Neurochemical Research (2021) 46:595–610
https://doi.org/10.1007/s11064-020-03195-9

ORIGINAL PAPER

Characterization of Glycosaminoglycan Disaccharide Composition in Astrocyte Primary Cultures and the Cortex of Neonatal Rats

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Received: 27 May 2020 / Revised: 12 November 2020 / Accepted: 4 December 2020 / Published online: 4 January 2021

Abstract

Astrocytes are major producers of the extracellular matrix (ECM), which is involved in the plasticity of the developing brain. *In utero* alcohol exposure alters neuronal plasticity. Glycosaminoglycans (GAGs) are a family of polysaccharides present in the extracellular space; chondroitin sulfate (CS)- and heparan sulfate (HS)-GAGs are covalently bound to core proteins to form proteoglycans (PGs). Hyaluronic acid (HA)-GAGs are not bound to core proteins. In this study we investigated the contribution of astrocytes to CS-, HS-, and HA-GAG production by comparing the makeup of these GAGs in cortical astrocyte cultures and the neonatal rat cortex. We also explored alterations induced by ethanol in GAG and core protein levels in astrocytes. Finally, we investigated the relative expression in astrocytes of CS-PGs of the lectican family of proteins, major components of the brain ECM, *in vivo* using translating ribosome affinity purification (TRAP) (in Aldh1l1-EGFP-Rpl10a mice. Cortical astrocytes produce low levels of HA and show low expression of genes involved in HA biosynthesis compared to the whole developing cortex. Astrocytes have high levels of chondroitin-0-sulfate (C0S)-GAGs (possibly because of a higher sulfatase enzyme expression) and HS-GAGs. Ethanol upregulates C4S-GAGs as well as brain-specific lecticans neurocan and brevican, which are highly enriched in astrocytes of the developing cortex *in vivo*. These results begin to elucidate the role of astrocytes in the biosynthesis of CS- HS- and HA-GAGs, and suggest that ethanol-induced alterations of neuronal development may be in part mediated by increased astrocyte GAG levels and neurocan and brevican expression.

Keywords Astrocytes · Chondroitin sulfate (CS) · Ethanol · Glycosaminoglycans (GAGs) · Heparan sulfate (HS) · Lecticans

Abbreviations

AMAC	2-Aminoacridone
ARSB	Arylsulfatase B
CS	Chondroitin Sulfate
ECM	Extracellular Matrix
FASD	Fetal Alcohol Spectrum Disorders
GAGs	Glycosaminoglycans
GALNS	Galactosamine (*N*-acetyl)-6 Sulfatase
HA	Hyaluronic Acid
HS	Heparan Sulfate
NDST	*N*-Deaceylase/*N*-sulfotransferases
PD	Postnatal Day
PGs	Proteoglycans
RRID	Research Resource Identifier
SUMF1	Sulfatase Modifying Factor 1
TRAP	Translating ribosome affinity purification

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11064-020-03195-9.

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Introduction

Neurons, astrocytes, and other brain cells are present in the brain parenchyma in close proximity, separated by the extracellular space, comprised of a highly organized extracellular matrix (ECM) [1]. The extracellular space accounts

for approximately 20% of the total volume of the mature rat brain and approximately 40% of the rat neonatal brain [2, 3].

During brain development, the ECM modulates cell proliferation, cell migration, the growth of dendrites and axons, and the formation of synapses, while in the adult brain the ECM provides stabilizing and structural support, interacts with membrane receptors, and modulates synaptic activity and plasticity [4, 5]. The ECM is highly dynamic and rapidly undergoes remodeling with ECM components being deposited, degraded, and modified [4, 5] and contributes to the molecular signals regulating neuronal plasticity, with ECM components both facilitating or inhibiting plasticity [6–8].

The brain extracellular space contains high levels of glycosaminoglycans (GAGs). These long, unbranched polysaccharides, consisting of repeating disaccharide units present on the cell surface and in the ECM, play major, though not yet fully elucidated, roles in modulating ECM functions both in the developing and adult brain [9]. Three major forms of GAGs that have been found to be highly relevant to neuronal plasticity are chondroitin sulfate (CS-GAGs), heparan sulfate (HS-GAGs), and hyaluronic acid (HA) [9–11].

CS-GAGs are covalently bound to core-proteins in the form of glycoconjugates called proteoglycans (PGs) and are formed by repeated glucuronic acid (or iduronic acid in the case of C4S-type B) and *N*-acetylgalactosamine disaccharides modified by sulfation in positions 2, 4, and/or 6, with the most common modifications in the brain at position 4 (C4S) and 6 (C6S) [12]. CS-PGs of the lectican family (neurocan, brevican, versican, and aggrecan) are the most abundant proteins of the CNS ECM and are characterized by the presence of binding sites for HA, ECM proteins, membrane proteins, and growth factors [13]. Lecticans are highly expressed in the developing brain where their presence in a given location constitutes a barrier to cell migration [14] and to the growth of axons and dendrites [6]. In the mature brain, lecticans are the major components of the perineuronal net, a highly condensed matrix responsible, in part, for the reduced plasticity of the mature brain [15, 16]. After CNS injury, reactive astrocytes upregulate the release of lecticans, which represent the major inhibitory components of the glial scar tissue that prevent axonal regeneration [15, 16]. Neurocan and brevican are CNS-specific CS-PGs, while aggrecan and versican are also expressed in other tissues.

HS-GAGs are composed of repeating disaccharide units of *N*-acetylglucosamine and glucuronic or iduronic acid that can be modified by *N*-sulfation and three *O*-sulfations in positions 2-*O*-, 3-*O*-, and 6-*O*-. HS-GAGs are covalently bound to core-proteins forming HS-PGs [17]. HS-PGs can be membrane-bound or secreted [18], and are involved in a wide range of cellular processes by direct interactions with different binding partners. Most of these interactions occur in a HS-dependent and specific manner [19] and HS-PGs are expressed in a brain region- and cell type-specific manner and are emerging as important players in the formation and function of synaptic connections [10].

HA is a large, unbranched, non-sulfated GAG formed by the repeating disaccharide unit *N*-acetylglucosamine and *N*-glucuronic acid. HA is the only GAG that is not covalently bound to a core protein and serves as scaffold for lecticans, all of which have HA binding sites. HA also has binding sites for membrane receptors. HA is a major component of the perineuronal net and is also present in the neural stem cell niche of the adult brain [9] and plays a critical role in stabilizing the ECM of the CNS [20].

During brain development, astrocytes contribute to the functional maturation of neurons and the formation of the brain architecture, as they are involved in axon pathfinding, axon and dendrite outgrowth and elaboration, and synaptogenesis [21–26]. Immature astrocytes are also implicated in altered brain development observed in neurodevelopmental disorders such as Fragile X syndrome, autism, and Rett Syndrome [27]. *In vitro*, astrocytes release numerous ECM proteins and modulators that can promote or inhibit neuronal development [28].

Ethanol abuse during pregnancy may lead to Fetal Alcohol Spectrum Disorders (FASD) characterized by structural brain abnormalities and life-long functional impairments of neurocognition, self-regulation, and adaptive functioning as well as development of mental illnesses in more than 90% of affected individuals [29, 30]. Clinical and preclinical studies indicate that neuronal plasticity and connectivity are affected by *in utero* alcohol exposure [31, 32].

Our laboratory has been pursuing the hypothesis that ethanol affects neuronal development in part by altering the levels of astrocyte mediated ECM expression, release, or/or degradation. Using shotgun proteomics we have found that most of the astrocyte-secreted proteins *in vitro* are ECM components or involved in the regulation of ECM proteolysis (proteases and protease regulators) and most of the identified proteins are involved in neuronal development and plasticity [28]. We have also reported that ethanol treatments in astrocytes inhibits neurite outgrowth in co-cultured hippocampal neurons. This effect is in part mediated by the upregulation of neurocan core-protein and in part by the inhibition of the enzyme arylsulfatase B (ARSB), which removes sulfate groups from CS-GAG associated with lecticans (including neurocan) in astrocyte cultures *in vitro* and after neonatal alcohol exposure *in vivo* [33].

In this study, we characterized for the first time CS-, HS-, and HA-GAG disaccharide composition in primary cortical astrocyte cultures, in the conditioned medium derived from the same cultures, and in the developing rat cortex by liquid chromatography-mass spectrometry (LC–MS). We also investigated the effects of ethanol on GAG disaccharides and neurocan and brevican core-proteins in astrocyte cultures. Finally, we analyzed the relative expression of lecticans in

astrocytes compared to the rest of brain cells *in vivo* in the neonatal cortex of Aldh1l1-EGFP-Rpl10a mice.

