Published in final edited form as:

Neuroscience. 2012 August 30; 218: 367–384. doi:10.1016/j.neuroscience.2012.05.055.

DECONSTRUCTING THE PERINEURONAL NET: CELLULAR CONTRIBUTIONS AND MOLECULAR COMPOSITION OF THE NEURONAL EXTRACELLULAR MATRIX

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Abstract

Perineuronal nets (PNNs) are lattice-like substructures of the neural extracellular matrix that envelop particular populations of neurons throughout the central nervous system. Previous work suggests that this structure plays a major role in modulating developmental neural plasticity and brain maturation. Understanding the precise role of these structures has been hampered by incomplete comprehension of their molecular composition and cellular contributions to their formation, which is studied herein using primary cortical cell cultures. By defining culture conditions to reduce (cytosine-β-D-arabinofuranoside/AraC addition) or virtually eliminate (elevated KCl and AraC application) glia, PNN components impacted by this cell type were identified. Effects of depolarizing KCl concentrations alone were also assessed. Our work identified aggrecan as the primary neuronal component of the PNN and its expression was dramatically up-regulated by both depolarization and glial cell inhibition and additionally, the development of aggrecan-positive PNNs was accelerated. Surprisingly, most of the other PNN components tested were made in a glial-dependent manner in our culture system. Interestingly, in the absence of these glial-derived components, an aggrecan- and hyaluronan-reactive PNN developed, demonstrating that these two components are sufficient for base PNN assembly. Other components were expressed in a glial-dependent manner. Overall, this work provides deeper insight into the complex interplay between neurons and glia in the formation of the PNN and improves our understanding of the molecular composition of these structures.

Keywords

chondroitin sulfate proteoglycans; lecticans; hyaluronan and proteoglycan link proteins; hyaluronan; aggrecan; extracellular matrix

1. Introduction

Within the central nervous system, enigmatic structures known as perineuronal nets (PNNs) envelop the cell soma and proximal neurites of particular populations of neurons in a lattice-

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like fashion (Hockfield and McKay, 1983; Zaremba et al., 1989; Guimaraes et al., 1990; Hockfield et al., 1990; Brückner et al., 1993; Celio and Blumcke, 1994; Celio et al., 1998). PNNs are specialized substructures of the neural extracellular matrix (ECM), which is molecularly distinct from other classical matrices, in that it contains low amounts of several traditional ECM components, such as: fibronectin, collagen and laminin (Miner and Sanes, 1994) and is uniquely enriched with chondroitin sulfate proteoglycans (CSPGs) and hyaluronan (for review, see Bandtlow and Zimmermann, 2000). Based on the coincident temporal appearance of PNNs with the maturation and stabilization of synapses (Zaremba et al., 1989; Guimaraes et al., 1990; Hockfield et al., 1990), they are postulated to impact these important developmental processes (Hockfield et al., 1990; for review, see Wang and Fawcett, 2012). However, despite the known existence of PNNs for more than a century, our understanding of their assembly and function has been elusive, due in part to an incomplete understanding of their molecular components and how they interact.

Previous work has identified members of the lectican family of CSPGs, hyaluronan (for review, see Yamaguchi, 2000), tenascins (Celio and Chiquet-Ehrismann, 1993; Wintergerst et al., 1996; Hagihara et al., 1999; Galtrey et al., 2008) and hyaluronan and proteoglycan link proteins (HAPLNs) (Hirakawa et al., 2000; Bekku et al., 2003; Carulli et al., 2006, 2007; Galtrey et al., 2008; Carulli et al., 2010; Kwok et al., 2010) all to comprise the PNN. There is also some evidence for both neuronal and glial contributions to PNN formation (Miyata et al., 2005; Carulli et al., 2006), but their distinct roles remain poorly understood. To understand the function of PNNs it is critical to dissect their molecular composition, which will then enable us to specifically disrupt these structures in a precise manner.

The goal of the present study was to unravel the molecular composition of PNNs to determine the cellular contributions to these structures, gain insights about the role of extrinsic factors such as neural activity and establish the minimal components required to form PNNs. Here we utilized dissociated cortical cultures to determine how chemical glial inhibition and chronic depolarization impacts the expression pattern of PNN components as well as the overall structure of PNNs. To achieve this, we performed immunocytochemistry, Western blotting and real-time PCR analyses. Taken together, our results reveal specific contributions by neurons and glia to the production of individual PNN constituents and provide a more complete understanding of PNN structure.

2. EXPERIMENTAL PROCEDURES

2.1 Animals

Timed pregnant female wild-type CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Brains were removed and used on embryonic day 16 (E16). All experiments were performed in accordance with protocols approved by the State University of New York Upstate Medical University Committee for the Humane Use of Animals.

2.2 Primary Neuronal Culture

Primary neuronal cultures were derived from brains of E16 CD-1 mice essentially as described (Giamanco et al., 2010). Cells were plated in neurobasal medium with 3x B-27, 1x penicillin-streptomycin, and 1x glutaMAX (all from Invitrogen) at a density of 1.4×10^6 cells/ml (500 μ l/well), 2.0×10^6 cells/ml in 100 mm dishes (10 ml/plate), 1.97×10^6 cells/ml in 60 mm dishes (4 ml/plate), or 1.78×10^6 cells/ml in individual wells within a 6-well plate (2 ml/well), depending on the experiment. Coverslips and plates were coated overnight at 37°C with 100 μ g/ml Poly-D-lysine (Sigma-Aldrich) and 5 μ g/ml laminin (Millipore, Billerica, MA, USA) in 1x Dulbecco's phosphate buffered saline. Cells were maintained at 37°C/5% CO₂ until fixation.

2.3 Culture Treatments

On one day *in vitro* (1 DIV) 20 mM potassium chloride (KCl) was added to appropriate plates to achieve a final concentration of 25 mM KCl. Additionally, 5 μ M–10 μ M cytosine- β -D-arabinofuranoside (AraC; Sigma-Aldrich) was added to appropriate plates to inhibit glial growth from 1–3 DIV. At 3 DIV a full media exchange was performed and thereafter, every three days a half media exchange was performed. There were four experimental groups denoted as follows: +KCl +AraC, +KCl –AraC, –KCl +AraC, –KCl –AraC.

2.4 Immunocytochemistry and Antibodies

Cells were fixed between 12–15 DIV with 4% paraformaldehyde in 1x sodium phosphate buffer pH 7.4 for 20 mins at room temperature (RT) and processed as previously described (Giamanco et al., 2010). Cells were stained overnight at 4°C with the following antibodies: anti-aggrecan (rabbit IgG, Millipore, cat. no. AB1031, 1:2000), anti-B50 (50 kDa cleavage fragment of brevican, 1:2000, Matthews et al., 2000), anti-HAPLN1 (goat IgG, R&D Systems, cat. no. AF2608, 1:100), anti-tenascin-R clone 619 (mouse IgG, R&D Systems, cat. no. MAB1624, 1:100), biotinylated hyaluronic acid-binding protein (HABP, EMD Biosciences, San Diego, CA, USA, cat. no. 385911, 1:500), anti-glial fibrillary acidic protein (GFAP, mouse IgG, Sigma-Aldrich, cat. no. G3893, 1:2000), anti-microtubule-associated protein 2 (MAP2, mouse IgG, clone HM2, Sigma-Aldrich, cat. no. M4403, 1:1000), and anti-active caspase-3 (rabbit IgG, R&D Systems, cat. no. AF835, 1:300). After incubation in primary antibody, cells were washed and then incubated with Alexa fluorescence-conjugated secondary antibodies at a concentration of 1:400 for 2 hours at RT. Cells were then washed and incubated with Hoechst 33342 (Molecular Probes, 1:50,000) to stain nuclei for 30 mins at RT.

2.5 Microscopy

Coverslips were imaged using a Nikon Eclipse TE200 (Nikon Instruments Inc., Melville, NY, USA) fluorescent microscope with Spot- RT camera and Spot imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) or on a Zeiss Axio imager A2 (Zeiss, Thornwood, NY, USA) with Nikon Elements Software package. Final images were assembled using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

2.6 Preparation of cell lysates and conditioned media

Cells were harvested for analysis on 8 and 12 DIV and conditioned media was collected as well. Cells were scraped and lysed in 1/3 sodium phosphate buffer pH 7.4 with 1 mini-EDTA free protease tablet (Roche, Indianapolis, IN, USA). Where appropriate, samples were diluted in water with 10x chondroitinase buffer (final concentration: 1x, 400 mM sodium acetate, 400 mM Tris pH 8, and 1 mini-EDTA free protease tablet (Roche)) and chondroitinase ABC protease free (0.2 mU/ μ l, Associates of Cape Cod Inc., Falmouth, MA, USA) for 8 hrs at 37°C. Next, samples were boiled at 100°C for 4 mins with 3x laemmli buffer (final concentration: 1x: 1 M Tris buffer pH 6.8, 1% bromophenol blue, 20% sodium dodecyl sulfate in glycerol).

