quantitative RT-PCR showed that aggrecan mRNA levels were higher in pups with IVH than controls without IVH at day 7 ($P<0.05$), but not at days 1, 3 and 14 (Supplemental Fig. 3).

Together, brevican, neurocan, phosphacan, and aggrecan levels were elevated in rabbit pups with IVH at day 14 of postnatal age and human data showed a concurrent trend.

NG2 and Decorin reduced in IVH

To determine NG2 levels in rabbit pups with IVH vs. pups without IVH, we quantified NG2 (NG2 isoforms at 300-275 kDa) levels in their forebrain at postnatal days 3, 7, and 14 by Western blot analyses. We found that NG2 expression was reduced in pups with IVH than controls at day 14 ($P<0.001$), but not at days 3 and 7 (Fig. 5A). In addition, NG2 levels increased as a function of postnatal age in pups without IVH (day 14 vs. day 3 and 7: $P=0.025$, 0.012), in contrast to pups with IVH where levels did not change significantly. NG2 immunoreactivity was less extensive in the periventricular white matter of pups with IVH compared with controls at day 14 (Fig. 5B), indicating a failure of expansion of NG2 (+) glial cells with the development of IVH. Double labeling of brain sections from rabbit-pups also showed that NG2 is expressed on GFAP (+) glial cells and on the cell membrane and processes encircling Olig2⁺ nuclear labeling of OL progenitors and, consistent with previous study (Kucharova, et al.) 2011; Fig. 5 and Supplementary Fig. 4). NG2 mRNA accumulation were similar in pups with and without IVH (Supplementary Fig. 3), suggesting that NG2 expression is regulated post-transcriptionally.

In human infants, Western blot analyses exhibited trends for lower NG2 levels in the germinal matrix and white matter of infants with IVH relative to controls without IVH (Fig. 5C). However, the difference was not significant. Accordingly, immunohistochemistry demonstrated that NG2 immunoreactivity was less abundant in the germinal matrix and deep white matter of infants with IVH relative to controls without IVH (Fig. 5D). NG2 was expressed in GFAP (+) glial cells of the germinal matrix, Olig2 (+) cells of OL lineage, and blood vessels (Fig. 5E), but not on DCX (+) cells. Thus, in both rabbits and humans, NG2 levels were downregulated after the development of IVH relative to controls.

Decorin levels were assessed by Western blot analyses in rabbit pups. This showed that decorin expression was significantly reduced in pups with IVH relative to controls at days 7 and 14 ($P=0.023$ and 0.003), but not at day 3 (Supplementary Fig. 5A). A satisfactory immunolabeling could not be obtained with the use of commercially available antibodies. Reduced decorin could be attributed either to its degradation or consumption.

Versican expression exhibited a biphasic trend in IVH

Versican is highly expressed in the white matter of the CNS and is closely linked to myelination. Quantification by Western blot analyses showed that versican level (versican isoforms 300–400 kDa and 75 kDa) was reduced at day 7 ($P=0.002$), but elevated at day 14 in rabbit pups with IVH relative to controls without IVH ($P=0.04$, Fig. 6A). Consistent with these findings, versican immunoreactivity was more abundant around the ventricles in pups with IVH compared to controls without IVH at postnatal day 14 (Fig. 6B). Versican was

expressed predominantly in the extracellular matrix and sparsely on glial cells of the maturing brain. Double immunolabeling revealed that versican was expressed on periventricular GFAP (+) astrocytes and Olig 2 (+) OL, but not on Tuj1 (+) neurons (Supplemental Fig. 4). Versican mRNA levels were similar between pups with and without IVH except for a transient elevation at day 1 in pups with IVH (Supplemental Fig. 3).

In humans, versican protein levels, assayed on Western blot analyses, were greater in the white matter and germinal matrix of premature infants with IVH than in controls ($P < 0.003$, 0.034 respectively, Fig. 6C). Double immunostaining experiments demonstrated that versican was expressed on numerous GFAP (+) astrocytes, few Tuj1 (+) immature neurons, some Olig 2 (+) OL (Fig. 6E), consistent with previous studies on developing brain (Gu, et al., 2007). In conclusion, rabbit pups with IVH exhibited a reduction at day 7 and elevation at day 14 in versican levels compared with controls without IVH, while versican levels were elevated in the germinal matrix and white matter of pups with IVH.