Materials and Methods

Animals

Time-pregnant Sprague–Dawley rats (gestational day 15) (Research Resource Identifiers, RRID: MGI:5651135) were purchased from Charles River (Wilmington, MA) and singly housed. For primary astrocyte cultures, on gestational day 21 dams were euthanized by CO_2 followed by decapitation. Neocortex tissue was dissected from post-natal day (PD) 9 female rats that were euthanized by intraperitoneal injections of Ketamine/Xylazine (100 mg/Kg and 10 mg/Kg respectively; 0.1 mL/10 g body weight), snap-frozen in in liquid nitrogen, and stored at −80 °C until used for GAG analysis. Sex determination of gestational day 21 fetuses (for astrocyte cultures) and PD9 pups was carried out by observation of the anogenital distance [34] and confirmed by *Sry* genotyping of tail biopsies after DNA isolation; only female fetuses and pups were used in this study. Adult hemizygous Aldh1l1-EGFP-Rpl10a transgenic mice (B6; FVB-Tg (Aldh1l1/EGFP/Rpl10a)JD130Htz/J) [35] purchased from the Jackson Laboratory (Stock N0: 030247) were bred with C57BL/6 J mice, also purchased from the Jackson Laboratory to obtain hemizygous offspring. Post-natal (PD)7 mice were genotyped by tail biopsy using a rapid DNA isolation protocol [36] followed by qPCR with primers targeting eGFP for wild-type/transgenic identification, *Sry* for sex identification [37, 38], and *Gapdh* as a positive control. All animals were housed at the VA Portland Health Care System Veterinary Medical Unit. All the animal procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the VA Portland Health Care System's Institutional Animal Care and Use Committee. Ethical approval was not required.

Female Astrocyte Cultures

Female primary cortical astrocytes were prepared from female rat fetuses at gestational day 21 as described previously [33, 39]. Astrocytes were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin under a humidified atmosphere of 5% CO_2, and 95% air at 37 °C. After 10 days in culture, astrocytes were plated in 100 mm dishes at cell density of 2.5×10^6 per dish for GAG analyses and protein expression determinations, and 6 well plates at cell density of 0.25×10^6 per well for gene expression determinations. This method consistently elicits cultures that are > 95% astrocytes by glial fibrillary acidic protein (GFAP) immunostaining (see Supplemental Fig. 1) consistent with what previously published by us [40, 41]. After four days in culture, astrocytes were switched to a serum-free medium (DMEM, 0.1% bovine serum albumin, BSA, 100 units/mL penicillin, and 100 mg/mL streptomycin) for 24 h followed by treatments in the same serum-free DMEM medium for another 24 h. Astrocyte treatments used in GAG analysis were carried out using serum-free and phenol red-free medium.

In vitro Ethanol Exposure

Astrocyte cultures were exposed to 75 mM ethanol in serum-free medium for 24 h. We previously reported that exposure to up to 100 mM ethanol did not cause cytotoxicity in astrocytes [42]. To reduce ethanol evaporation, cultures were placed in sealed chambers filled with a 5% CO_2/95% air gas mixture and a reservoir tray containing water supplemented with ethanol at the same concentration used in the culture medium, as previously described [33, 43].

GAG Disaccharide Analysis by Liquid Chromatography/Mass Spectrometry (LCMS)

At the end of astrocyte treatments, the medium was collected for GAG analysis. The monolayer was washed twice in 5 mL PBS w/o Ca^{++} and Mg^{++} and 5 mL of PBS w/o Ca^{++} and Mg^{++} supplemented with 10 mM EDTA was added to the cells, the cells were scraped, and the cell suspension was collected in a 15 mL tube. 500 µl of cell suspension was transferred to an Eppendorf tube and centrifuged at 200 g; the supernatant was removed and the pelleted cells were sonicated (5x, 5 s each, at 30% power) in 500 µl of water to extract proteins; debris were pelleted at 20,000 g for 10 min; protein content was determined in the supernatant fraction by the Bradford method. The remaining 4.5 mL of cell suspension was centrifuges at 200 g to pellet the cells; the supernatant was discarded and the cell pellet was frozen at −80° C until used for GAG analysis.

As previously described [44], samples undergoing GAG disaccharide analysis were first defatted by the treatment with 0.2 mL acetone for 30 min; samples were then vortexed and the acetone supernatants were discarded. The remaining tissue samples were dried in the hood and then lyophilized. Dried sample (2 mg) were subjected to proteolysis at 55 °C with 10% actinase E (10 mg/mL) until all tissue was dissolved (36 h). GAGs were purified by Mini Q spin columns. Samples eluted from Mini Q spin column were desalted by passing through a 3 kDa molecule weight cut off spin column and washed three times with distilled water. The casing tubes were replaced before 150 µL of digestion buffer (50 mM ammonium acetate containing 2 mM calcium

chloride adjusted to pH 7.0) was added to the filter unit. Recombinant heparin lyase I, II, III (pH optima 7.0–7.5) and recombinant chondroitin lyase ABC (10 mU each, pH optimum 7.4) were added to each sample and mixed well. The samples were all placed in a water bath at 37 °C for 12 h, after which enzymatic digestion was terminated by removing the enzymes by centrifugation. The filter unit was washed twice with 300 µL of distilled water and the filtrates containing the disaccharide products were dried via vacuum centrifuge. Half of the dried samples were 2-aminoacridone (AMAC)-labeled by adding 10 µL of 0.1 M AMAC in dimthylsulfoxide/acetic acid (17/3, V/V) and incubated at room temperature for 10 min, followed by addition of 10 µL of 1 M aqueous sodium cyanoborohydride and incubation for 1 h at 45 °C. A mixture containing all 17-disaccharide standards prepared at 0.5 ng/µL was similarly AMAC-labeled and used for each run as an external standard. After the AMAC-labeling reaction, the samples were centrifuged and each supernatant was recovered. LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 µm, 3.0 × 50 mm) column. The mobile phase A (MPA) was a 50 mM ammonium acetate aqueous solution, and the mobile phase B (MPB) was methanol. The mobile phase passed through the column at a flow rate of 300 µL/min. The gradient was 0–10 min, 5–45% B; 10–10.2 min, 45–100%B; 10.2–14 min, 100%B; 14–22 min, 100–5%B. Injection volume was 5 µL. A triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific, San Jose, CA) was used as detector. The following disaccharides were quantified by mass spectrometry: HA; Total CS; Total HS; CS TriS; CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S; HS-TriS; HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; HS 0S.

Quantification of GAG Disaccharide Concentrations

The disaccharide quantification was performed by comparing the integrated disaccharides peak area with the external disaccharide standard peak area. The annotated profile of the LC multiple reaction monitoring (LC-MRM) spectra for each of the disaccharides analyzed is shown in Supplemental Fig. 2. The data analysis was performed in Thermo Xcalibur software. For astrocytes samples, the protein content from an aliquot of cell lysates was determined by the Bradford method. The final GAG disaccharide concentrations were calculated by dividing the GAG disaccharides concentrations in ng/ml by the protein concentration of each sample (in mg/ml) and expressed as ng/mg protein. For all the tissue samples, 2 mg of the dry tissues were used for GAGs analysis. The final GAGs concentrations were reported in ng/mg dry weight values: total amount of each disaccharide divided by 2 mg (ng/mg), as previously described [44].There were a total eight HS, eight CS and one HA disaccharides standards analyzed. The percentage distribution of each disaccharide was calculated by the amount of each disaccharide divided by the total amount of all the disaccharides.

Quantitative PCR

RNA was isolated using the Trizol reagent (ThermoFisher Scientific, Inc. Waltham, MA) in conjunction with the Direct-zol RNA MiniPrep Plus kit (Zymo Research, Orange, CA), including the DNase treatment for removal of residual genomic DNA, according to the manufacturer's recommendations. RNA concentration and purity were determined by UV absorption at 260 nm, with 260/280 ratios between 1.9 and 2.1. Quantitative RT-PCR (qPCR) was carried out using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA) with 10 ng of RNA per reaction using a CFX96 Touch thermocycler (Bio-Rad Laboratories, Hercules, CA). Relative expression was determined using the ΔΔCt method after normalizing expression to total RNA measured with the Quant-iT RiboGreen kit (ThermoFisher Scientific, Inc. Waltham, MA). Table 1 lists all the primers used in this study; confirmation of specific, efficient amplification was carried out in our laboratory before their use.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were scraped in water, sonicated (5x, 5 s each), and centrifuged at 1000 g to pellet cell debris and membranes. The supernatant was collected and used for protein determination by the Pierce™ BCA Protein Assay Kit and for neurocan protein levels determination using a commercially available ELISA kit (Antibodies-Online; catalogue number: ABIN432581) following the directions of the manufacturer. Briefly, samples were diluted 1:9 in water; 100 µl of the diluted samples were added to wells pre-coated with the neurocan antibody and incubated for 2 h at 37 °C followed by an incubation with the biotin-conjugated secondary antibody for 1 h at 37 °C and by three washes. Avidin-conjugated horseradish peroxidase (HRP) was then added to each microplate well and incubated for 30 min followed by 5 washes to reduce non-specific binding. The TMB substrate solution was then added for 10–20 min at 37 °C; the reaction was stopped by the addition of a sulfuric acid solution. The color intensities were measured with a plate reader (SPECTROstar NANO) at the wavelength of 450 nm. For each experiment we generated a standard curve which included a blank (control without sample or neurocan standard) and 6 concentrations of neurocan standard. The concentration of neurocan in the samples was interpolated on the regression curve and normalized to protein content.