2.7 Western blotting

Samples were subjected to electrophoresis using 8% sodium dodecyl sulfate-polyacrylamide gels and analyzed by standard methodologies. The following antibodies were used: anti-aggrecan (1:1000), anti-B50 (1:5000), anti-HAPLN1 (1:400), anti-brevican (mouse IgG, BD Biosciences, cat. no. 610895, 1:500), anti-tenascin-R clone 619 (1:500), anti-GFAP (1:2000) and anti- β -actin (mouse IgG, Sigma, cat. no. MABA544, 1:5000). Blots were incubated in primary antibody overnight at 4 °C and then incubated with horseradish peroxidase-coupled secondary antibodies (The Jackson Laboratory, Bar Harbor, ME, USA) at a dilution of

1:2000 for 2 hours at RT. Blots were then washed, incubated for 5 mins at RT with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and exposed to Kodak BioMax MR Film (Sigma-Aldrich).

2.8 RNA Isolation

RNA was isolated from primary cortical cultures at 8 and 12 DIV using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions from $n=3$ separate CD-1 litters harvested on E16. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol, with slight modification. RNA and cDNA concentrations were measured using the Eppendorf Photometer (Eppendorf, Hauppauge, NY, USA). cDNA was diluted to 50 ng/ μ l for use in real-time PCR in molecular biology grade water.

2.9 Real-time PCR

Real-time PCR reactions were carried out using the SYBR Green Master Mix (Roche). Primers were synthesized and purchased at 10 nmole with HPSF purification from Eurofins MWG Operon (Huntsville, AL, USA) and used at a final concentration of 0.5 μ M in each reaction (primer sequences available upon request). For each reaction, 16 μ l of the master mix was combined with 4 μ l (200 ng total) in appropriate wells in a 384-well plate. Each sample was run in duplicates, including a positive and negative control. The following PCR reaction was used: Preincubate (95°C for 10 mins), PCR, 40 cycles (Denature 95°C for 10 sec; Anneal 58–61°C for 20 sec; Extend 72°C for 30 sec), Melting (95°C for 5 sec; 65°C for 1 min; 97°C continuous), and Cooling (40°C for 20 sec).

2.10 Reverse Transcriptase PCR (RT-PCR)

Mixed astrocytes from CD-1 mice were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA) and propagated according to standard protocol and recommendations by the manufacturer. RNA was isolated from mixed astrocytes from CD-1 mice and cDNA was generated as described 2.8. cDNA was diluted to 1 μ g/ μ l in molecular biology grade water for use in reverse transcriptase PCR (RT-PCR). Each reaction contained the following: 10x PCR buffer (2.5 μ l), 10 mM dNTP mix (0.5 μ l), 50 mM magnesium chloride (0.75 μ l), 10 μ M forward and reverse primers (0.5 μ l each), Platinum Taq (0.2 μ l) and 500 ng cDNA in a final volume of 25 μ l. Samples were run for 30 cycles and analyzed by electrophoresis using 8% polyacrylamide non-denaturing gels stained with ethidium bromide.

2.11 Cell Counts

Cells stained for GFAP and activated caspase-3 were counted at 10X in 5 non-overlapping fields per coverslip at both 8 and 12 DIV in all conditions. Counts were averaged and the number divided by the area (in mm²) of a counting field. Cells with aggrecan-reactive PNNs were counted in 8 non-overlapping fields per coverslip at 20X and otherwise the numbers were calculated as above. Statistical analyses were then conducted as described below.

2.12 Data Analysis

Western blot films were scanned using an Epson Expression 1600 Scanner (Epson, Long Beach, CA, USA) at 8-bit grayscale and 600 dots per inch. Final figures were assembled using Adobe Photoshop 7.0 (Adobe Systems). Real-time PCR reactions were run using three biological replicates for each primer pair. Generated Ct values were first normalized to the geometric mean of the control genes, 18S and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), to yield Δ Ct values. Next, for GFAP, aggrecan, brevican, and HAPLN1 results were normalized to the 8 DIV untreated

control (–KCl –AraC) in order to determine the $\Delta\Delta$ Ct value. For the remaining genes, results were normalized to the 12 DIV untreated control (–KCl –AraC). For GFAP, aggrecan, brevican, and HAPLN1 a 2-way repeated measures ANOVA was performed with a $p < 0.05$ considered significant. For the remaining genes, data was analyzed using a 1-way repeated measures ANOVA with a $p < 0.05$ considered significant. Lastly, *post-hoc* analyses were carried out using a pair-wise comparison Bonferroni *t*-test, where a $p < 0.05$ was deemed significant. For presentation purposes, the $\Delta\Delta$ Ct values were used to calculate the mean fold change by employing the following formula: $2^{(-\Delta\Delta Ct)}$. Standard error measurements were calculated with $n=3$. Paired ANOVAs were also utilized to analyze the cell counting data and *post-hoc* test conducted as described above.

3. RESULTS

3.1 Media addition of AraC plus elevated KCl most effectively reduces glia

Our first goal was to define conditions to inhibit glial cell growth in our established primary cortical culture model (Giamanco et al., 2010) to better understand the contribution of glia to PNN formation. This was achieved using AraC alone (Wallace and Johnson, 1989; Martin et al., 1990; Dessi et al., 1995), or AraC in combination with elevated KCl. Untreated control cultures maintained for 12 DIV developed a network of GFAP-positive cells, consistent with growth and proliferation of astrocytic glia (Fig. 1B, F). As expected, application of AraC (from 1–3 DIV) dramatically reduced GFAP-immunoreactivity and the number of glial cells. The number of GFAP-reactive cells was decreased by more than 90% at both 8 and 12 DIV (Fig. 1A). The few remaining GFAP-positive cells that remained were found in isolated pockets and appeared morphologically different, ie: less branching (Fig. 1C, G). Cells grown in elevated KCl alone exhibited ample GFAP-reactivity and the branching was more extensive in comparison to untreated control cultures (Fig. 1D, H), but there was no change in the number of GFAP-reactive cells (Fig. 1A). Importantly, the combination of elevated KCl plus AraC virtually abolished GFAP-immunoreactivity (Fig. 1A, E, I). Therefore, we used AraC alone to substantially reduce glia in the cultures and co-application of elevated KCl plus AraC was utilized to eliminate glia. Moreover, since medium supplementation with elevated KCl has been used to model *in vivo* bioelectric activity (Lasher and Zagon, 1972; Miller and Johnson, 1996; Fickbohm and Willard, 1999), we reasoned it could accelerate neuronal maturation and activity-dependent expression of PNN components and therefore, included a group receiving elevated KCl alone.

To corroborate our immunocytochemical findings, we performed Western blot and real-time PCR analyses. At both 8 and 12 DIV GFAP immunoblot reactivity was eliminated with AraC treatment, irrespective of the co-application of elevated KCl (Fig. 1J). Similarly, real-time PCR analyses showed dramatic reductions in GFAP mRNA with AraC addition either with or without elevated KCl at 12 DIV (–5.21-fold, $p=0.031$ and –5.6-fold, $p=0.024$ respectively) and at 8 DIV with the combination of AraC and KCl (–4.84 fold, $p=0.042$; Table 1) relative to the respective untreated control cultures. Consistent with our immunocytochemistry studies, GFAP mRNA was not reduced as dramatically by AraC alone at 8 DIV (–2.98 fold, $p=0.265$). Together, our data strongly demonstrates that elevated KCl and AraC administration virtually abolishes the GFAP-positive population of cells.