***In vivo* Chondroitiase ABC degraded brevican, neurocan, aggrecan, but not NG2**

CSPGs consist of a core-protein and variable number GAG chains. *In vivo* ChABC treatment removes GAG chains; and allows the CSPG core protein to be removed by plasmin proteolytic system. Thus, we treated the rabbits pups with either 3 doses of intracerebroventricular ChABC (days 3, 5 and 7) or vehicle. We found that ChABC treatment reduced brevican, neurocan, aggrecan, and versican levels at day 14 ($P < 0.015$, 0.04 , 0.001 , 0.001 respectively, Fig 7). However, ChABC administration did not significantly affect NG2, phosphacan and decorin expression (Fig. 7 and Supplementary Fig. 5B). Since our antibodies identify the core-protein, significant reduction of CSPGs in ChABC treated pups compared to vehicle (Western blot analyses, Fig. 7) confirmed that most of the CSPG core-proteins were removed from the ECM in the treatment group. Consistent with Western blot analyses, the immunolabeling showed that neurocan and other CSPGs were significantly reduced in the periventricular white matter (data on neurocan shown, Supplementary Fig. 1). In conclusion, ChABC treatment led to the removal of not only the GAG chain, but also allowed clearing of the CSPG core-protein.

***In vivo* ChABC neither enhanced myelination nor reduced gliosis in pups with IVH**

ChABC treatment might promote myelination by enhancing axonal growth and interaction between axons, OL and growth factors. We, therefore, assessed myelination in three sets of P14 rabbit pups: (1) glycerol-treated pups without IVH; (2) vehicle-treated pups with IVH; (3) ICV ChABC-treated pups with IVH. Stereological analyses of immunolabeled sections revealed that the volume fraction (load) for myelin basic protein (MBP) in both corpus callosum and corona radiata was significantly reduced in pups with IVH relative to controls without IVH. However, MBP load was comparable between ChABC-treated and vehicle-treated pups with IVH (Fig. 8 A and B). Accordingly, Western blot analyses showed that there was no significant difference in the expression of MBP between ChABC-treated pups with IVH and vehicle-treated controls (Fig. 8 C, D). To further confirm the effect of ChABC treatment on myelination, we compared the expression of myelin associated glycoprotein (MAG)—another key component of myelin—between pups without IVH, ChABC-treated pups with IVH, vehicle-treated controls with IVH (Fig. 8 C, E). MAG levels were reduced in pups with IVH compared to controls without IVH, but ChABC treatment did not enhance MAG expression in pups with IVH. To confirm whether ChABC treatment enhances myelination at postnatal day 21, we compared the expression of MBP and MAG between ChABC treated and vehicle treated pups with IVH. There was no significant difference between the two groups in these protein levels on Western blot analyses (Supplemental Fig. 6).

We next compared gliosis among three sets of P14 rabbit pups: 1) pups without IVH, 2) ChABC-treated pups with IVH, and 3) vehicle-treated controls with IVH. GFAP-labeled brain sections were assessed by unbiased stereology; and quantification of GFAP in homogenates from brain slice (midseptal nucleus level) was performed by Western blot analyses (Fig. 9 A, B, C). Both the methods revealed that GFAP levels were greater in pups with IVH compared to controls without IVH. However, there was no significant difference between the ChABC-treated and vehicle-treated pups with IVH. Together, ChABC treatment neither enhanced myelination nor reduced gliosis in rabbit pups with IVH.

To determine whether ChABC inhibits myelination, we treated healthy E29 rabbit pups with 20 μ l ChABC or vehicle at days 2, 4 and 6. We compared myelin basic protein in the two groups using Western blot analyses and found the levels to be comparable (data not shown). This suggests that ICV ChABC treatment does not inhibit myelination in healthy pups.

No effect of Chondroitinase ABC on proliferation and maturation of cells of oligodendrocyte lineage

Inability of OL to myelinate can be attributed to multiple reasons, including failure in OL proliferation, differentiation, or interaction between neurons and OL. Studies in culture experiments have shown that ChABC treatment enhances proliferation of cells of OL lineage (Gu, Li 2009). Importantly, ChABC treatment enhances migration of OL, activates formation of OL process outgrowth, and breaks the barrier between axons and OL. Therefore, we sought to assess the effect of ChABC on proliferation and maturation of OL.

To this end, we double labeled brain sections with Ki67 and Olig2 antibodies, and quantified Ki67 (+) and both Olig2 and Ki67 positive cells in ChABC-treated pups with IVH compared with vehicle-treated controls. We found that ChABC treatment affects neither the density of proliferating Olig2 (+) cells nor the number of total proliferating neural cells (Fig. 10A & B). Quantification of Olig2 (+) cells revealed that Olig2 (+) cells displayed a trend for higher expression in corona radiata and corpus callosum of ChABC treated pups compared to vehicle controls ($P < 0.05$), however the difference was not significant.