Table 1 Primer sequences

Gene	Species	Forward	Reverse
Acan	*Mus musculus*	AGGTGTCGCTCCCCAACTAT	CTTCACAGCGGTAGATCCCAG
Bcan	*Mus musculus*	CTGCGCGTCAAGGTAAACG	AGAGACACATCCGTGAGCGAT
Ncan	*Mus musculus*	GCTGGGGATCAGGACACAC	CAGTCTGAACCTTAGTCCACTTG
Vcan	*Mus musculus*	TGGCCCAGAACGGAAATATCA	ACTAGCCCGGAGTTTGACCAT
Arsb	*Rattus norvegicus*	CCTCCTGGACGAAGCAGTGGG	CCCGCCGTTGTCTGTGGAGA
Galns	*Rattus norvegicus*	CCATCGACGCCACACACGCA	GCATCGCCATAACGCCCTCG
Sumf1	*Rattus norvegicus*	AAACGCTCAACCCAAAAGGC	GCACAGCGATACCTGTAGCA
Has1	*Rattus norvegicus*	CCACTGCACATTTGGGGATG	AAGGAAAGAGGAGGGCGTCT
Has2	*Rattus norvegicus*	AGGAGCTGAACAAGATGCATTG	AAAGGCACCATACAGCCCAA
Has3	*Rattus norvegicus*	TTCTTTCTTGCCACCGGGAA	ACCGGCATCCTGCAAGC
Ndst1	*Rattus norvegicus*	CAGAAAACAGGCACCACAGC	GTCAGAGGTGGTGTTGGAGG
Ndst2	*Rattus norvegicus*	CCCACTCCACCGGATATGAAG	AAGTGGATGGCCGTTAGTGG
Ndst3	*Rattus norvegicus*	GGGATGACTGTCTGTTGGGG	GCCATGCTCTAAGGCGAACT
Ndst4	*Rattus norvegicus*	GGCAAGAGGCTGACACTATCC	TCTTCAGTCCCTGTGTGGTA

Western Blot Analysis

Astrocyte extracts for brevican level determinations by Western blot were prepared by solubilizing cells in lysis buffer supplemented by a protease inhibitor cocktail. Western blot analysis was carried out as described previously [43]. Total cellular protein content was quantified by the BCA assay. 20 µg of proteins were loaded on a 3–8% SDS-PAGE gel subjected to electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, and labeled overnight with a mouse monoclonal antibody against brevican (1:500, EMD Millipore, catalogue number: MABN491) followed by an HRP-conjugated goat anti-mouse secondary antibody (BD Biosciences, catalogue number 554002; RRID: AB_395198) for 1.5 h at room temperature. Membranes were stripped and reprobed for ß-actin (Abcam, catalogue number: ab8226; RRID: AB_306371) with detection by the HRP-conjugated goat anti-mouse secondary antibody. Specific bands were detected by electrochemiluminescence (ECL) using the Pierce ECL Plus Substrate (ThermoFisher Scientific, Inc. Waltham, MA); PVDF membranes were then exposed to X-ray films (BioExpress, Kaysville, UT), which were developed with a Hope Micro-Max processor. The relative optical density (OD) of brevican bands was determined using the OptiQuant image analysis software (Version 04.00) and normalized by dividing the OD of the brevican band to the OD of ß-actin in the same sample. Each membrane was exposed for different times to multiple (2–4) X-ray films; only films showing non-saturated bands were chosen for quantification; quantification was carried out on one film/protein/experiment.

Isolation of Astrocyte RNA by the Translating Ribosome Affinity Purification (TRAP) Procedure

The cortex from PD7 Aldh1l1-EGFP-Rpl10a TRAP mice were dissected, snap frozen in liquid nitrogen, and stored at −80 °C until processing. The TRAP procedure to isolate astrocyte-enriched RNA was carried out as previously described [35] with modifications described in Sanz et al. [45]. using anti-GFP antibodies (purchased from the Memorial-Sloan Monoclonal Antibody Facility). Following the final wash of the RNA-Antibody-Bead complex, RNA was isolated using TRIzol Reagent and Direct-zol RNA MicroPrep kit from input and pull-down samples. qPCR was carried out as described [46] on a Bio-Rad CFX96 Real-Time PCR Detection System (RRID:SCR_018064).

Statistical Analysis and Blinding Conditions

No sample size calculations were performed but experimental group sizes were based on previous studies of astrocyte cultures and neonatal rat pup intubation studies. Samples for GAG analyses were generated in Dr. Guizzetti's laboratory at OHSU/Portland VA and then shipped to the laboratory of Dr. Linhardt at Rensselaer Polytechnic Institute for unbiased LC–MS analysis; for qPCR, Western Blot, and ELISA analyses, no blinding was performed. Student's *t*-test was performed to determine significant differences between control and ethanol-treated astrocytes in Figs. 7 and 9 using the software GraphPad Prism, Version 8.0.2 (RRID:SCR 002798). One data point in the control group of Fig. 9a was removed as it was identified as outlier by the Grubb's test.

Normal distributions of the data were verified using the Shapiro–Wilk test in GraphPad Prism.

Results

CS- HS- and HA-GAG Disaccharides Levels in Cortical Astrocyte Cultures, Astrocyte-conditioned Medium, and the Cortex of PD9 Rats

All the results presented in this study were carried out in female primary cultures and in the cortex of neonatal female rats and mice to reduce possible sex-related variability.

Glycomic profiling of CS-, HS, and HA-GAG disaccharide levels in primary astrocyte cultures and astrocyte-conditioned medium was compared to glycomic analyses of GAG disaccharides in the cortex of PD9 rat pups (a stage of brain development corresponding to the third trimester of human gestation). The age of primary astrocyte cultures, prepared from gestational day 21 fetuses (just before birth) and maintained for 10 days before being sub-cultured for experiments, was comparable to the PD9 cortex *in vivo*, with the caveat that astrocyte development *in vitro* may not fully recapitulate their development *in vivo*. Another caveat of these comparisons is that primary culture results were normalized to mg protein, while the whole cortex results were normalized to mg of dry tissue. Despites these limitations we believe these comparisons to provide useful information about what are the GAG disaccharides mainly produced by developing cortical astrocyte in culture versus the GAG disaccharides present in the whole developing cortex.

GAG disaccharide content in astrocyte cell lysates (representing intracellular, not yet released GAGs, GAGs covalently bound to membrane proteins, and GAGs present in the ECM strongly anchored to cell surface proteins) was 2590 ± 170 ng/mg protein. The most abundant GAG

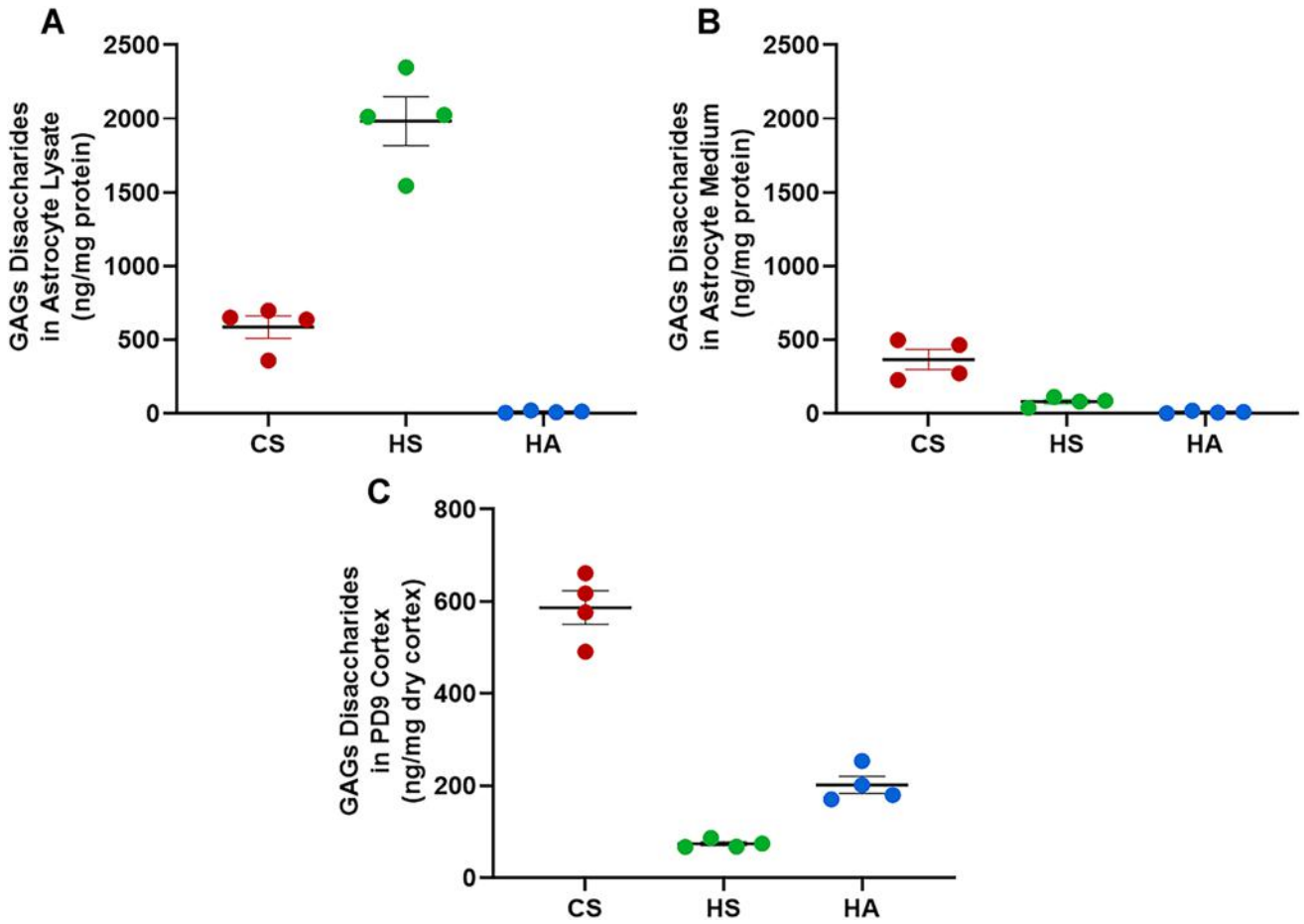

Fig. 1 Quantification of CS- HS- and HA-GAG disaccharides in cortical astrocyte cell lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a** Concentration (ng/mg protein of CS-, HS-, and HA-GAG disaccharides in astrocyte cell lysate. **b** Concentration (ng/mg protein) of CS-, HS-, and HA-GAG disaccharides in astrocyte-conditioned medium. **c**: Concentration (ng/mg dry tissue) of CS-, HS-, and HA-GAG disaccharides in the neocortex of PD9 rats. **a**, **b**: n = 4 independent cell culture preparations; **c**: n = 4 neocortices from 4 PD9 rats