3.2 Neurons are not damaged by KCl and AraC application

Since we had established conditions in which glial cells were inhibited and cells were chronically depolarized, we next investigated the impact of these treatments on neuronal cell health. To evaluate this, cells were stained for MAP2, to visualize the basic morphology of the neurons and for active caspase-3 to assess cell death (Fig. 2A–D). Neurons in all groups appeared healthy and exhibited normal morphology (Fig. 2A–D). To further analyze

neuronal viability, cells labeled for active caspase-3 were analyzed quantitatively by cell counting. Overall, the staining showed a low level of ongoing cell death (less than 0.5% of the cells) in control conditions and there were no significant changes in the number of caspase-3-labeled cells in any experimental conditions at either 8 or 12 DIV (Fig. 2E). Furthermore, the rate of cell death remained constant across experimental time points. Therefore, we established experimental conditions that enabled us to reduce glial contributions to matrix and chronically depolarize neurons that do not negatively impact neuronal cell viability.

3.3 Aggrecan expression is neuron specific, attenuated by glia, and enhanced by elevated KCl

Our previous studies identified aggrecan as a key activity-dependent component of PNNs (McRae et al., 2007; Giamanco et al., 2010). Herein, we determined the effects of glial inhibition and/or elevated KCl application on aggrecan expression, secretion, and incorporation into PNNs. PNNs reactive for aggrecan were barely detectable in 12 DIV control cultures (Fig. 3A) thereby indicating that at this early developmental stage little protein was made and aggrecan-positive PNNs were just beginning to assemble. Interestingly, application of AraC or elevated KCl alone or in combination dramatically increased the overall intensity of aggrecan-staining and the number of neurons with aggrecan-reactive PNNs (Fig. 3B–D). These results were quantified by cell counts at 12 DIV (Fig. 3E). Note other than the absence of staining in the control group there were no significant difference in the number of reactive cells between groups.

The observed increase in aggrecan-labeled PNNs could have resulted from increased aggrecan assembly with or without a concomitant increase in aggrecan expression. To better understand the precocious formation of aggrecan-reactive PNNs, first we analyzed aggrecan protein levels in cell lysates versus the levels secreted into the culture media by Western blotting analysis. At 8 and 12 DIV AraC treatment with or without elevated KCl dramatically increased aggrecan protein levels in both cell lysates and conditioned media (Fig. 3F, G). Treatment with elevated KCl alone also led to increases in both cell lysates and conditioned media samples at 12 DIV, but only in the media at 8 DIV. To further understand the effects on aggrecan expression we performed real-time PCR analysis. Consistent with the other work, treatment of cultures with elevated KCl alone and AraC alone or in combination resulted in robust increases in aggrecan message at both 8 DIV (22.83-fold, $p < 0.001$; 55.94-fold, $p < 0.001$; 46.09-fold, $p < 0.001$, respectively) and 12 DIV (62.19-fold, $p < 0.001$; 76.58-fold, $p < 0.001$; 93.82-fold, $p < 0.001$, respectively) relative to the respective untreated controls (Table 1). Altogether, these data indicate that aggrecan expression and incorporation into PNNs are neuron-specific events that are accelerated when glia are reduced or virtually absent and also are enhanced by neural activity. The profound induction of aggrecan mRNA in response to elevated KCl or addition of AraC further indicates that it is transcriptionally regulated.

3.4 Brevican and HAPLN1 are expressed in a glial-dependent manner

3.4.1 Brevican—Brevican, like aggrecan, belongs to the lectican family of CSPGs. Similar to aggrecan it has been detected in PNNs (Bekku et al., 2003; Carulli et al., 2006, 2007), but in contrast with aggrecan, it is thought to be primarily made by glia instead of neurons (Yamada et al., 1994, 1997; Carulli et al., 2006; John et al., 2006). Therefore, we next asked how brevican expression and incorporation into PNNs is affected by the reduction and virtual elimination of glia. The presence of AraC, with or without elevated KCl, completely eliminated brevican-reactivity and no neurons with brevican-reactive PNNs were observed, relative to untreated control cultures (Fig. 4D, B, and A, respectively). In contrast, cells treated with elevated concentrations of KCl alone demonstrated brevican-positive PNNs that

were indistinguishable from untreated control cells and moreover, there was no obvious change in the number of neurons with brevicin-based PNNs (Fig. 4C).

The essentially complete elimination of brevicin-reactive PNNs could be explained by either reduced expression of brevicin protein or impaired ability to bind brevicin to the cell surface and to assemble into PNNs. To distinguish between these two possibilities, we performed Western blot analyses. All brevicin-reactive bands were eliminated in both the cell lysates and the conditioned media with AraC treatment, with or without the co-application of elevated KCl, at both 8 and 12 DIV (Fig. 4E, F). These findings were corroborated by real-time PCR, which essentially showed the elimination of brevicin mRNA with AraC at both timepoints (more than 20-fold reduction; Table 1). Elevated KCl alone did not dramatically alter expression of any form of brevicin at either time point in both cell lysates and conditioned media, except for a slight increase in the 50 kDa cleavage fragment in the cell lysates at 8 DIV. One conspicuous difference was the low or absent staining for the 50 kDa cleavage fragment within the cell lysates of samples not treated with AraC at 8 DIV versus the abundant staining at 12 DIV, which may suggest a developmental shift in brevicin cleavage, which is known to influence its function (Zhang et al., 1998; Matthews et al., 2000; Hu et al., 2008). Contrasted with the aggrecan findings, these data reveal the complex differences in the cellular source of different components in PNN assembly, even when these components are from the same family of proteins.

3.4.2 HAPLN1—HAPLN1s are a family of molecules that are thought to strengthen the interaction between CSPGs, chiefly, the lectican family members, and the hyaluronan scaffold. HAPLN1, in particular plays an important role in PNN formation (Carulli et al., 2010; Kwok et al., 2010), therefore, we studied the expression in detail in our system. Immunostaining of untreated cortical cultures at 12 DIV with anti-HAPLN1 antibodies revealed the formation of HAPLN1-positive PNNs (Fig. 5A). Application of AraC or elevated KCl alone did not change the expression of HAPLN1 appreciably (Fig. 5B, C). Interestingly, the co-administration of elevated KCl and AraC led to the complete abolishment of neurons with HAPLN1-reactive PNNs (Fig. 5D). Therefore, only in instances where glial cells were eliminated were HAPLN1-positive PNNs affected. While brevicin-reactivity was more sensitive to AraC treatment, these data also indicate that the expression of HAPLN1 is glial-dependent.

Consistent with our immunostaining data, Western blot analyses of cell lysates at 12 DIV revealed no detectable protein in cells treated with elevated KCl and AraC (Fig. 5E). At 8 DIV there was also no HAPLN1-reactivity in doubly treated cells, however, it was also absent from control cells (Fig. 5E). Chronic depolarization with KCl increased HAPLN1 protein expression, as surprisingly, did AraC treatment at 8 DIV, however, by 12 DIV, there was no significant difference between these groups and control cells (Fig. 5E). We did not find HAPLN1 protein within our conditioned media samples (data not shown) in any condition at any time point, indicating that detected changes in HAPLN1 are not the result of differences in surface binding or assembly, but direct differences in protein expression. Consistent with the protein analysis, robust glial inhibition, through application of elevated KCl and AraC, significantly reduced HAPLN1 transcript at 12 DIV (-5.08 -fold, $p=0.05$; Table 1), relative to untreated control cultures. Therefore, virtual elimination of glia led to dramatic decreases in HAPLN1 protein and message, supporting our conclusion that HAPLN1 is likely made in a glial-dependent manner.

3.5 Hyaluronan expression is likely expressed in both a glial- and neuronal-dependent manner

Hyaluronan serves as the backbone of the neural ECM and specifically, as the backbone of the PNN and therefore, we next analyzed expression of hyaluronan in our culture system. Hyaluronan localization was visualized utilizing a biotinylated HABP. In untreated control cells, HABP bound diffusely throughout the cultures and additionally was found in pericellular aggregates surrounding subsets of neurons (Fig. 6A). Cells that were treated with elevated KCl alone or AraC alone showed no dramatic alterations in HABP-reactivity or the number of cells with pericellular aggregates that expressed hyaluronan (Fig. 6C, B, respectively). However, interestingly, co-application of elevated KCl and AraC resulted in the elimination of the diffuse component of hyaluronan, while the pericellular aggregates persisted (Fig. 6D). Therefore, in the complete absence of glial cells, HABP still detected PNNs, indicating that this component of hyaluronan is likely synthesized by neurons. However, this staining pattern was different than the pattern noted when glia were present, indicating that perhaps components of hyaluronan have different cellular origins. In sum, these results indicate that both neurons and glia play a role in the production of hyaluronan.