We next double labeled the brain sections with O4 and APC antibodies and quantified these cells in corpus callosum and corona radiata of ChABC treated pups compared to vehicle-treated controls. We found that the density O4 (+) and APC (+) cells were comparable between the two groups. In addition, pre-OL ($O4^+APC^-$) were also similar between the two sets of animals (Fig. 10 C). This suggests that ChABC treatment did not enhance proliferation and maturation of cells of OL lineage.

***In vivo* Chondroitinase ABC did not enhance clinical recovery**

To determine whether ChABC treatment alleviates neurological impairment, we performed a neurobehavioral evaluation among three sets of premature rabbit pups at postnatal day 14 based on our previously described scoring system (Chua et al., 2009): (1) glycerol-treated pups without IVH; (2) pups with IVH treated with ICV ChABC; and (3) pups with IVH treated with ICV vehicle (Table 2). The severity of IVH, measured by head ultrasound, in ChABC-treated pups was similar to those of vehicle-treated controls. We observed significant weakness in the hind leg of 3 ChABC-treated and 2 vehicle-treated pups with IVH. The weakness resulted in adduction, internal rotation of back legs and significant asymmetry in gait. The mean score for gait was similar in the ChABC- and vehicle-treated pups with IVH. The motor activity of back legs and ability to lift the rear end, although significantly less than pups without IVH, were also similar in the two groups of pups with IVH. The latency to slip down a ramp pitched at a slope of 60° also was comparable between ChABC- and vehicle- treated pups with IVH. There was no significant difference in

the tests evaluating cranial nerves in the three sets of rabbit pups. Importantly, we did not observe any apparent adverse effect attributable to ChABC treatment in this group of animals.

***In vivo* Chondroitinase ABC did not reduce ventriculomegaly**

The cross-sectional areas of the ventricles were measured in Nissl-stained brain sections (at 2 coronal levels: midseptal–nucleus and ventral posterolateral nucleus of the thalamus) of ChABC- and vehicle-treated pups with IVH. There was no significant difference in the ventricle size between the two groups (midseptal nucleus level: 6.09 ± 1.75 vs. 4.76 ± 0.9 mm²; posterolateral nucleus of thalamus level: 26.34 ± 4.4 vs. 24.9 ± 7.1 mm²). On a separate subset of pups, we observed that ICV injection of ChABC did not significantly relieve hydrocephalus compared to controls which did not receive ICV injection. This was probably because of thin bore of the needle used (27gauge), administration of ChABC over short period (2 minute) and no ICV treatment given between day 7–14. Together, ChABC treatment did not reduce ventriculomegaly in premature rabbit pups with IVH.

DISCUSSION

CSPGs constitute a major component of ECM and are elevated in animal models of brain injury. CSPGs inhibit migration, formation of process-outgrowth, and differentiation of OL progenitor cells. Compositional changes in ECM in models of brain injury also impact movement of extracellular fluid in the brain that might contribute to hydrocephalus. Thus, we determined whether CSPG levels were elevated in rabbit pups with IVH and if degradation of CSPGs by ChABC treatment would enhance OL maturation, myelination, and clinical recovery in treated pups compared with controls. We found that CSPGs, including brevican, neurocan, aggrecan, phosphacan and versican were elevated, whereas NG2 levels were reduced in pups with IVH compared to controls without IVH. However, degradation of CSPGs by *in vivo* ChABC treatment neither promoted myelination nor enhanced neurologic recovery in pups with IVH. Moreover, the ChABC treatment did not reduce gliosis and ventriculomegaly in pups with IVH.

In the present study, CSPGs, including brevican, neurocan, aggrecan and phosphacan, were elevated at day 14 in rabbit pups with IVH compared to controls without IVH. Accordingly, expressions of these CSPGs were higher in the SVZ and deep white matter of human infants with IVH compared with controls without IVH. To our knowledge, CSPGs have not been studied in premature infants or any animal model of IVH of prematurity. Studies in neonatal rat models of hypoxia-ischemia have revealed that brevican, phosphacan and neurocan levels increase in the cerebral hemisphere after a few days of the insult (Leonardo, et al., 2008, Matsui, et al., 2005, Shen, et al., 2008). However, brevican expression exhibits an initial decrease during the first 4 days of hypoxic-ischemic insult with a subsequent increase, unlike phosphacan and neurocan (Leonardo, et al., 2008). In adult rat model of spinal cord injury, brevican, neurocan, aggrecan and phosphacan levels increase within days after spinal cord injury, peaking at day 14 and remain high for at least 2 months (Jones, et al., 2003, Tang, et al., 2003). Animal models of traumatic and inflammatory brain injury also display in a similar elevation in CSPG levels after the insult (Harris, et al., 2009, Rauch, 2004). Our real-time qPCR studies demonstrated that elevation in protein levels of CSPGs—phosphacan, versican, aggrecan and neurocan—did not precisely coincide with the upregulation in their transcription, suggesting involvement of other post-transcriptional or post-translational mechanisms (Gris, et al., 2007). Together, brevican, neurocan, aggrecan and phosphacan levels significantly increase in preterm human infants and rabbit pups with IVH, similar to other models of brain injury.