disaccharides were HS-GAGs (1983 ± 165.4 ng/mg protein), followed by CS-GAGs (585.5 ± 76.6 ng/mg protein); HA-GAGs (11.4 ± 3.5 ng/mg) were detectable, but at much lower levels (Figs. 1a, 2a). Astrocyte-conditioned media contained overall less GAG disaccharides (454.6 ± 81 ng/mg protein corresponding to 15% of the total astrocyte GAG disaccharides) compared to the cellular GAGs. The highest GAG disaccharides in the medium were CS (364.7 ± 68.1 ng/mg protein), while HS (79.1 ± 15.6 ng/mg protein) were much lower in the medium than in the cellular compartment (only 4% of the astrocyte HS-GAGs), and HA-GAG content was also very low in astrocyte conditioned medium (9.2 ± 3.6 ng/mg protein) (Figs. 1b, 2b).

The major GAG disaccharides in the developing (PD9) cortex were CS (586.6 ± 36.3 ng/mg protein), while the major GAG disaccharides in astrocyte cell lysate, HS, were the least represented in the developing cortex (73.9 ± 4.4 ng/mg protein) (Figs. 1c, 2c). HA-GAGs were present at an intermediate level in the cortex (201.3 ± 18.6 ng/mg protein) (Figs. 1c, 2c). The very low levels of HA-GAGs in astrocytes but higher representation in the whole cortex was correlated to a significantly lower expression of *Has1* and *Has2*, encoding for HA synthases (HAS) 1 and 2, in astrocyte cultures compared to the whole cortex (Table 2).

Table 2 Comparison of gene expression in PD9 cortex and primary astrocyte cultures

Gene	Cortex	Astrocytes	p-value
Arsb	1.000 ± 0.075	1.200 ± 0.146	0.1798
Galns	1.000 ± 0.046	2.288 ± 0.191	6.18E-06
Sumf1	1.000 ± 0.062	3.179 ± 0.258	3.12E-06
Has1	1.000 ± 0.087	0.236 ± 0.054	1.52E-04
Has2	1.000 ± 0.070	0.302 ± 0.084	3.81E-04
Has3	1.000 ± 0.064	0.798 ± 0.160	0.2254
Ndst1	1.000 ± 0.040	1.063 ± 0.088	0.4389
Ndst2	1.000 ± 0.070	1.686 ± 0.328	0.0424
Ndst3	1.000 ± 0.035	0.142 ± 0.025	7.87E-08
Ndst4	1.000 ± 0.171	0.011 ± 0.002	0.0047

Levels of CS-GAG Disaccharide Types in Cortical Astrocyte Cultures, Astrocyte-conditioned Medium, and the Cortex of PD9 Rats

CS-GAG disaccharides can be modified by sulfation in positions 2, 4, and/or 6 [12] generating eight combinations of sulfated CS disaccharides: TriS (CS 2S4S6S), 2S4S, 2S6S, 4S6S, 4S, 6S, 2S, and 0S. The major sulfated CS-GAG disaccharides in astrocytes were C4S (188.9 ± 62.6 ng/mg protein) and C6S (96.32 ± 20.7 ng/mg protein), as previously reported in the brain [12]. However, the most abundant CS disaccharide type overall was the non-sulfate form (C0S) (275.2 ± 40.9 ng/mg protein). The other five CS-GAG disaccharide types together comprised only about 4% of the astrocyte CS-GAGs (Figs. 3a, 4a).

In astrocyte-conditioned medium, by far the major type of CS-GAG disaccharides was C0S (290.8 ± 59.6 ng/mg protein). C4S was the second most abundant disaccharide (56.6 ± 7.0 ng/mg protein), while C6S was present at much lower levels (2.7 ± 0.7 ng/mg protein) and, together with the other 5 CS types, comprised only about 5% of the total conditioned medium CS-GAGs (Figs. 3b, 4b).

In the whole cortex, the most abundant CS-GAG disaccharide type was C4S (70% of total CS-GAGs; 409.7 ± 10.2 ng/mg protein), followed by C6S (26% of the

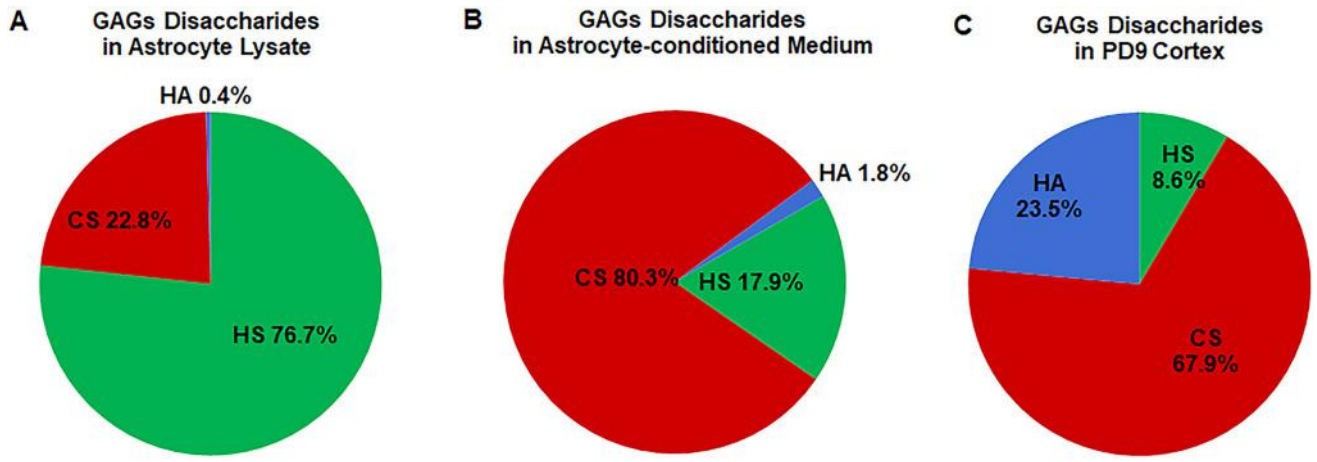

Fig. 2 Relative distribution of CS- HS- and HA-GAG disaccharides in cortical astrocyte cell lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a**: Relative distribution (expressed as percentage of total GAGs of CS-, HS-, and HA-GAGs in astrocyte cell lysate. **b** Relative distribution (expressed as percentage of total GAGs) of CS-, HS-, and HA-GAGs in astrocyte-conditioned medium. **c** Relative distribution (expressed as percentage of total GAGs) of CS-, HS-, and HA-GAGs in the neocortex of PD9 rats