3.6 Tenascin-R is expressed in a glial-dependent manner

Like HAPLN1, tenascin-R is believed to be a key component in the formation and structure of PNNs (Brückner et al., 2000) and therefore, we examined how tenascin-R expression was affected by glial inhibition and chronic depolarization. We analyzed tenascin-R-immunoreactivity at 15 DIV instead of 12 DIV, the time point at which we analyzed other components, due to the fact that tenascin-R-reactive PNNs develop somewhat later in our culture system than the aforementioned PNN constituents. Samples were additionally stained with HABP to show the co-localization of tenascin-R on HABP-stained PNNs. At 15 DIV cultures treated with AraC alone showed dramatic reductions in the intensity of tenascin-R-reactivity, relative to the untreated controls, but there was no effect on the number of neurons surrounded by a tenascin-R-positive PNN (Fig. 7A, B). Still however, the tenascin-R reactivity co-localized with HABP both in controls and in the AraC-treated cells. The combinatorial treatment of elevated KCl and AraC resulted in the abolishment of tenascin-R-reactivity and no neurons with tenascin-R-positive PNNs were detected, even though PNNs detected with HABP were still present (Fig. 7D). Treatment with elevated KCl alone had no effect (Fig. 7C). Protein analysis revealed that at 8 DIV low levels of tenascin-R protein were detected in cell lysates of untreated samples, while reactivity was reduced in samples treated with AraC alone and completely eliminated in samples that were treated with elevated KCl and AraC (Fig. 7E). Importantly, tenascin-R protein was not detectable in conditioned media (data not shown). To further investigate the regulation of tenascin-R in our system we turned to real-time PCR (Table 2). Because of the late expression of tenascin-R in our cultures, we focused our analysis on 12 DIV. Generally, at 12 DIV there were decreases in message levels following elevated KCl and AraC application (−1.47 fold) and AraC application alone (−1.44 fold), compared to the untreated control, but there was no overall significant treatment effect on tenascin-R transcript levels.

3.7 Effect of chronic depolarization and glial inhibition on PNN components by real-time PCR

To analyze a broader spectrum of molecules we turned to real-time PCR. The goal of this work was to determine if we could identify a molecule or group of molecules that are critical for the organization of the PNN at the cellular surface. To date, this identification still eludes us and is a critical question that remains unanswered in understanding how PNN construction occurs.

3.7.1 Expression of CSPGs—First we wanted to determine how the other members of the lectican family, neurocan and versican, which have also been identified in PNNs (for review, see Yamaguchi, 2000) were altered by chronic depolarization and glial inhibition. AraC alone or the combination of elevated KCl and AraC, caused appreciable decreases in neurocan transcript levels, relative to the untreated control (−1.63-fold change, $p=0.031$; −1.83-fold change, $p=0.022$, respectively, Table 2). Treatment with elevated KCl or AraC alone or in combination also showed a trend towards reducing levels of versican transcript at 12 DIV (−1.23-fold, −1.51-fold, and −3.58-fold, respectively), but these results were not significant ($p=0.214$, Table 2). Our results do highlight a potential role of glial cells in the production of neurocan and versican, however, since transcript levels were not significantly reduced, we cannot rule out a role for neurons in production of versican.

We also examined the expression pattern of phosphacan, another CSPG. Treatment of the cultures with AraC alone or elevated KCl and AraC resulted in a trend towards a decrease in phosphacan transcript levels (−5.41-fold and −6.70-fold, respectively, Table 2), but the overall treatment effect did not reach significance ($p=0.097$). Interestingly, there was also a trend in a decrease of phosphacan transcript levels following administration of depolarizing concentrations of KCl (−2.65-fold change).

These studies provided us with two main conclusions. First, aggrecan is the only CSPG investigated that is dramatically up-regulated by chronic depolarization and glial inhibition. Second, most of the other CSPGs are predominantly expressed by glia. The combined treatment of elevated KCl and AraC generally reduces or eliminates all CSPGs assayed, but aggrecan.

3.7.2 Expression of HAPLNs—Previous studies showed that PNNs do not form appropriately in the absence of HAPLN1 (Carulli et al., 2010). As we detailed above with glial inhibition HAPLN1-reactivity was essentially eliminated, but aggrecan- and hyaluronan-reactive PNNs still formed. We therefore, next aimed to determine if another HAPLN family member (HAPLN2-4) could take the place of HAPLN1 in cultures in which glia were inhibited, thereby contributing to PNN formation. While HAPLN2 could be a strong candidate for replacing the function of HAPLN1, there were not detectable levels of the HAPLN2 transcript in our samples (data not shown). Therefore, within our defined culture system, HAPLN2 cannot compensate for HAPLN1 in its absence.

We next investigated the expression of HAPLN3 and HAPLN4 in our culture system. Treatment with AraC alone or in combination with elevated KCl significantly reduced the expression of HAPLN3 message (−5.77-fold, $p=0.008$; −4.61-fold, $p=0.017$, respectively, Table 2), relative to the untreated control cultures. Treatment with AraC alone or in combination with elevated KCl demonstrated a trend towards a reduction in HAPLN4 message (−1.90-fold and −2.31-fold respectively, $p=0.25$, Table 2).

Overall, our data suggest that all expressed HAPLN family members in our culture system are likely made in a glial-dependent manner and that none can likely compensate for the loss of HAPLN1 with the combined treatment of elevated KCl and AraC. It therefore, seems aggrecan- and hyaluronan-based PNNs can form in the absence of an associated HAPLN.

3.7.3 Effect of chronic depolarization and glial inhibition on the expression of HAS3—Three hyaluronan synthases (HAS1-3) are responsible for the production of hyaluronan. It has been postulated that HAS3 plays a critical role in tethering hyaluronan to the cellular surface in neurons and therefore, may be critical for the formation of PNNs (Carulli et al., 2006, 2007; Kwok et al., 2010). Given the suggested role this enzyme may play in PNN formation, we analyzed the message levels of HAS3 in our culture system

(Table 2). We found that at 12 DIV application of elevated KCl (2.23-fold), AraC (2.09-fold), or the combination of elevated KCl and AraC (1.74-fold), did not lead to statistically significant alterations in the message levels of HAS3 ($p=0.329$). Therefore, from this work, it seems that glial inhibition did not alter HAS3 transcript levels, thereby indicating, that this enzyme is likely to be synthesized in a neuron-dependent manner. Our immunocytochemistry results demonstrated that glial inhibition resulted in alterations in the expression pattern of hyaluronan, in that the diffuse process staining was eliminated, while the PNN staining remained. In light of our real-time results, these data suggest that perhaps HAS3 could contribute to the neuronal component of hyaluronan.

3.8 Expression of PNN components by glia

3.8.1 Real-time PCR analysis of cellular markers—Due to the fact that we detected such robust alterations in aggrecan protein and transcript levels, we next aimed to determine if the cell types that are normally decorated with a PNN were altered by our treatments. First, we sought to analyze parvalbumin transcript levels. Parvalbumin is the classic marker of PNN-bearing neurons in the cerebral cortex (Celio and Chiquet-Ehrismann, 1993; Härtig et al., 1999, 2001). Unfortunately, at 12 DIV parvalbumin transcript was not detected by PCR in our cultures (data not shown). Instead we analyzed expression of Kv3.1. Kv3.1 is a voltage-gated potassium channel and it has been reported that parvalbumin-immunoreactivity and Kv3.1b, a subunit of voltage-gated potassium channels, immunoreactivity overlap (Härtig et al., 1999). Importantly, mRNA for Kv3.1b co-localizes with the mRNA for parvalbumin and glutamic acid decarboxylase (GAD) (Lenz et al., 1994). We determined that at 12 DIV our culture treatments (elevated KCl and AraC, elevated KCl, and AraC) did not alter the Kv3.1 transcript levels, most likely indicating that the number of Kv3.1-positive cells or cells that would be enwrapped by PNNs were not altered (Table 2; $p=0.168$).