We demonstrated that versican level exhibited a biphasic change—a decrease at day 3 and 7, and subsequent increase at day 14 in animals with IVH. Interestingly, versican expression displays a similar trend—an initial reduction during first 2–3 weeks with subsequent elevation—in neonatal model of hypoxia-ischemia (Leonardo, et al., 2008). However, versican levels are consistently reduced for an extended period in adult rat model of spinal cord injury (Tang, et al., 2003). Given that versican is predominantly produced by glial cells and their progenitors, a reduction in versican level indicates injury of these cells. An elevation in versican level after an initial decline suggests an initial injury with subsequent proliferation and hypertrophy of cells of glial—OL and astrocyte—lineage. In contrast to other CSPGs, NG2 levels were consistently reduced at both days 7 and 14 in pups with IVH compared to controls. This is consistent with the findings in the neonatal rat model of hypoxia-ischemia (Aya-ay, et al., 2005). However, NG2 levels are elevated in adult rat models of spinal cord injury (Jones, et al., 2002, Tang, et al., 2003). The exact mechanism of depletion in NG2 in our model of IVH is unclear, however a plausible explanation would be reduced production by the damaged cells of OL lineage. Collectively, IVH results in a reduction of NG2 expression and a biphasic change in levels of versican, which is similar to other models of neonatal brain injury.

In vivo ChABC treatment results in cleavage of GAG chain, and generation of the deglycosylated core-protein in the ECM. In brain, tissue plasminogen activator (tPA) is secreted by the neurons and microglia, which are activated by nonproteolytic pathway to eliminate the core-protein from the matrix (Bukhari, et al., 2011, Nolin, et al., 2008, Tsirka, et al., 1997). Thus, *in vivo* ChABC not only cleaves GAG chain, but also allows tPA to clear the CSPG core-protein. We demonstrated that antibodies used in the present experiment identify CSPGs independent of the GAG chain, and that intracerebroventricular ChABC treatment significantly cleared CSPGs from the periventricular brain in both immunohistochemistry and Western blot analyses. We also showed that ChABC treatment does not interfere with myelination in healthy rabbit pups. Together, we confirmed that ChABC digested and significantly cleared CSPGs from the forebrain of rabbit pups with IVH, and that ChABC treatment did not hinder myelination in healthy pups. These findings are critical to our conclusion that degradation of CSPGs by ChABC treatment did not enhance maturation of OL and myelination in pups with IVH.

In our preterm rabbit model, the onset of IVH induces apoptosis, reduces proliferation, and arrests maturation of oligodendrocytes (Dummula, et al., 2011). We expected that degradation of CSPGs would enhance maturation of OL and myelination. However, we found that *in vivo* ChABC treatment did not enhance differentiation of OL, subsequent myelination and neurological recovery in rabbit pups with IVH. This was unanticipated as both *in vitro* and *in vivo* studies have shown that ChABC treatment enhances migration, formation of process outgrowth and differentiation of OL (Fok-Seang, et al., 1995, Levine, et al., 2001, Siebert and Osterhout, 2011, Siebert, et al., 2011). Moreover, ChABC is known to improve plasticity, enhance axonal regeneration and thereby promote clinical recovery in animal models of spinal cord and nigrostriatal tract injury (Bradbury, et al., 2002, Garcia-Alias, et al., , Moon, et al., 2001). In our model, we did not observe significant clinical recovery in ChABC treated pups compared to controls without IVH. A possible explanation could be that axons are relatively preserved in pups with IVH (Chua, 2008) and CSPG may not be a significant inhibitor of OL maturation and myelination. ChABC also did not impact OL proliferation in rabbit pups with IVH. This is in agreement with an *in vivo* study on adult rat model of spinal cord injury (Siebert, et al., 2011). However, ChABC enhances proliferation of neuronal progenitor cell in culture experiments. (Gu, et al., 2009). Taken together, degradation of CSPGs by ChABC, even though promotes axonal regeneration in other animal models, does not promote OL maturation and subsequent myelination in rabbit pups with IVH, and by extension in premature infants with IVH.