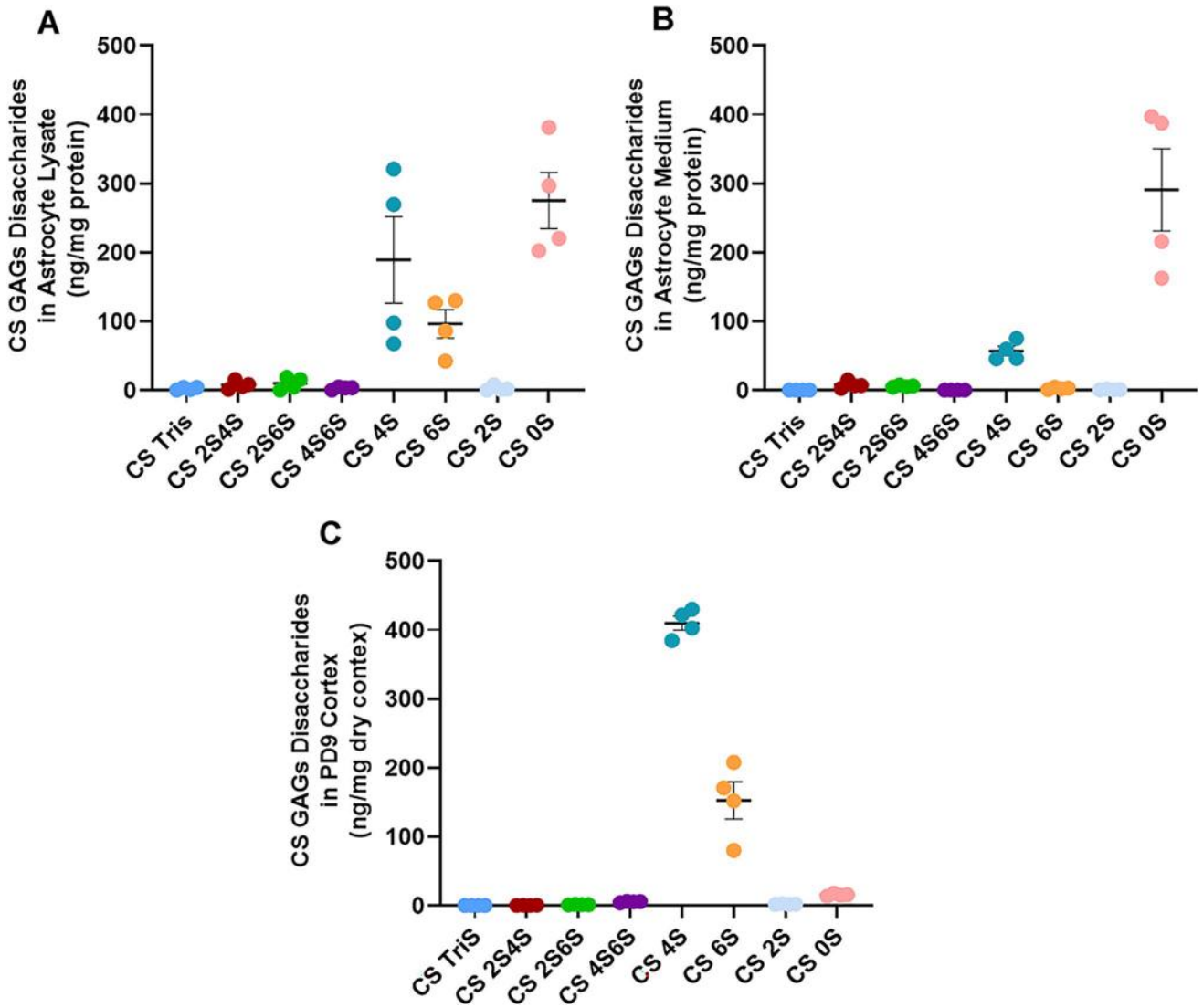

Fig. 3 Quantification of CS-GAG disaccharides in cortical astrocyte cell lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a** Concentration (ng/mg protein) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in astrocyte lysate. **b** Concentration (ng/mg protein) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in astrocyte-conditioned medium. **c** Concentration (ng/mg dry tissue) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in the neocortex of PD9 rats. **a**, **b** n = 4 independent cell culture preparations; **c**: n = 4 neocortices from 4 PD9 rats

total CS-GAGs; 152.5 ± 26.9 ng/mg protein). All the other CS forms, including C0S, were present in very low amounts that together accounted for only 4% of the total CS-GAGs.

The high levels of C0S-GAG disaccharides in astrocytes and the very low levels in the whole cortex was consistent with the high expression of *Galns* and *Sumf1* in astrocyte cultures compared to the whole cortex (Table 2). *Galns*, encodes for the enzyme Galactosamine (*N*-acetyl)-6 Sulfatase (GALNS) which removes sulfates selectively from C6S and *Sumf1* is the gene for the enzyme Sulfatase Modifying Factor 1 (SUMF1), an enzyme necessary for the activation of several sulfatases, including ARSB and GALNS. *Arsb*, which encodes for the enzyme arylsulfatase B (ARSB), was similarly expressed in astrocyte cultures and in the whole cortex (Table 2).

Levels of HS-GAG Disaccharide Types in Cortical Astrocyte Cultures, Astrocyte-conditioned Medium, and the Cortex of PD9 Rats

In astrocyte cell lysate the vast majority of HS-GAGs were non-sulfated (H0S; 87% of total HS-GAGs; 1731 ± 139.9 ng/mg protein) while the second most abundant HS-GAGs, NS (113.3 ± 9.2 ng/mg protein), was only

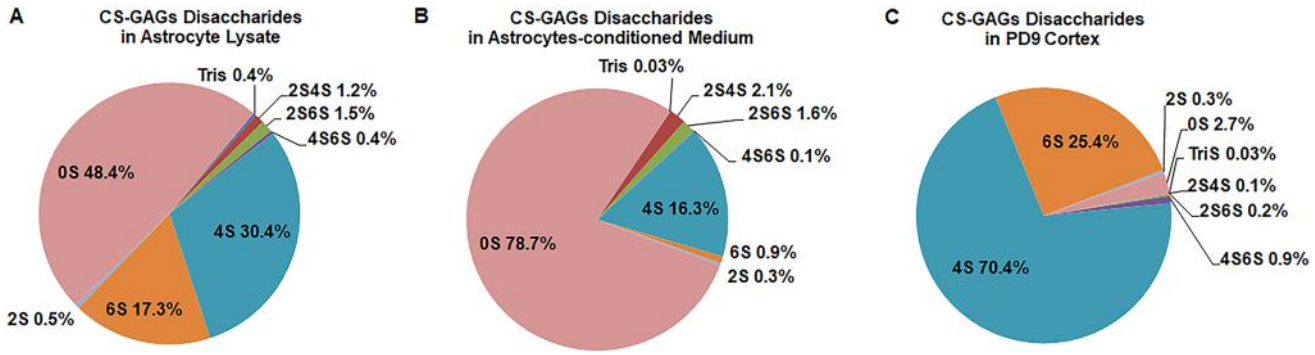

Fig. 4 Relative distribution of CS-GAG disaccharides in cortical astrocyte lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a**: Relative distribution (expressed as percentage of total CS-GAGs) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in astrocyte lysate. **b**: Relative distribution (expressed as percentage of total CS-GAGs) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in astrocyte-conditioned medium. **c**: Relative distribution (expressed as percentage of total CS-GAGs) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides in the neocortex of PD9 rats

6% of total HS-GAGs (Figs. 5a, 6a). HS-GAG disaccharides in the medium were detectable (Figs. 5b, 6b); however, their levels were only 4% of the HS-GAG disaccharides in astrocyte lysates.

In the cortex of PD9 rats, H0S was also the major type of HS-GAG disaccharide (41.0 ± 1.5 ng/mg protein; 56% of total HS-GAGs). NS6S (5.9 ± 0.6 ng/mg protein; 8% of total HS-GAGs), NS2S (15.5 ± 1.2 ng/mg protein; 21% of total HS-GAGs), and NS (7.3 ± 0.5 ng/mg protein; 10% of total HS-GAGs) were the most abundant other HS-GAG disaccharides present (Figs. 5c, 6c). Overall, there was a higher proportion of *N*-sulfation in HS-GAG disaccharides in the cortex compared to astrocytes (Fig. 6), which corresponds with much higher levels of expression of *Ndst3* and *Ndst4* in the cortex than in astrocytes (Table 2). *Ndst3* and *Ndst4* are two of the four *N*-deacetylase/*N*-sulfotransferases (NDSTs) that are able to catalyze HS-GAG *N*-sulfation.

Effect of Ethanol on CS- HS- and HA-GAG Disaccharide Levels in Astrocytes

Ethanol significantly upregulated the total levels of CS-GAG disaccharides ($p = 0.0289$, 45% increase; Fig. 7a) and 4S-GAGs ($p = 0.0225$, 123% increase; Fig. 7b) in astrocyte cell lysates. The levels of all the other types of CS-GAGs were not significantly affected by ethanol (Fig. 7b). Total HS-GAGs displayed a trend toward upregulation in astrocyte cell lysates exposed to ethanol in comparison to control cells ($p = 0.0574$, 40% increase; Fig. 7c). NS-GAGs displayed a significant increase after ethanol treatments in astrocyte cell lysates ($p = 0.0499$; 56% increase; Fig. 7d). HA-GAG levels in astrocyte cell lysate and CS-GAG, HS-GAG, and HA-GAG levels in astrocyte-conditioned medium were not affected by ethanol (not shown).

Relative Expression of Lecticans in Astrocytes *in vivo*

PGs of the lectican family (neurocan, brevican, versican, and aggrecan) are conjugated to CS-GAGs and are the most abundant proteins of the CNS ECM [13]. We used Aldh1l1-EGFP-Rpl10a mice to determine the relative expression of the four lecticans in neonatal cortical astrocytes and the bulk cortex *in vivo* [35]. The Aldh1l1-EGFP-Rpl10a mice express a modified Rpl10a ribosomal protein with an EGFP tag under the transcriptional control of the astrocytic marker Aldh1l1. This allows for the purification from brain tissue of RNA that is actively being transcribed (attached to EGFP tagged ribosomes) in astrocytes.

Aggrecan is expressed at the lowest level of the lecticans in the cortex of neonatal Aldh1l1-EGFP-Rpl10a mice while brevican, neurocan and versican are all expressed at high levels (not shown). In Fig. 8, the $\log_2$ expression of lecticans expressed as fold/input shows that brevican is about seven-fold and neurocan is about five-fold more expressed in astrocytes than in the whole cortex similar to what previously reported in a RNA-Seq study [47]. These results indicate that astrocytes are the main, and, possibly, the only cell types expressing neurocan and brevican in the developing cortex *in vivo*.

Effect of Ethanol on Lectican Gene and Protein Expression in Astrocyte Cultures

Ethanol increased neurocan protein ($p = 0.0327$, 25% increase; Fig. 9a) and gene expression ($p = 0.0197$, 22% increase; Fig. 9d) in astrocyte cultures, confirming our previous results obtained in mixed-sex astrocyte cultures [33]. Ethanol also upregulated brevican protein ($p = 0.0153$, 74% increase; Fig. 9b–c) and gene expression ($p = 0.0386$, 29% increase; Fig. 9e).