We next analyzed message levels for GAD67, which is the rate-limiting enzyme responsible for the synthesis of GABA (for review, see Huang, 2009) and therefore, is a good marker of the maturation and functional state of interneurons. Our experiments showed that elevated KCl, AraC, as well as the combination of treatments led to robust increases in GAD67 transcript levels (5.22-fold change, $p=0.013$; 5.95-fold change, $p=0.008$; 4.14-fold change, $p=0.022$, respectively, Table 2), relative to untreated control cultures. Expression of GAD67 is regulated in an activity-dependent manner in interneurons (Patz et al., 2003; Kinney et al., 2006) and is up-regulated as GABAergic neurons mature. Interestingly, all the statistically significant changes were relative to the control and we did not find any significant alterations between our treatment groups. These results match quite well with the prominent increases in aggrecan message within our three treatment groups and perhaps indicate that acceleration of the maturation of GAD67-positive interneurons is coordinated with the maturation of aggrecan-positive PNNs.

3.8.2 PCR analysis of PNN markers—To more definitively determine if certain PNN components are expressed by glia, we isolated RNA from mouse mixed astrocytes and analyzed transcript levels of known PNN components through RT-PCR. We used GFAP as a positive control and then determined that these astrocytic glial cells express HAPLN1, tenascin-R, brevican, neurocan, versican, and phosphacan transcripts (Fig. 8). These results are in corroboration with the data presented demonstrating that most assayed PNN components are produced in a glial-dependent manner. We were unable to detect any aggrecan transcript in these astrocytes, thereby affirming our conclusion that aggrecan is likely made by neurons. Interestingly, in support of our real-time PCR data, HAS3 transcripts were undetectable in mixed astrocytes, thereby indicating that this HAS is likely responsible for the production of the neuronal component of hyaluronan. Therefore, analysis

of mixed astrocytes confirmed that most assayed PNN components are likely produced by glia, while uniquely aggrecan and HAS3 are made by neurons.

4. Discussion

To date a thorough analysis of the cellular contributions to PNN formation has been lacking. Additionally, the precise determination of how these components aggregate at the cellular surface has eluded us. Therefore, in this study we utilized methods to inhibit glia and mimic key features of cellular activity through chronic depolarization to determine the cellular source of specific components found in PNNs and to better understand the activity-dependent nature of PNN expression by modifying our previously defined culture model (Giamanco et al., 2010). Given the complexity of the extracellular environment *in vivo*, many laboratories have opted to utilize culture models to study PNN formation and development (Brückner and Grosche, 2001; Miyata et al., 2005; John et al., 2006; Dityatev et al., 2007; Reimers et al., 2007). More specifically, use of dissociated cortical cultures derived from embryonic rodent cortical tissue has been exploited to examine the molecular composition and development of PNNs (Miyata et al., 2005). Our results demonstrate an intriguing and complex interplay between neurons and glia in the formation of these structures and suggest that aggrecan is likely the key activity-dependent component of the PNN. It is important to note, that in the work presented herein, we have focused on PNN formation and development around GABAergic interneurons due to the fact that within the cerebral cortex PNNs are predominantly found surrounding this subclass of neurons (Celio and Chiquet-Ehrismann, 1993; Härtig et al., 1994, 1999; Schüppel et al., 2002). However, within particular areas of the neocortex of rodents, PNNs have been found to surround pyramidal neurons (Alpár et al., 2006).

4.1 Aggrecan is made by neurons and is expressed in a highly regulated manner

Our previous studies indicated that aggrecan is made primarily by neurons (Matthews et al., 2002) and is a key activity-dependent component of the PNN (McRae et al., 2007). Here we add support to this hypothesis and extend this work by analyzing the formation of aggrecan-reactive PNNs in the virtual absence of glia through chronic depolarization and administration of AraC. More specifically, we suggest that aggrecan is profoundly up-regulated and the most responsive component of PNNs to physiological perturbations that mimics aspects of neuronal activity. While chronic depolarization can lead to a number of molecular changes (Vallano et al., 1996; Oberdorf et al., 1997; Mellor et al., 1998; Tremper-Wells et al., 2002; Bui et al., 2003; Moulder et al., 2003), one of the key effects is altered calcium homeostasis that mimics bioactivity in neurons (Franklin et al., 1992; Fickbohm and Willard, 1999). Therefore, in this context one could interpret our data in terms of a modulation of cellular activity. Consistent with this hypothesis, GAD67 transcript levels were appreciably increased following elevated KCl and AraC application. Work from a number of laboratories has demonstrated that GAD67 is regulated in a highly activity-dependent manner (Patz et al., 2003; Kinney et al., 2006; for review, see Huang, 2009). Therefore, in light of our results, it is possible that we are accelerating the maturation of synapses following application of elevated KCl and AraC, which is potentially coordinated with the formation of aggrecan-based PNNs. It is also interesting to note that inhibition of glia with AraC alone also produced a similar magnitude of change in aggrecan expression and GAD67 levels, which is surprising, given the role of glia in supporting synaptic maturation. Understanding the mechanism by which glial inhibition versus chronic depolarization enhances aggrecan expression will be a focus of future work.

The dramatic alterations in the expression of different PNN components in our studies were generally correlated with changes in mRNA expression and, therefore, we have interpreted these results primarily in terms of altered transcriptional regulation. However, it is important

to note that the ECM molecules that comprise PNNs are also regulated by a number of post-translational mechanisms (for review, see Viapiano and Matthews, 2006). For example, ECM remodeling via proteolytic processing is particularly important in regulating the developmental functions of the ECM (for review, see Ethel and Ethel, 2007). Therefore, it is possible that inhibition of glial cells and/or chronic depolarization altered the ECM via post-transcriptional mechanisms in addition to the transcriptional mechanisms noted in our work.

4.2 Use of AraC and depolarizing concentrations of KCl to inhibit glial growth

Interestingly, we found that the combination of depolarizing concentrations of KCl and AraC inhibited glia much more effectively and completely than AraC alone. While the mechanism of action for this is unclear, we believe AraC inhibits glial cell growth and the remaining glia that do survive are likely detrimentally effected by the presence of depolarizing concentrations of KCl. This finding enabled us to complete a more thorough analysis of the neuronal versus glial contributions to PNN structure and therefore, became a key tool in this study.

4.3 Glial contribution to perineuronal net formation

With the notable exceptions and HAS3 (discussed below) and aggrecan, our data suggested that most of the other PNN components were expressed in a partially or completely glial-dependent manner. Consistent with this work we found that all transcripts other than aggrecan and HAS3 are expressed by glia. Therefore, the most likely explanation for our findings is the direct glial-expression of these PNN components, either from astroglia, which we have focused on in this manuscript, or oligodendrocytes or oligodendrocyte precursor cells, which likely also contribute in our culture system. However, we cannot exclude the alternative explanation that glial cells instead induce neuronal expression of certain matrix components, and therefore in the absence of glial cells, neurons no longer express these matrix molecules. Future studies will be directed at distinguishing between these different possibilities. However, we identified three basic patterns of glial-dependent expression, the first being proteins and transcripts virtually completely abolished by AraC alone, highlighted by brevican and HAPLN3; the second being proteins and transcripts reduced by AraC, but more effectively eliminated by the combination of KCl and AraC, highlighted by tenascin-R, neurocan, and phosphacan; the final being proteins and transcripts relatively unaffected by AraC alone, but dramatically reduced by the combination of elevated KCl and AraC, highlighted by HAPLN1. Overall, our data indicate that the neuronal ECM is made in a largely co-dependent manner with glial cells, with these individual cell types contributing different key components of the structure to the surface of neurons.

In an attempt to understand how components arrange to form PNNs and the manner in which they are attached to the cell surface, investigators have hypothesized that HAS3, one of the enzymes responsible for the synthesis of hyaluronan, is the critical molecule responsible for tethering hyaluronan to the surface (Carulli et al., 2007; Galtrey et al., 2008; Kwok et al., 2010). Due to the fact that our culture treatments did not alter the transcript levels of HAS3, we cannot rule out that HAS3 is the nucleating factor responsible for the organization of hyaluronan and therefore, organization of the PNN. Furthermore, from these results it seems likely that HAS3 is the neuronal source of hyaluronan.

4.4 Formation of perineuronal nets in vitro

One of the main goals of this study was to elucidate the source of different components in PNNs in order to better understand its molecular structure. Previous studies have shown, for example, that tenascin-R and HAPLN1 play important roles in the formation of PNNs (Brückner et al., 2000; Carulli et al., 2010). However, in our study the combination of elevated KCl and AraC essentially eliminated the expression of both of these components of

PNNs. Interestingly, in these conditions aggrecan-reactive pericellular aggregates still formed in our cultures suggesting that neither component is required for the nucleating event in PNN formation.