Another key finding of our study was that ChABC treatment did not reduce hydrocephalus. Studies on animal model of hydrocephalus have shown that there is increased tissue density and reduction in extracellular compartment in the cerebral cortex of these brains (Del Bigio and Bruni, 1987, Grasso, et al., 2002, Tamaki, et al., 1990). In addition, MR imaging has demonstrated a reduction in flow of extracellular fluid in the cerebral cortex of rat model of kaolin-induced hydrocephalus (Massicotte, et al., 2000, Shoesmith, et al., 2000, Sykova, et al., 2001). This indicates that there are compositional changes in the extracellular compartment that might contribute to hydrocephalus. Hence, we expected that degradation of CSPGs would enhance extracellular fluid movement and consequent CSF absorption, resulting in restoration of ventricle size. However, no significant reduction in ventricle size was observed. This suggests that either CSPGs do not contribute to flow of extracellular fluid in the cerebral cortex of the developing brain, or an alternate route of CSF circulation—lymphatics or ventricular system—is more important for CSF flow than movement of extracellular fluid in the brain.

Conclusion

The development of IVH elevated neurocan, brevican, versican, aggrecan, phosphacan levels and reduced NG2 expression in rabbit pups and infants with IVH; and degradation of CSPGs by *in vivo* ChABC treatment did not promote differentiation of OL, myelination and clinical recovery in the survivors. Moreover, ChABC treatment did not reduce gliosis and ventriculomegaly in these infants. The study refutes the speculation of several *in vitro* and *in vivo* studies that ChABC treatment might enhance myelination and clinical recovery in brain injuries. However, *in vivo* ChABC might be effective in enhancing myelination in brain injury paradigms involving significant axonal injury, unlike our preterm rabbit model of IVH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

1. Intraventricular hemorrhage (IVH) induces deposition of proteoglycans in cerebrum
2. Intracerebroventricular chondroitinase degrades cerebral proteoglycans in IVH
3. Contrary to the current notion, degradation of proteoglycans does not enhance myelination
4. Chondroitinase ABC treatment does not promote clinical recovery in IVH
5. Chondroitinase does not reduce gliosis or ventriculomegaly in IVH

Figure 1. Brevican expression elevated in both rabbit pups and human infants with IVH

A) Representative Western blot analyses for brevicin in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7, and 14. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values are normalized to β actin levels. Bar chart shows mean $\pm$ s.e.m (n=6 each). Brevican (145 and 75 kDa) levels were higher in pups with IVH than without IVH at day 14. *P<0.05 for the comparison between pups with and without IVH. B) Representative immunofluorescence of cryosections from 14 day old E29 pups with and without IVH labeled for brevicin specific antibody. Note higher expression of brevicin in the brain region around the lateral ventricle of rabbit pups with IVH compared with controls without IVH. Scale bar, 40 μ m. C) Representative Western blot analyses for brevicin in human premature infants with and without IVH. Values are normalized to β actin levels. The bars are mean $\pm$ s.e.m (n = 4 each group). Brevican levels were higher in the germinal matrix of infants with IVH compared to infants without IVH. *P<0.05 for the comparison of germinal matrix between pups with and without IVH. D) Cryosection from 23 week premature infant (postnatal age 10–15 days) labeled with brevicin antibody. Note higher immunoreactivity for brevicin in VZ and SVZ of infants with IVH than controls without IVH. E) Representative immunofluorescence of cryosections from 23 week premature infant with IVH double-labeled with brevicin (red) and DCX or GFAP (green) specific antibodies. Note brevicin expression co-localized with several GFAP (+) astrocytes (arrowhead), but not with DCX (+) neurons of the SVZ. Inset shows immunolabeling under high magnification from a brain region in the SVZ. Scale bar, 40 μ m.

Figure 2. IVH induces elevation of neurocan in premature rabbits and humans

A) Representative Western blot analyses for neurocan in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7, and 14. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values were normalized to β actin levels. Data are mean $\pm$ s.e.m (n=6 each). Neurocan (300 and 200 kDa) levels were higher in pups with IVH than without IVH at day 14. *P<0.05 for the comparison between pups with and without IVH. B) Cryosections from E29 pups of 14 day postnatal age were labeled for neurocan specific antibody. Note higher immunoreactivity of neurocan in the periventricular region of rabbit pups with IVH compared with controls without IVH. Scale bar, 40 μ m. C) Representative Western blot analyses for neurocan in human premature infants with and without IVH. Values are normalized to β actin levels. Data are mean $\pm$ s.e.m (n = 4 each group). Neurocan levels show a trend for higher levels in the three brain region of infants with IVH than controls without IVH. D) Representative immunofluorescence of cryosections from 23 week premature infant (postnatal age 10–15 days) labeled with neurocan antibody. Note higher immunoreactivity for neurocan in VZ and SVZ of infants with IVH relative to controls without IVH. E) Cryosections from 23 week premature infant were double-labeled with neurocan (red) and DCX or GFAP (green) specific antibodies. Note neurocan immunoreactivity co-localized with numerous GFAP (+) astrocytes (arrow), and DCX (+) neurons (arrow). Inset shows immunolabeling under high magnification from a brain region in the SVZ. Scale bar, 40 μ m