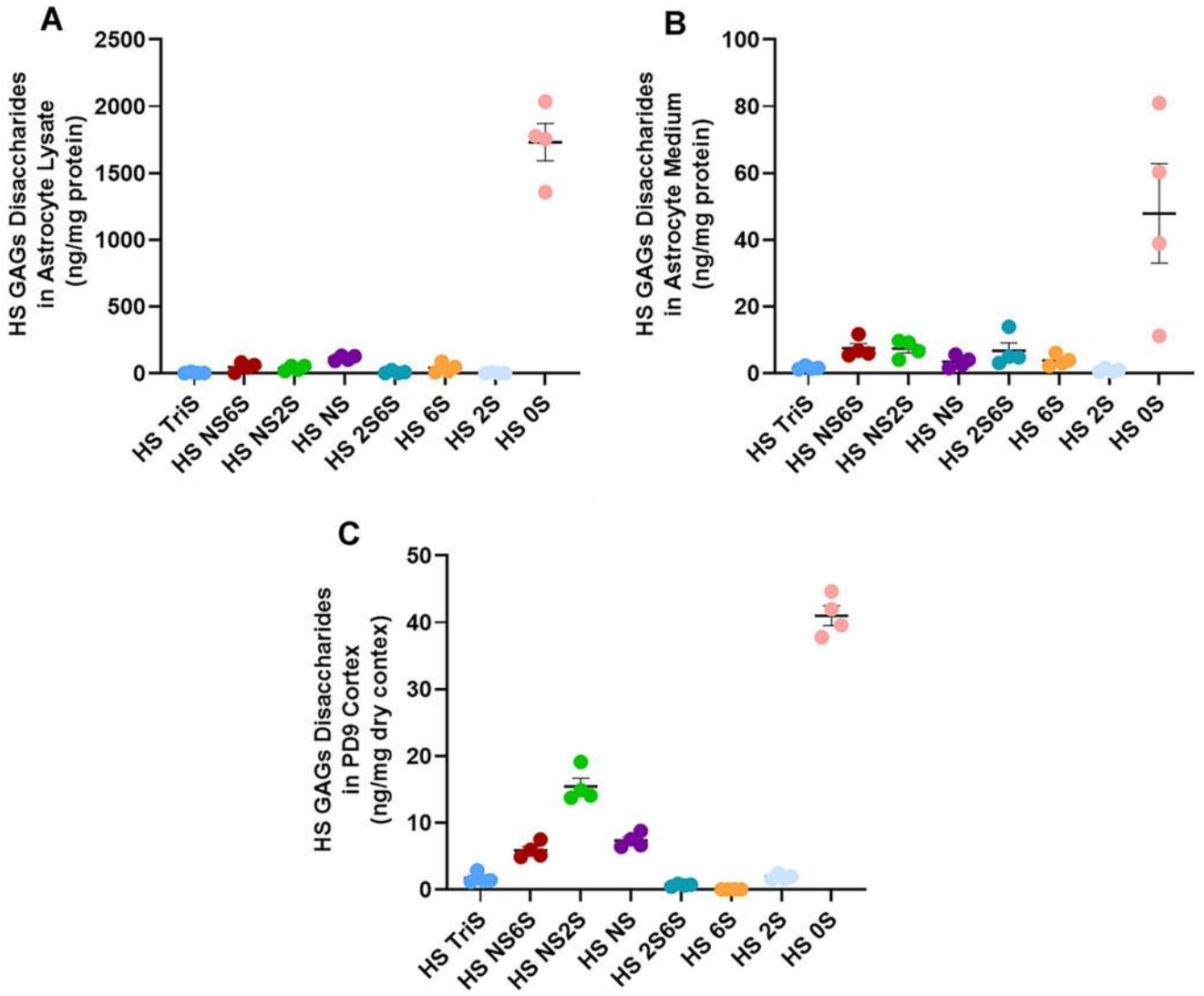

Fig. 5 Quantification of HS-GAG disaccharides in cortical astrocyte cell lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a**: Concentration (ng/mg protein) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in astrocyte lysate. **b**: Concentration (ng/mg protein) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in astrocyte-conditioned medium. **c**: Concentration (ng/mg dry tissue) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in the neocortex of PD9 rats. **a**, **b** n = 4 independent cell culture preparations; **c**: n = 4 neocortices from 4 PD9 rats

Discussion

One of the major goals of this study was to characterize CS-, HS-, and HA-GAG disaccharides in developing cortical astrocytes and the developing cortex. Figures 1–6 summarize the levels and percentage distributions of GAG disaccharides in astrocyte cell lysates, astrocyte conditioned media, and cortex of neonatal rats.

A major difference between astrocyte and cortex GAG disaccharide composition is the very low levels of HA-GAGs in astrocytes (0.4% of total astrocyte GAGs), while in the cortex, HA-GAGs constitute 23.5% of total GAGs (Figs. 1, 2). The main enzymes responsible for the biosynthesis of HA-GAGs are HAS1, HAS2, and HAS3 [9]. We found that the genes encoding for two of these enzymes, *Has1* and *Has2*, are expressed at much lower levels in astrocytes than in the cortex (Table 1). These data suggest that the low levels of HA in astrocytes are due to the low expression of HA synthesizing enzymes. Our data are in agreement with a paper reporting that, in the adult mouse brain *Has1*, *Has2*, and *Has3* are expressed mainly by neurons, with *Has3* being expressed also by endothelial cells in the hippocampus and cerebral cortex [48]; in contrast, increased transcription of *HAS1*, but not *HAS2* and *HAS3,* has been reported in reactive astrocytes of the aging brain of non-human primates [49].

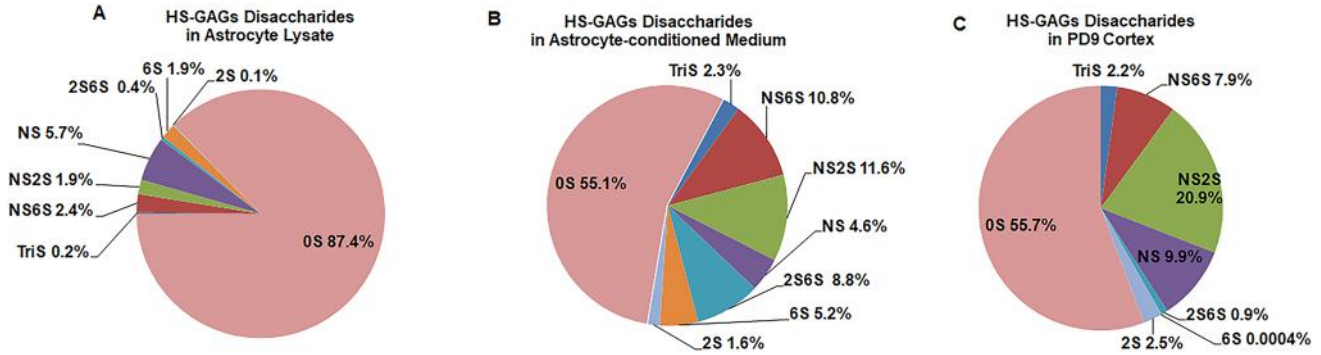

Fig. 6 Relative distribution of HS-GAG disaccharides in cortical astrocyte lysate, astrocyte-conditioned medium, and the cortex of PD9 rats. **a** Relative distribution (expressed as percentage of total HS-GAGs of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in astrocyte lysate. **b** Relative distribution (expressed as percentage of total HS-GAGs) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in astrocyte-conditioned medium. **c** Relative distribution (expressed as percentage of total HS-GAGs) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides in the neocortex of PD9 rats

Another major difference between the developing cortex and astrocyte cultures is the large percentage of C0S-GAGs present in astrocyte cultures but not in the whole cortex (Figs. 3, 4), which we hypothesize is a result of the higher expression of sulfatase enzymes in astrocyte cultures than in the whole cortex (Table 2). Sulfatase enzymes remove sulfate groups from GAGs in a highly specific manner; for instance, ARSB selectively removes sulfate groups from C4S and GALNS selectively removes sulfate groups from C6S [50]. Sulfatases are synthesized in an inactive form and require a unique, sulfatase-specific post-translational modification consisting of the oxidation of a crucial cysteine residue, and the generation of a Cα formylglycine (FGly) residue; this reaction is catalyzed by the enzyme SUMF1, also known as FGly-generating enzyme (FGE) [51]. Our findings that *Galns* and *Sumf1* (but not *Arsb*) are expressed at a significantly higher level in astrocyte cultures than in the whole cortex (Table 2) are consistent with the hypothesis that the high levels of C0S in astrocytes are the result of higher GALNS activity and higher sulfatase activation by SUMF1.