The formation of aggrecan-based pericellular coats in the absence of HAPLN1 and tenascin-R was a surprise. We hypothesized that perhaps another HAPLN family member could be expressed in the absence of HAPLN1 to serve to aggregate these aggrecan-reactive structures. We determined that under conditions in which cells were treated with elevated KCl and AraC to effectively inhibit glial cell growth, no HAPLN family member compensated for HAPLN1 in its absence. Thus, aggrecan is able to condense on the cell surface without the presence of a HAPLN molecule. Similarly, brevican was lost by the treatment of AraC alone or in combination with elevated KCl and we found the up-regulation of no other CSPG, but aggrecan that could compensate for its loss.

We therefore determined to define what known components of PNNs were still present in the doubly treated cultured neurons in addition to aggrecan. The only other PNN component we could still detect was hyaluronan. While the amount of hyaluronan detected was clearly reduced by elevated KCl and AraC, hyaluronan and aggrecan formed pericellular coats with the classic appearance of PNNs remaining unchanged, thereby indicating that these two molecular components are sufficient to form the base structure of PNNs. It should be noted that while elimination of HAPLN1 (Carulli et al., 2010) and tenascin-R (Brückner et al., 2000) both were previously shown to affect PNN structure, our data that aggrecan and hyaluronan pericellular coats form in their absence is not necessarily in conflict with these studies. While PNN staining is reduced and structurally altered in these knockouts the cell surface binding of PNN components is not altered (Brückner et al., 2000; Carulli et al., 2010). Our studies focused on the nucleating event in PNN formation, which appears to depend on aggrecan and hyaluronan; however, it is likely that HAPLN1 and tenascin-R are critically involved in contributing to the further aggregation and elaborating the three-dimensional structure of PNNs, which could not be assessed in detail in our studies.

Work on PNNs *in vivo* and *in vitro* has highlighted the molecular diversity of these structures (Lander et al., 1997, 1998; Matthews et al., 2002; John et al., 2006) and certainly such diversity is found in our culture system. For example, we observed that brevican is always detected on HAPLN1-reactive neurons, while aggrecan-reactivity partially overlaps with HAPLN1 but rarely with brevican (data not shown). Therefore, we believe there are at least two molecularly distinct PNN substructures within our culture system: one that is brevican-immunoreactive and the other that is immunoreactive for aggrecan. Interestingly, brevican and HAPLN1 are the first molecular constituents to be expressed within this system, while tenascin-R and aggrecan temporally appear later in development of these cultures. While these temporal patterns of expression do not perfectly match *in vivo* data, they do provide important insights into PNNs development. The studies presented herein revealed that in the virtual absence of glia, only two molecular components of PNNs persist: aggrecan and hyaluronan, and highlights a particularly important role for hyaluronan in the formation of the base PNN structure.

Our previous work in dissociated cultured neurons called into question the current model of PNN molecular structure (Giamanco et al., 2010). This work suggested that hyaluronan is bound directly to the cell surface, in contrast to the prevailing model, which indicates that a lectican and an associated HAPLN bind hyaluronan to the cell surface (for review, see Viapiano and Matthews, 2006). Given our current work we are therefore left with the question of how and to what hyaluronan binds to on the surface of a subset of interneurons. One hypothesis is that HAS3, one of the enzymes responsible for the synthesis of hyaluronan, is the critical molecule responsible for tethering hyaluronan to the surface

(Carulli et al., 2006, 2007; Galtrey et al., 2008; Kwok et al., 2010). As noted above our data are consistent with this conclusion. Therefore, in its absence of another viable mechanism for tethering hyaluronan to the cell surface, our data supports the concept that HAS3 may be the nucleating factor for PNNs. Proof of this hypothesis will be the goal of future studies.

Acknowledgments

We would like to thank Dr. Mary Lou Vallano for her advice in experimental design, helpful discussions, and comments during the preparation of the manuscript. We also acknowledge Dr. Eric Olson for advice and comments during the writing of this manuscript. Additionally, we would like to thank Wendi Burnette for her technical assistance. This work was supported by NIH grant R01NS069660.

Abbreviations

AraC	cytosine- β -D-arabinofuranoside
B50	50 kDa cleavage fragment of brevican
CS	chondroitin sulfate
CSPGs	chondroitin sulfate proteoglycans
DIV	days <i>in vitro</i>
E	embryonic day
ECM	extracellular matrix
GAD	glutamic acid decarboxylase
GFAP	glial fibrillary acidic protein
HABP	hyaluronic acid-binding protein
HAPLN	hyaluronan and proteoglycan link protein
HAS	hyaluronan synthase
KCl	potassium chloride
MAP2	microtubule-associated protein 2
PNN	perineuronal net
RT	room temperature
RT-PCR	reverse transcriptase PCR
YWHAZ	tyrosine 3-monooxygenase tryptophan 5-monooxygenase activation protein, zeta polypeptide

Glossary

Chondroitin sulfate proteoglycan	Specialized glycoproteins that consist of a protein core that is decorated with chondroitin sulfate (CS) chains. These CS chains are comprised of repeating sugar units: N-acetyl galactosamine and glucuronic acid. Importantly, these CS chains can be sulfated in a variety of positions, further contributing to the molecular heterogeneity of CSPGs.
Hyaluronan	Provides the scaffolding of the neuronal extracellular matrix and by extension, the perineuronal net. Hyaluronan is a polymer consisting of repeating sugar units of glucosamine and glucuronic acid.

Hyaluronan and proteoglycan link proteins (HAPLNs)	A family consisting of four members that play an integral role in strengthening the interaction between lectican family members and the scaffolding of hyaluronan.
Hyaluronan synthases (HASs)	Transmembrane enzymes responsible for the synthesis of hyaluronan. These three enzymes are postulated to extrude hyaluronan and retain hyaluronan at the cellular surface, thereby organizing the extracellular space.
Lectican	Family of CSPGs with four members: aggrecan, neurocan, brevican, and versican. These CSPGs bind to hyaluronan at their N-termini and cell-surface molecules such as tenascins at their C-termini.
Perineuronal nets (PNNs)	Specialized substructures of the neuronal extracellular matrix. This unique structure enwraps the cell soma and proximal neurites of patterned subsets of neurons within the central nervous system.
Tenascins	Tenascins are cell-surface molecules and interact with lectican family members, thus serving to organize the extracellular space.

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***Highlights**

- Most assayed PNN components are produced in a glial-dependent manner.
- Aggrecan and subcomponents of hyaluronan are produced by neurons.
- Aggrecan is produced in an activity-dependent manner.
- Aggrecan and hyaluronan are necessary for the base formation of PNNs.

Fig. 1.

Application of 25 mM KCl and AraC effectively inhibits glial cell growth. (A) The number of GFAP-reactive cells at 8 and 12 DIV treated with or without AraC and KCl are shown graphically. Treatment with AraC alone reduced GFAP-reactivity by more than 90% at both 8 and 12 DIV and the combination of AraC and elevated KCl effectively eliminated GFAP-reactive cells. There were no differences in the number of GFAP-reactive cells with KCl treatment alone at either time point. Note the number of GFAP-reactive cells increased significantly between all groups between 8 and 12 DIV. In no condition was there a change in the number of Hoechst-stained cells, indicating no change in total cell numbers due to our various treatments (data not shown). * $p < 0.001$ compared to control, # $p < 0.01$ compared to treatment with AraC alone, $N = 5$. (B–E) Low-magnification images and (F–I) high-magnification images, where the white boxes outlined in B–E indicate the portion that is shown in F–I. (B, F) A network of GFAP-positive cells (green) were found in the untreated

controls and the Hoechst-immunoreactivity (blue) highlights nuclei to outline the total cell population. (C, G) AraC treatment as noted above dramatically the number of GFAP-reactive cells, but isolated positive cells persisted. Interestingly, the GFAP-positive cells that persisted displayed truncated branches and were phenotypically different than GFAP-immunoreactive cells that were present under the other culture conditions. (D, H) Elevated KCl treatment did not alter GFAP-immunoreactivity. (E, I) Treatment with elevated KCl and AraC eliminated GFAP-reactivity. Scale bars: 20 μ m. (J) Cell lysates were harvested at 8 and 12 DIV and subjected to Western blot analyses. GFAP protein was detected in untreated control samples as well as in samples where cells were treated with elevated KCl alone, but was absent from those treated with AraC alone or with elevated KCl. β -actin (42 kDa) was used as a loading control. Abbreviations: -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 2.