Figure 3. Phosphacan expression higher in IVH than controls without IVH

A) Representative Western blot analyses for phosphacan in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7, and 14. Each lane indicates lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values are normalized to β actin levels. Bar chart shows mean $\pm$ s.e.m (n=6 each). Phosphacan (400 and 250 kDa) levels were higher in pups with than without IVH at day 14. *P<0.05 for the comparison between pups with and without IVH. B) Typical immunofluorescence of phosphacan in subventricular zone of 14 day old E29 pups with and without IVH. Note higher expression of phosphacan in the brain region around the lateral ventricle of rabbit pups with IVH compared with controls without IVH. Scale bar, 40 μ m. C) Cryosection from 23 week premature infant (postnatal age 10–15 days) immunolabeled with phosphacan antibody. Note higher expression of phosphacan in VZ and SVZ of infants with IVH than controls without IVH. D) Typical appearance of phosphacan in the VZ and SVZ of 23 week premature infant with IVH double-labeled with phosphacan (red) and DCX or GFAP (green) specific antibodies. Note phosphacan expressed extensively on GFAP (+) astrocytes (arrowhead) and moderately on DCX (+) neurons (arrowhead). Inset shows immunolabeling under high magnification from a brain region in the SVZ. Scale bar, 40 μ m.

Figure 4. IVH induces elevation of aggrecan in both premature rabbits and humans

A) Representative western blot analyses for aggrecan in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7 and 14. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values are normalized to β actin levels. Bar chart shows mean $\pm$ s.e.m (n=6 each). Aggrecan (300–370 kDa) levels were higher in pups with than without IVH at day 14. *P<0.05 for the comparison between pups with and without IVH. B) Typical appearance of aggrecan in subventricular zone of 14 day old E29 pups with and without IVH. Note higher immunoreactivity of aggrecan in the periventricular brain region of rabbit pups with IVH compared with controls without IVH. Scale bar, 40 μ m. C) Representative immunofluorescence of cryosections from 23 week premature infant (postnatal age 10–15 days) labeled with aggrecan antibody. Note higher expression of aggrecan in VZ and SVZ of infants with IVH relative to controls without IVH. D) Typical appearance of aggrecan in the VZ and SVZ of 23 week premature infant with IVH double-labeled with aggrecan (red) and DCX or GFAP (green) specific antibodies. Note aggrecan is expressed on several GFAP (+) astrocytes (arrow) and few DCX (+) neurons (arrowhead). Inset shows immunolabeling under high magnification from a brain region in the SVZ. Scale bar, 40 μ m.

Figure 5. NG2 expression reduced in both rabbit pups and human infants with IVH

A) Representative Western blot analyses for NG2 in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7, and 14. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values were normalized to β actin levels. Data are mean $\pm$ s.e.m (n=6 each). NG2 (275–300 kDa) levels were less in pups with IVH than without IVH at day 14. * $P < 0.05$ for the comparison between pups with and without IVH. B) Cryosections from E29 pups of 14 day postnatal age were labeled for NG2 specific antibody. Note less extensive NG2 immunoreactivity in the periventricular region of pups with IVH compared with controls without IVH. Scale bar, 40 μ m. C) Representative Western blot analyses for NG2 in human premature infants with and without IVH. Values are normalized to β actin levels. Data are mean $\pm$ s.e.m (n = 4 each). NG2 levels showed a trend for lower levels in the germinal matrix of infants with IVH than controls without IVH. D) Representative immunofluorescence of cryosections from 23 week premature infant (postnatal age 10–15 days) labeled with NG2 antibody. Note a typical NG2 immunoreactivity in glial cells (arrowhead) and blood vessels (arrow) in the VZ and SVZ of infants with and without IVH. E) Cryosections from 23 week premature infant were double-labeled with NG2 (red) and Olig2 or GFAP (green) specific antibodies. Note NG2 immunoreactivity co-localized with Olig2 (+) cells and GFAP (+) astrocytes (arrowhead). Inset shows immunolabeling under high magnification from a brain region in the SVZ. Scale bar, 40 μ m.