HS-GAGs are much more abundant in astrocyte cell lysates (77% of the total GAGs) than in the PD9 cortex (only 9% of the total GAGs) (Fig. 2), suggesting that astrocytes are the major producers of HS-GAGs in the developing brain. By far the major form of HS-GAGs in the astrocyte cell lysate was the non-sulfated form, H0S (almost 90%). In PD9 cortex, besides H0S, which is also the major form, the HS-GAGs containing N sulfation were well represented (NS6S: 8%, NS2S: 21%; NS: 10%) (Figs. 5, 6). We found that of the four genes encoding for enzymes responsible for *N*-sulfation of HS-GAGs (*Ndst1-4*), *Ndst3* and *Ndst4* are expressed at much lower levels in astrocytes than in the neonatal cortex suggesting that NDST3 and NDST4 may be responsible for the very low *N*-sulfation observed in astrocyte cultures. It is possible that *Ndst3* and *Ndst4* in astrocytes are inducible enzymes that require signals from other cell types or environmental stimuli in order to be expressed. In support of this hypothesis we show that ethanol treatments induce a significant increase in NS disaccharides (Fig. 7d).

In astrocyte cultures, CS-GAG disaccharides were similarly distributed between cell lysate and astrocyte-conditioned medium, while HS-GAG levels were much higher in the cell lysate fraction than in the medium fraction. HA was present at low levels in both astrocyte cell lysate and astrocyte-conditioned medium (Fig. 1a, b). The higher representation of CS-GAGs compared to HS-GAGs in the conditioned medium (Fig. 2b) is consistent with the fact that lecticans, which are the major family of CS-PGs in the brain, are secreted proteins [13]; conversely, only one of the three main subfamilies of HS-PGs are secreted, while the other two subfamilies (syndecans, and glypicans) are membrane-bound [10]. The vast majority of CS-GAGs in the medium (almost 80%) were non-sulfated suggesting that the presence of negatively charged sulfate groups contribute to the anchoring of CS-GAGs to cell surface proteins, while, in the absence of sulfation, CS-GAGs are more likely to be found unbound in the medium.

Interestingly, the percentage composition of GAG disaccharides in neonatal rat cortex (Figs. 1–6) did not dramatically differ from what has been found in the medial prefrontal cortex (mPFC) of adult rats [52]. Indeed, in both the adult mPFC and neonatal cortex, CS are the major GAG disaccharides followed by HA, and lastly, HS. The distribution of the differentially sulfated HS was also similar in the adult mPFC and neonatal cortex. The only major difference between developing and adult cortex was that in the adult mPFC, 90% of CS-GAG disaccharides are C4S and 2–3% are C6S, while in the developing cortex C4S represented 70% and C6S represented 26% of the total CS-GAG

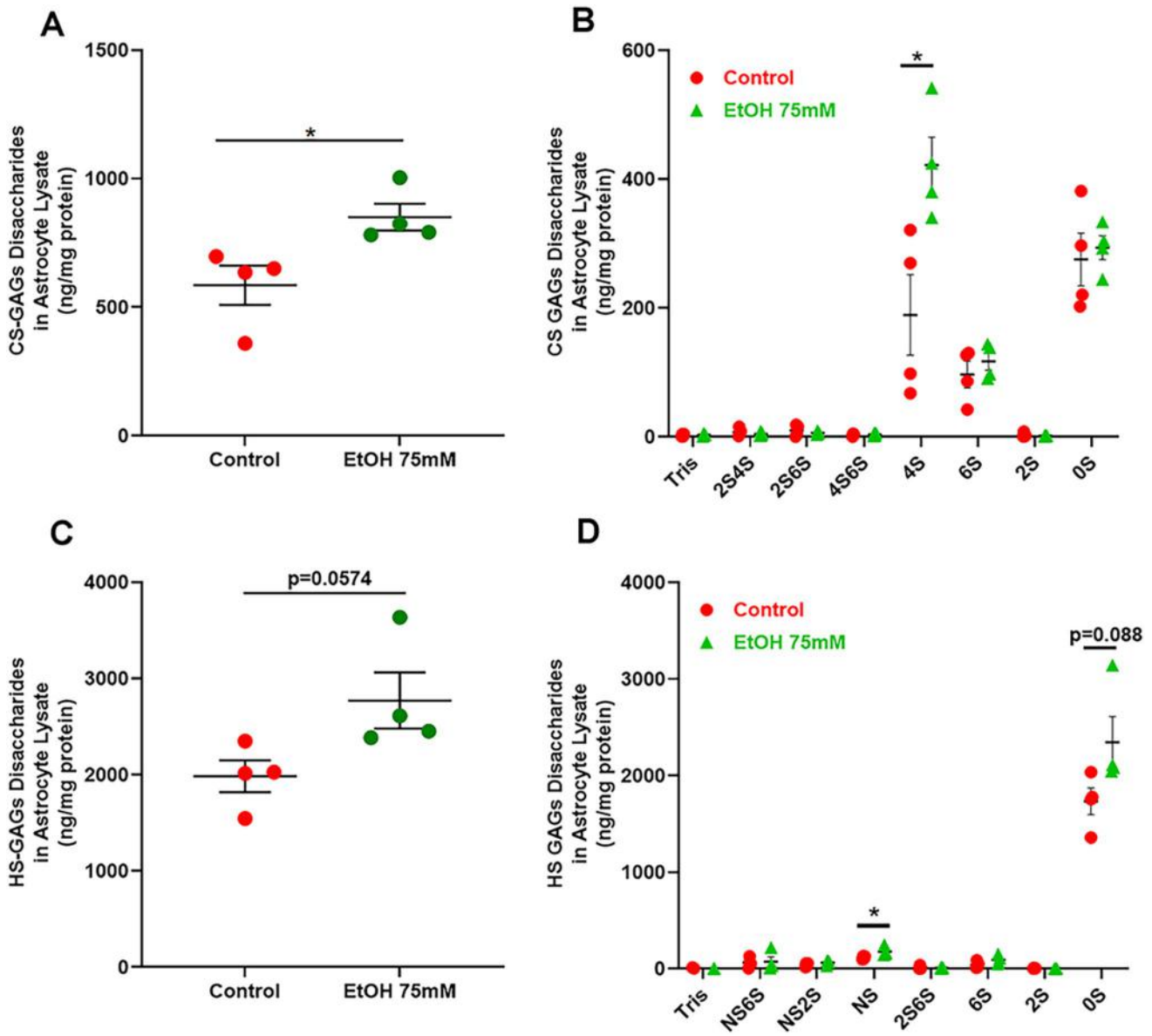

Fig. 7 Effect of ethanol on CS- and HS-GAG disaccharides in cortical astrocyte cell lysate. Primary rat astrocytes were incubated for 24 h in the presence or absence of 75 mM ethanol. **a** Concentration (ng/mg protein) of total CS-GAG disaccharides from control and ethanol-treated astrocytes. **b** Concentration (ng/mg protein) of CS-2S4S6S (TriS); CS-2S4S; CS-2S6S; CS-4S6S; CS-2S; CS-4S; CS-6S; CS-0S disaccharides from control and ethanol-treated astrocytes. **c**: Concentration (ng/mg protein) of total HS-GAG disaccharides from control and ethanol-treated astrocytes. **d** Concentration (ng/mg protein) of HS-NS2S6S (TriS); HS-NS6S; HS-NS2S; HS-NS; HS-2S6S; NS 6S; HS 2S; and HS 0S disaccharides from control and ethanol-treated astrocytes. * $p < 0.05$ by the Student's t test ($n = 4$ independent cell culture preparations)

disaccharides. The ten-fold difference in the representation of C6S-GAGS between the developing and adult brain is in agreement with a study showing that the expression of chondroitin 6-sulfotransferase (C6ST), the enzyme responsible for the 6-O-sulfation of CS, is developmentally regulated and decreases toward the end of development in embryonic chicken brain [53].

A limitation of these studies is that, because of the challenges of distinguishing *in vivo* GAGs produced by astrocytes from GAGs produced by other cell types, we compared GAG disaccharide levels in rat primary cortical astrocyte cultures *in vitro* and in the developing rat cortex *in vivo*. Using *in vitro* approaches similar to the one used in these studies, we have discovered several astrocyte functions impacting neuronal development and their alterations induced by ethanol [28, 33, 39, 54, 55] and other groups have identified exciting roles of astrocytes in synaptogenesis [21–23, 25]. Untill recenlty, tools to reliably and selectively

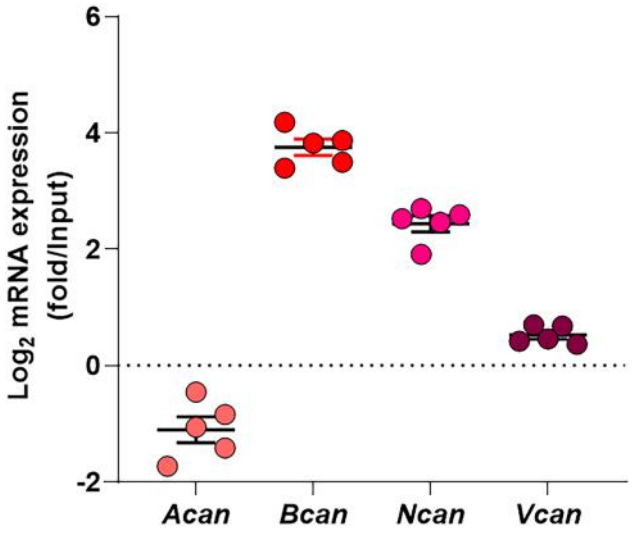

Fig. 8 *In vivo* astrocyte lectican expression in neonatal Aldh1l1-EGFP-Rpl10a mice. The relative *in vivo* expression of aggrecan, versican, brevican, and neurocan from PD7 astrocytes from the cortex of Aldh1l1-EGFP-Rpl10a mice was measured by qPCR after the TRAP procedure to enrich for astrocyte RNA. Relative expression of astrocyte lecticans was compared to expression in the whole neocortex (input) Shown is the $\log_2$ of the fold-input (mean ± SEM) expression data normalized total RNA. n = 5 PD7 pups from 3 different litters (1 male and 1 female per litter with the exception of a litter that did not have a female transgenic animal)

study astrocytic functions in the vertebrate CNS *in vivo* were lacking [56]. In this study we used the recently developed Ald1l1-EGFP-Rpl10a mouse model and assessed relative gene expression of components of the astrocyte ECM *in vivo* in astrocytes (Fig. 8). However, a cell-type specific approach to determine GAG disaccharide levels specifically produced by astrocytes *in vivo* is not technically possible and *ex vivo* approaches that rely on brain tissue digestion by proteolytic enzymes are unsuitable to answer questions about astrocyte GAGs because they cleave extracellular and membrane proteins, leading to the loss of ECM GAGs.