Treatment with AraC and KCl did not disrupt neuronal development and survival. (A–D) Cells at 12 DIV were stained for MAP2 (red) and activated caspase-3 (green) to assess the gross morphology of neurons and the rate of cell death in each treatment condition. MAP2 staining showed that in all conditions neurons maintained a normal morphology and appeared healthy. To further assess cells health cultures were co-stained for activate caspase-3 as a marker of cell death. We found relatively sparse cells labeled for caspase-3 (arrows) and therefore, no obvious difference in the number of caspase-3 labeled cells in any condition. (E) To quantify the number of caspase-3-reactive and presumably dying cells, cell counts were conducted at 8 and 12 DIV. At both time points and in all conditions we found relatively low numbers of caspase-3-labeled cells and importantly no difference between groups or between time points. These numbers correspond to less than 0.5% of the total cells

labeling with caspase-3, N=3. Scale bar represents 20 μ m. Abbreviations: -KCl -AraC (-K -A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 3.

Glial inhibition led to robust increases in aggrecan-immunoreactivity. (A) At 12 DIV in the untreated control cells, no aggrecan-reactivity (green) was noted, indicating that this developmental stage in the culture preceded the development of aggrecan-positive PNNs. (B) AraC treatment caused the condensation of aggrecan-reactivity to form PNNs. (C) Treatment of cultures with depolarizing concentrations of KCl similarly led to an enhancement of aggrecan-reactivity and evolution of aggrecan-positive PNNs. (D) Application of elevated KCl and AraC resulted in precocious development of aggrecan-positive PNNs at 12 DIV. Therefore, all of the culture treatments led to the precocious development of aggrecan-positive PNNs and led to the striking increase in the number of neurons with aggrecan-positive PNNs. Scale bar: A, 20 μ m. (E) To quantify these findings cell counts were performed at 12 DIV and the data is presented in graphical form. There were no statistically significant differences in the number of aggrecan-reactive PNNs in groups treated with AraC or KCl alone or in combination. However, at 12 DIV we found virtually no aggrecan-reactive PNNs in control cells. * $P < 0.001$ against cells treated with AraC or KCl alone or in combination. Cortical cultures were generated and cell lysates were harvested at 8 and 12 DIV for Western blotting analyses. Additionally, conditioned media was collected from these cultures. (F) In support of our immunocytochemistry results, there was a clear enhancement of total aggrecan protein (at > 400 kDa) in the cell lysates at both 8 and 12 DIV when cultures were treated with elevated KCl plus AraC, elevated KCl alone, and AraC alone. Also, there was an increase in total protein levels in the untreated controls between 8 and 12 DIV. (G) Analysis of the conditioned media harvested from cortical

cultures demonstrated clear increases in total aggrecan protein levels at 8 and 12 DIV, in each culture treatment groups. Abbreviations: -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 4.

Inhibition of glial cells within cortical cultures resulted in an elimination of brevican-reactivity. (A) Brevican-positive PNNs (green) populated the untreated control cultures at 12 DIV. (B) AraC treatment led to a complete elimination in the number of neurons enwrapped with brevican-reactive PNNs. (C) Treatment of cortical cultures with depolarizing concentrations of KCl did not alter brevican-reactivity, instead, ample brevican-positive PNNs were detected, enveloping the cell soma and proximal neurites of populations of cells. There was no noted difference in the number of neurons with brevican-based PNNs in cultures that were treated with elevated KCl in relation to untreated control cultures. (D) Under conditions in which glial cells are most effectively inhibited, elevated KCl and AraC

application, brevicin-PNN-reactivity was completely lost. These results indicate that any form of glial inhibition resulted in a complete elimination of brevicin-reactivity, reinforcing the critical role glial cells have in the production of brevicin. Scale bar: A, 20 μ m. (E) AraC treatment, irrespective of co-application of elevated KCl, led to complete elimination of brevicin reactivity (the full-length protein, 150 and 130 kDa and the 50 kDa cleavage fragment of brevicin) in cell lysates. The Δ g form of brevicin is present at 130 kDa, while the full-length form is present at 150 kDa. Developmentally, there were modest increases in the full-length form of brevicin and more robust increases in B50 protein noted between 8 and 12 DIV, suggesting a developmental change in brevicin cleavage. Additionally, there was an enhancement of B50-reactivity at both 8 and 12 DIV following elevated KCl application, relative to the untreated controls at 8 DIV. (F) Providing further support that brevicin is of glial origin, analysis of conditioned media samples treated with AraC, irrespective of elevated KCl administration, led to a complete elimination of brevicin-reactivity (full-length and the 50 kDa cleavage fragment). Abbreviations: -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 5.

The combined treatment of elevated KCl and AraC results in the loss of HAPLN1-reactivity at 12 DIV. (A) In untreated control cultures, HAPLN1-reactivity (green) was found enwrapping particular subpopulations of cells in a manner characteristic of PNNs. (B) Interestingly, when AraC was applied to cortical cultures without depolarizing concentrations of KCl, there was no noted effect on HAPLN1-reactivity and similar numbers of neurons surrounded by HAPLN1-positive PNNs were observed, relative to the untreated controls. (C) Elevated KCl treatment did not alter HAPLN1-staining, as neurons with HAPLN1-positive PNNs were found throughout these cultures. Moreover, there was no obvious difference in the number of neurons enveloped with HAPLN1-reactive PNNs as a result of AraC application. (D) Under conditions where glial cells are most effectively

inhibited, there is a concomitant loss of HAPLN1-immunoreactivity and no cells with HAPLN1-positive PNNs were detected. Therefore, these results indicate that HAPLN1 is made in a glial-dependent manner. Scale bar: A, 20 μ m. (E) Glial inhibition led to a decrease in HAPLN1 protein levels. Cell lysates were harvested from cortical cultures at 8 and 12 DIV and were subjected to Western blot analysis. At 8 DIV no HAPLN1 protein (40 kDa) was detected in cells that were treated with elevated KCl and AraC as well as in the untreated controls. There was an increase in HAPLN1-expression following elevated KCl application as well as AraC administration, relative to the untreated control. Overall, at 12 DIV there was a developmental increase in HAPLN1 protein levels. No protein was detected in samples that were treated with both depolarizing concentrations of KCl and AraC, affirming the fact that HAPLN1 is made in a glial-dependent manner. Abbreviations: -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 6.

Hyaluronan is produced in both a neuronal and glial-dependent manner. Cells were harvested at 12 DIV and immunostained with biotinylated hyaluronic acid-binding protein (HABP), a specific marker of hyaluronan. (A) Untreated cells exhibited ample HABP-reactivity (green) at 12 DIV with both the diffuse staining pattern and PNNs noted. (B) Under conditions in which cells were treated AraC, and thus, glial cells were moderately inhibited, both components of HABP-immunoreactivity were intact and there were no alterations in the number of neurons with pericellular aggregates. (C) Elevated KCl application did not alter the staining pattern of HABP. More specifically, the diffuse staining and PNN staining was observed, with a similar number of neurons reactive for hyaluronan. (D) Following application of elevated KCl and AraC the diffuse staining that is considered a hallmark of HABP staining was eliminated, while the PNN staining persisted. These results indicate that there is a component of hyaluronan that is produced in a neuronal-dependent manner, while there is also a component that is produced in a glial-dependent manner. These results indicate that hyaluronan is produced in both a neuronal and glial-dependent manner. Scale bar: A, 20 μ m.