Figure 6. Versican expression exhibits a biphasic change in rabbit pups with IVH

A) Typical Western blot analyses for versican in the forebrain of premature rabbit pups with and without IVH at postnatal days 3, 7 and 14. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values are normalized to β actin levels. Bar chart shows mean $\pm$ s.e.m (n=6 each). Versican (300–400 and 75 kDa) levels were lower at day 7 and higher at day 14 in pups with IVH compared with controls without IVH. *P<0.05 for the comparison between pups with and without IVH. B) Typical appearance of versican in subventricular zone of 14 day old E29 pups with and without IVH. Note higher immunoreactivity of versican in the periventricular brain region of rabbit pups with IVH relative to controls without IVH. Scale bar, 40 μ m. C) Representative Western blot analyses for versican in human premature infants with and without IVH. Values are normalized to β actin levels. Data are mean $\pm$ s.e.m (n = 4 each). Versican levels were higher levels in the germinal matrix and white matter of infants with IVH than controls without IVH. *P<0.05 for the comparison between germinal matrix of infants with and without IVH. ##P<0.01 for the comparison white matter of infants with and without IVH. D) Representative immunofluorescence of cryosections from 23 week premature infant (postnatal age 10–15 days) labeled with versican antibody. Note higher expression of versican in VZ and SVZ of infants with IVH relative to controls without IVH. E) Typical appearance of versican in the VZ and SVZ of 23 week premature infant with IVH double-labeled with versican (red) and β tubulin (Tuj 1) or GFAP (green) specific antibodies. Note versican is expressed in ECM and on several GFAP (+) astrocytes (arrow) and few Tuj1 (+) neurons. Scale bar, 40 μ m.

Figure 7. ChABC treatment degrades brevican, neurocan, phosphacan, aggrecan, but not NG2
Representative Western blot analyses for brevican, neurocan, versican, aggrecan and NG2 in the forebrain of vehicle and ChABC treated pups with IVH, and pups without IVH. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates undigested lysate from one brain. Values were normalized to β actin levels. Data are mean $\pm$ s.e.m (n=5 each). Brevican, neurocan, aggrecan and versican levels were reduced in ChABC treated pups compared with vehicle treated controls. However, ChABC treatment did not reduce NG2 level significantly. * $P < 0.05$, *** $P < 0.001$ for the comparison between pups with and without IVH.

Figure 8. ChABC treatment does not enhance myelination

A) Representative immunofluorescence of cryosections from 14 day old pups labeled with myelin basic protein (MBP) specific antibody. Note reduced immuno-reactivity to MBP in periventricular zone (corona radiata) of pups with IVH than controls without IVH and no difference in expression of myelin basic protein between ChABC- and vehicle treated pups with IVH. The bars are mean $\pm$ s.e.m (n = 5–6 pups each time point). Scale bar=100 μm. B) Volume fraction of MBP was similar in corona radiata (CR) and corpus callosum (CC) of ChABC-treated and vehicle-treated pups with IVH. Volume fraction of MBP was significantly higher in pups without IVH compared to pups with IVH in both brain regions. *P<0.05 for the comparison between pups with and without IVH. C) Representative Western blot analyses for MBP and MAG in the forebrain of premature rabbit pups in 3 sets of pups as indicated. A healthy rat brain used as positive control. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. Arrow indicates rat brain lysate taken as positive control. D) The bars are mean $\pm$ s.e.m (n = 5–6 each group). MBP expression was comparable in ChABC-treated and vehicle treated pups with IVH. E) The data in the bar diagram are mean $\pm$ s.e.m (n = 5 each group). MAG expression was similar in ChABC-treated and vehicle-treated pups with hemorrhage. #P<0.05 for the comparison between vehicle treated pups with IVH and pups without IVH.

Figure 9. ChABC- treatment does not affect gliosis

A) Representative labeling of cryosections from 14 day old rabbit pups labeled with GFAP antibody. Note abundant abundant hypertrophic astrocytes—with large cell body and numerous processes making a dense network—in the periventricular zone of pups with IVH (arrow) and no change in these astrocytes in ChABC treated pups. Inset shows hypertrophic astrocytes. B) The bars are mean $\pm$ s.e.m (n = 5–6 each group). The total volume fraction of astrocyte cell body and their fibers on stereological evaluation were comparable in ChABC- and vehicle-treated pups with IVH in corona radiata and corpus callosum. *P<0.05 for the comparison between vehicle treated pups with IVH and pups without IVH. C) Representative Western blot analyses for GFAP in the forebrain of premature rabbit pups in 4 groups of pups as indicated. Arrow indicates rat brain lysate taken as positive control. D) The data are mean $\pm$ s.e.m (n = 5 each group). A healthy rat brain used as positive control. Each lane represents lysate from whole coronal slice taken at the level of midseptal nucleus of one brain. GFAP expression was similar in ChABC-treated and vehicle-treated pups. *P<0.05 for the comparison between vehicle treated pups with IVH and pups without IVH. Scale bar=25 μ m.