A second major goal of this study was the characterization of the effects of alcohol on astrocyte-expressed GAG disaccharides and on their conjugated proteoglycans. We have reported that the pretreatment of astrocytes with ethanol *in vitro* reduces neurite outgrowth in co-cultured neurons and identified several alterations in the ECM in astrocytes as consequence of ethanol treatments [33, 39, 57]. In this study we find that total CS-GAG disaccharides are significantly increased by ethanol treatments (Fig. 7a); an effect driven by a selective increase in C4S-GAG disaccharides (Fig. 7b). CS-GAGs, and in particular C4S-GAGs are inhibitors of neuronal adhesion, neurite outgrowth, and axonal growth and regeneration [15, 58–60]. These results are in agreement with what we previously reported using an antibody-based method to measure C4S-GAG levels [33]. In the present study, we determined that astrocytes are by far the main source of neurocan and brevican in the developing cortex *in vivo* using the TRAP technology in Aldh1l1-EGFP-Rpl10a mice (Fig. 8). Finally, we found that neurocan and brevican core-protein and RNA expression are upregulated by ethanol in astrocytes (Fig. 9). Together, these results indicate that ethanol increases C4S-GAG disaccharides and neurocan and brevican mRNA and protein levels in astrocytes, thus creating an environment that is inhibitory for neuronal development, consistent with our previous observation that ethanol-pretreated astrocytes inhibit neurite outgrowth in co-cultured hippocampal neurons [33]. The alcohol concentration used in this study, 75 mM corresponding to 0.35 g/dl, has been found in the blood heavy drinkers and is therefore clinically relevant and is within the range of concentrations recommended for *in vitro* studies [33, 61, 62]. Heavy ethanol drinking eliciting blood alcohol concentrations in the range of the concentration used in this study (http://www.clevelandclinic.org/health/interactive/alcohol_calculator.asp) has been reported also in pregnant women [63].

In summary, our data suggest that: (1) cortical astrocytes produce low levels of HA disaccharides compared to the whole developing cortex and show low expression of HA biosynthetic enzymes *Has1*, *Has2*, and *Has3*. (2) Astrocytes have high levels of C0S disaccharides compared to the whole cortex, possibly because of a higher sulfatase enzyme expression. (3) Astrocytes are major producers of HS-GAGs, but, under basal conditions (unstimulated), N-sulfation of HS-GAG disaccharides is very low. (4) Ethanol upregulates C4S-GAG disaccharides as well as brain-specific CS-PGs neurocan and brevican; two lecticans that are expressed mostly (or exclusively) by astrocytes in the developing cortex *in vivo*. Together, these results begin to elucidate the role of astrocytes in the biosynthesis of CS- HS- and HA-GAG disaccharides and further solidifies the evidence that ethanol exposure alters the astrocyte GAG disaccharide composition in the ECM; an effect likely involved in ethanol-treated astrocyte-mediated altered neuronal development [33, 55] and in the alterations in neuronal development caused by *in utero* alcohol exposure.

Acknowledgement We thank Mr. Nicholas Margolies for the critical review of the manuscript and Ms. Shelley H. Bloom and Dr. Clare J. Wilhelm for their technical assistance with astrocyte cultures and treatments.

Author Contributions MG conceived the study; MG, XZ, and RJL designed experiments. Material preparation, data collection and analysis were performed by XZ, JGH, XH and FZ; MG and XZ wrote the paper with suggestions from JGH and RJL. All authors read and approved the final manuscript.

Funding This work was supported by the Department of Veterans Affairs Merit Review BX001819, and National Institutes of Health

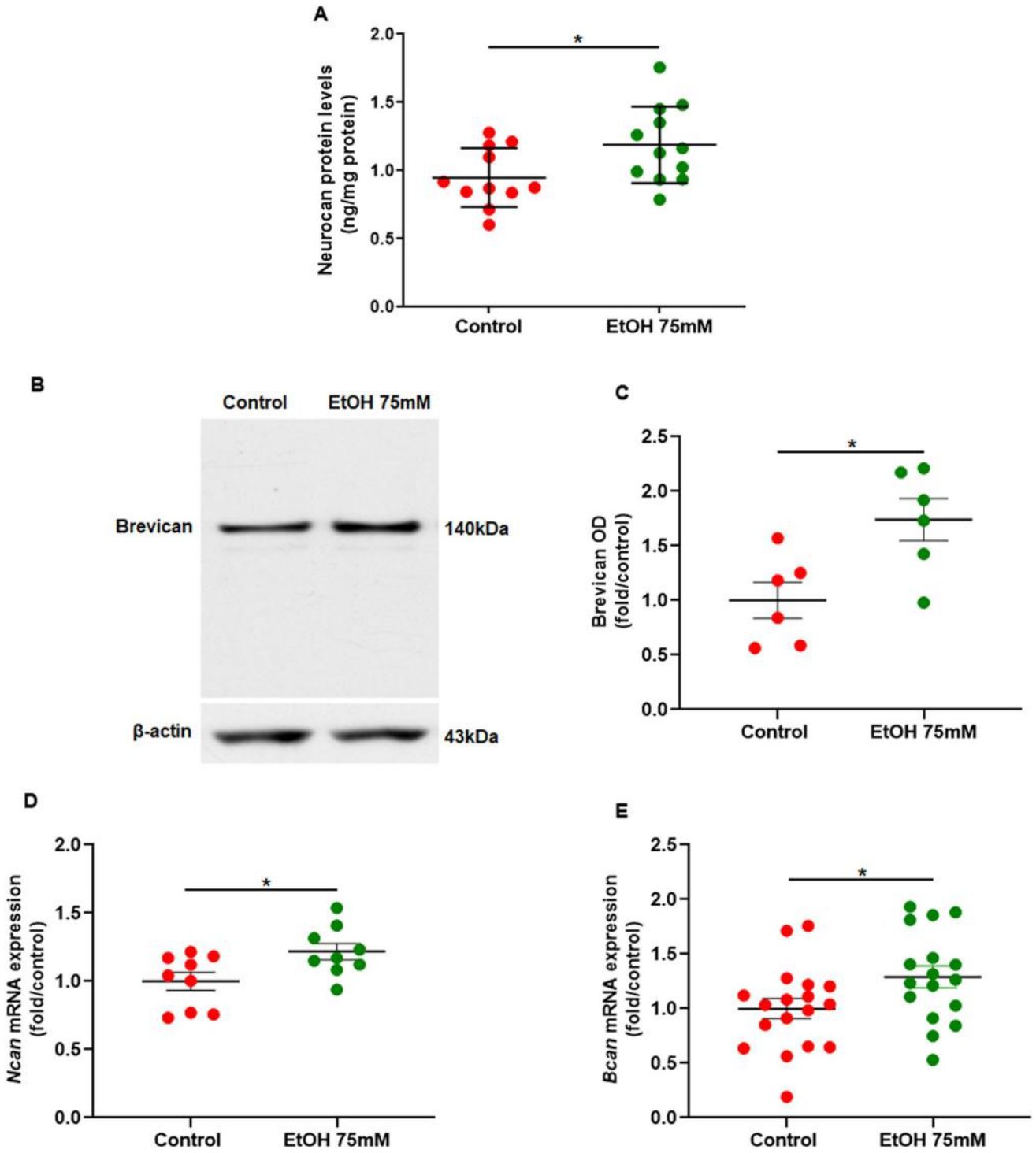

Fig. 9 Effect of ethanol on Neurocan and Brevican protein and gene expression in astrocyte cultures. Primary rat astrocytes were incubated for 24 h in the presence or absence of 75 mM ethanol. **a**: Neurocan protein levels in astrocyte lysates quantified by ELISA. **b**: Representative immunoblots of brevican and β-actin. **c**: Densitometric analysis of brevican protein immunoblots. Brevican was normalized to β-actin and expressed as fold over control. **d**: Neurocan mRNA levels were quantified by qPCR. **e**: Brevican mRNA levels were quantified by qPCR. *, $p < 0.05$ by the student's *t* test (n = 6–18 independently cultured astrocyte dishes prepared from the fetuses of three pregnant rats)

R01AA022948 (MG) and the New York State Spinal Cord Injury Research Board (SCRIB) Grant DOH01-PART2-2017 (RJL).

Data Availability The authors confirm that the data support their published claims and comply with field standards.

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to disclose.

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