Fig. 7. Tenascin-R-reactivity is altered following glial inhibition with elevated KCl and AraC. Cells were fixed at 15 DIV and analyzed for tenascin-R-reactivity (red). Additionally, cells were co-stained with HABP (green) to show their co-localization in PNNs. (A) At 15 DIV in untreated cortical cultures ample tenascin-R-positive PNNs were observed. There was an intricate network of tenascin-R-immunoreactive PNNs as well as diffuse staining found throughout the cultures co-localized with HABP. (B) Glial inhibition through with AraC treatment decreased the intensity of tenascin-R-staining, but there was no obvious difference in the number of cells with tenascin-R-immunopositive PNNs. (C) Application of depolarizing concentrations of KCl did not alter tenascin-R-reactivity. (D) Cells that were treated with elevated KCl and AraC exhibited no tenascin-R-PNN-reactivity, indicating that glial cells are critical in the production of tenascin-R, however, HABP-reactive PNNs

remained. Scale bar: A, 20 μ m. (E) At 8 DIV there was no tenascin-R protein (dimers at 160 and 180 kDa) in samples generated from cells that were treated with elevated KCl and AraC, indicating that tenascin-R protein cannot tightly aggregate at the cell surface as a result of glial inhibition. There was a modest increase in protein following elevated KCl application, while there was a decrease in protein as a result of AraC application, relative to the untreated control samples. At 12 DIV, there was a notable increase in tenascin-R protein in untreated cultures as well as in cultures that experienced administration of elevated KCl. There was little protein detected in samples that were treated singly with AraC and no protein was observed in cells that were treated with elevated KCl and AraC. These results indicate that tenascin-R is likely made in a glial-dependent manner. Abbreviations: -KCl -AraC (-K -A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Fig. 8.

Transcripts of most known PNN components are expressed by mouse astrocytes. RT-PCR reactions were run for 30 cycles to analyze expression of GFAP and known PNN components. Abundant levels of GFAP, brevican, and neurocan transcripts were expressed by astrocytic glial cells. Additionally, HAPLN1, tenascin-R, versican, and phosphacan transcripts were detected as well. Importantly, we were unable to detect aggrecan or HAS3 message. Abbreviations: glial fibrillary protein (GFAP, 198 bp), hyaluronan and proteoglycan link protein 1 (HAPLN1, 156 bp), tenascin-R (TnR, 159 bp), aggrecan (Acan, 147 and 261 bp), brevican (Bcan, 187 bp), neurocan (Ncan, 111 bp), versican (Vcan, 180 bp), phosphacan (PTPRZ1, 137 bp) and hyaluronan synthase 3 (HAS3, 166 bp).

Table 1

$\Delta\Delta$ Ct values and fold changes for PNN components at 8 and 12 DIV. Values are presented for $\Delta\Delta$ Ct with standard error measurements indicated with $n=3$. Equivalent fold changes are listed in parentheses underneath and p -values for the group effects by 2-way repeated measures ANOVAs are shown. *Post hoc* analyses of treatment effects within each time point were conducted utilizing Bonferroni pair-wise t -tests and resulting p -values are noted in the table below. A p -value that is statistically significant is indicated in bold ($p<0.05$). There was no overall significance for time and therefore *post-hoc* tests were not conducted across time points (data not shown). Abbreviations used: Acan, aggrecan; Bcan, brevican; -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Gene	GFAP	Acan	Bcan	HAPLN1
8 DIV	-K -A	0 $\pm$ 0.90 (1.36)	0 $\pm$ 0.57 (1.18)	0 $\pm$ 0.58 (1.15)
	-K +A	1.57 $\pm$ 0.92 (-2.98)	-5.72 $\pm$ 0.35 (55.95)	-2.07 $\pm$ 0.10 (4.23)
	+K -A	-0.53 $\pm$ 0.58 (1.70)	-4.34 $\pm$ 0.48 (22.83)	-2.00 $\pm$ 0.34 (4.22)
	+K +A	2.28 $\pm$ 0.66 (-4.84)	-5.42 $\pm$ 0.40 (46.09)	1.91 $\pm$ 0.52 (-3.76)
12 DIV	-K -A	0 $\pm$ 0.53 (1.12)	-1.61 $\pm$ 0.80 (3.95)	-1.25 $\pm$ 0.70 (2.94)
	-K +A	2.38 $\pm$ 0.76 (-5.21)	-5.91 $\pm$ 0.69 (76.58)	-2.22 $\pm$ 0.73 (5.73)
	+K -A	-0.10 $\pm$ 0.31 (1.12)	-5.61 $\pm$ 0.69 (62.19)	-1.89 $\pm$ 0.69 (4.71)
	+K +A	2.49 $\pm$ 0.68 (-5.60)	-6.29 $\pm$ 0.68 (93.82)	2.35 $\pm$ 0.77 (-5.08)
<i>p</i> -value	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$

Gene	GFAP	Acan	Bcan	HAPLN1
8 DIV	-K-A vs -K+A	0.265	<0.001	0.22
	-K-A vs +K-A	1	1	0.218
	-K-A vs +K+A	0.042	<0.001	1
	-K+A vs +K-A	0.066	<0.001	1
	-K+A vs +K+A	1	0.9	0.066
12 DIV	+K-A vs +K+A	0.01	0.076	0.066
	-K-A vs -K+A	0.031	<0.001	0.321
	-K-A vs +K-A	1	<0.001	1
	-K-A vs +K+A	0.024	<0.001	0.05
	-K+A vs +K-A	0.024	1	1
	-K+A vs +K+A	1	1	0.008
	+K-A vs +K+A	0.018	0.527	0.031

$\Delta\Delta$ Ct values and fold changes for PNN components at 12 DIV. Values are presented for $\Delta\Delta$ Ct with standard error measurements indicated with $n=3$. Equivalent fold changes are listed in parentheses underneath and p -values for the group effects by one-way repeated measures ANOVAs are shown. A p -value that is statistically significant is indicated in bold ($p < 0.05$). *Post hoc* analyses of treatment effects within each time point were conducted utilizing Bonferroni pair-wise t -tests and resulting p -values are noted in the table below. A p -value that is statistically significant is indicated in bold ($p < 0.05$). Note these analyses were only conducted when the results identified a significant treatment effect by one-way ANOVAs. Abbreviations: tenascin-R (TNR); neurocan (Ncan); versican (Vcan); phosphacan (PTPRZ1); -KCl -AraC (-K-A); -KCl +AraC (-K+A); +KCl -AraC (+K-A); +KCl +AraC (+K+A).

Table 2

Gene	TnR	Ncan	Vcan	PTPRZ1	Kv3.1
12 DIV	-K -A	0 $\pm$ 0.39 (1.08)	0 $\pm$ 0.23 (1.03)	0 $\pm$ 0.58 (1.19)	0 $\pm$ 0.53 (1.15)
	-K +A	0.53 $\pm$ 0.60 (-1.44)	0.71 $\pm$ 0.09 (-1.63)	0.60 $\pm$ 0.65 (-1.51)	2.44 $\pm$ 0.62 (-5.41)
	+K -A	-0.50 $\pm$ 0.23 (1.45)	-0.82 $\pm$ 0.33 (1.86)	0.30 $\pm$ 0.27 (-1.23)	1.40 $\pm$ 1.28 (-2.65)
	+K +A	0.55 $\pm$ 0.30 (-1.45)	0.87 $\pm$ 0.13 (-1.83)	1.84 $\pm$ 0.74 (-3.58)	2.74 $\pm$ 0.30 (-6.70)
<i>p</i> -value	$p=0.158$	$p<0.001$	$p=0.214$	$p=0.097$	$p=0.168$
Gene	HAS3	HAPLN3	HAPLN4	GAD67	
12 DIV	-K -A	0 $\pm$ 0.60 (1.16)	0 $\pm$ 0.14 (1.01)	0 $\pm$ 0.75 (1.34)	0 $\pm$ 0.17 (1.01)
	-K +A	-0.61 $\pm$ 0.93 (2.09)	2.53 $\pm$ 0.81 (-5.77)	0.93 $\pm$ 0.86 (-1.90)	-2.31 $\pm$ 0.66 (5.94)
	+K -A	-1.04 $\pm$ 0.43 (2.23)	0.32 $\pm$ 0.52 (-1.25)	2.27 $\pm$ 0.49 (-4.84)	-2.10 $\pm$ 0.69 (5.22)
	+K +A	-0.53 $\pm$ 0.62 (1.74)	2.20 $\pm$ 0.33 (-4.61)	1.21 $\pm$ 0.62 (-2.31)	-1.90 $\pm$ 0.48 (4.14)
<i>p</i> -value	$p=0.329$	$p=0.003$	$p=0.25$	$p=0.005$	

Gene	Ncan	HAPLN3	GAD67
12 DIV	-K-A vs -K+A	0.031	0.008
	-K-A vs +K-A	0.06	0.013
	-K-A vs +K+A	0.022	0.022
	-K+A vs +K-A	0.001	1
	-K+A vs +K+A	1	1
	+K-A vs +K+A	<0.001	1