Figure 10. ChABC treatment does not affect proliferation and maturation of OL progenitors
A) The bar graph depict mean $\pm$ s.e.m (n = 5 each). The density of proliferating Olig2 cells (Ki67+, Olig2+) in VZ and SVZ was similar in ChABC- and vehicle-treated pups with IVH at day 7. The total number of proliferating cells (Ki67+) in VZ and SVZ was also comparable in the two comparison groups. B) The bar graph depict mean $\pm$ s.e.m (n = 5 each). The density of Olig2 cells in corona radiata and corpus callosum at day 7 and 14 were similar. At day 14, Olig2 cells were comparable in corona radiata and corpus callosum of ChABC treated pups compared to vehicle controls. C) Representative double labeling with O4 (red) and APC (green) antibodies of cryosections from 7 day old pup. Note that O4(+) APC(+) (arrowhead) and O4(+) APC(-) OL (arrow) are comparable in density between vehicle and ChABC treated pups with IVH. Scale bar, 25 μ m. The data are mean $\pm$ s.e.m. All the three--O4 (+), APC(+), and O4(+)APC(-) cells--were similar in density between vehicle and ChABC treated pups with IVH.

Table 1

Characteristics of human infants with and without IVH

Postconceptional age (weeks)	sex	Birth weight (Kg)	IVH / No IVH	Cause of death
64-26	Male	0.810	IVH grade 3	Clinical sepsis
74-23	Male	0.57	IVH grade 2	Clinical sepsis
65-23	Female	0.58	grade 3 IVH	Respiratory failure
86-24	Male	0.64	grade 4 IVH	Pulmonary hemorrhage
66-23	Male	0.45	no IVH	RDS, Respiratory failure
61-24	Male	0.61	no IVH	Clinical sepsis
83-25	Male	0.73	no IVH	Metabolic acidosis, resp. failure
63-27	Female	0.56	no IVH	Respiratory failure

Table 2

Neurobehavioral evaluation of ChABC-treated pups compared to vehicle-treated controls with IVH and pups without IVH at postnatal day 14

System	Test	No IVH (n=14)	IVH-ChABC (n=14)	IVH-Vehicle (n=13)
Cranial Nerve	Aversive response to alcohol	3 (3,3)	3 (3,3)	3 (3,3)
	Sucking and Swallowing	3 (3,3)	3 (3,3)	3 (3,3)
	Vision	3 (3,3)	3 (3,3)	3 (3,3)
Motor	Motor activity			
	Head	3 (3,3)	3 (3,3)	3 (3,3)
	Fore Legs	3 (3,3)	3 (3,3)	3 (3,3)
	Hind Legs	3 (3,3)	3 (3,3)	3 (2,3)*
	Righting reflex ^a	5 (5,5)	5 (3,5)	3 (3,5)
	Locomotion on 30° inclination ^b	3 (3,3)	3 (3,3)	3 (2,3)
	Tone ^c : Forelimb	0 (0,0)	0 (0,0)	0 (0,0)
	Tone: Hind Limb	0 (0,0)	0 (0,0)	0 (0,0)
	Inability to hold their position at 60° inclination for 15 seconds or more (latency to slip down the slope if <15 seconds)	0	23	28.6
	Inability to walk less than 40 inches in one minute (%)	0	21	16
	Pups with falls during more than 5% of steps on walking (%) rolls over	0%	28%	16%
	Gait^d	4 (4,4)	4 (2.5,4)	3.5 (3, 4)
	Motor Impairment			
	Weakness in extremities, %	0%	21%	16%
Sensory	Facial touch	3 (3,3)	3 (3,3)	3 (3,3)
	Pain	3 (3,3)	3 (3,3)	3 (3,3)

Values are median and interquartile range. Zero is the worst response and 3 is the best response.

^aScore (range, 1–5): no. of times turns prone within 2 seconds when placed in supine out of 5 tries.

^bScore (range, 0–3) 0, does not walk; 1, takes a few steps (less than 8 inches); 2, walks for 9–18 inches; 3, walks very well beyond 18 inches.

^cScore (range, 1–3): 0, no increase in tone; 1, slight increase in tone; 2, considerable increase in tone; 3, limb rigid in flexion or extension.

^dGait was graded as 0 (no locomotion), 1 (crawls with trunk touching the ground for few steps and then rolls over), 2 (walks taking alternate steps, trunk low and cannot walk on inclined surface), 3 (walks taking alternate steps, cannot propel its body using synchronously the hind legs, but walks on 30° inclined surface), 4 (walks, runs, and jumps without restriction, propels the body using synchronously the back legs, but limitation in speed, balance, and coordination manifesting as clumsiness in gait), or 5 (normal walking).