

Semaphorin 3A Binds to the Perineuronal Nets via Chondroitin Sulfate Type E Motifs in Rodent Brains*

Received for publication, May 31, 2013, and in revised form, July 20, 2013. Published, JBC Papers in Press, August 12, 2013, DOI 10.1074/jbc.M111.310029

Gunnar Dick^{‡§}, Chin Lik Tan[‡], Joao Nuno Alves[‡], Erich M. E. Ehlert[¶], Gregory M. Miller^{||}, Linda C. Hsieh-Wilson^{||}, Kazuyuki Sugahara^{**}, Arie Oosterhof^{††}, Toin H. van Kuppevelt^{††}, Joost Verhaagen^{¶§§}, James W. Fawcett[‡], and Jessica C. F. Kwok^{‡¶}

From the [‡]John van Geest Centre for Brain Repair, University of Cambridge, Cambridge CB2 0PY, United Kingdom, the [§]Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, 0316 Oslo, Norway, the [¶]Netherlands Institute for Neuroscience, Institute of the Royal Netherlands Academy of Arts and Sciences, 1105 BA Amsterdam, The Netherlands, the ^{||}California Institute of Technology and Howard Hughes Medical Institute, Division of Chemistry and Chemical Engineering, Pasadena, California 91125, the ^{**}Laboratory of Proteoglycan Signaling and Therapeutics, Graduate School of Life Science, Faculty of Advanced Life Science, Hokkaido University, 001-0021 Sapporo, Japan, the ^{††}Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P. O. Box 9101, 6500 HB Nijmegen, The Netherlands, and the ^{§§}Centre for Neurogenomics and Cognitive Research, Vrije Universiteit Amsterdam, 1081HV Amsterdam, The Netherlands

Background: Semaphorin3A (Sema3A) is an axon guidance molecule present in the CNS extracellular matrix and on the perineuronal nets (PNNs).

Results: Sema3A interacts with chondroitin sulfate E (CS-E) for anchoring to the PNNs.

Conclusion: The binding of Sema3A to CS in the PNNs presents a novel mechanism of PNNs in restricting plasticity.

Significance: This finding suggests a novel candidate for intervention in promoting CNS recovery.

Chondroitin sulfate (CS) and the CS-rich extracellular matrix structures called perineuronal nets (PNNs) restrict plasticity and regeneration in the CNS. Plasticity is enhanced by chondroitinase ABC treatment that removes CS from its core protein in the chondroitin sulfate proteoglycans or by preventing the formation of PNNs, suggesting that chondroitin sulfate proteoglycans in the PNNs control plasticity. Recently, we have shown that semaphorin3A (Sema3A), a repulsive axon guidance molecule, localizes to the PNNs and is removed by chondroitinase ABC treatment (Vo, T., Carulli, D., Ehlert, E. M., Kwok, J. C., Dick, G., Mecollari, V., Moloney, E. B., Neufeld, G., de Winter, F., Fawcett, J. W., and Verhaagen, J. (2013) *Mol. Cell. Neurosci.* 56C, 186–200). Sema3A is therefore a candidate for a PNN effector in controlling plasticity. Here, we characterize the interaction of Sema3A with CS of the PNNs. Recombinant Sema3A interacts with CS type E (CS-E), and this interaction is involved in the binding of Sema3A to rat brain-derived PNN glycosaminoglycans, as demonstrated by the use of CS-E blocking antibody GD3G7. In addition, we investigate the release of endogenous Sema3A from rat brain by biochemical and enzymatic extractions. Our results confirm the interaction of Sema3A with CS-E containing glycosaminoglycans in the dense extracellular matrix of rat brain. We also demonstrate that the

combination of Sema3A and PNN GAGs is a potent inhibitor of axon growth, and this inhibition is reduced by the CS-E blocking antibody. In conclusion, Sema3A binding to CS-E in the PNNs may be a mechanism whereby PNNs restrict growth and plasticity and may represent a possible point of intervention to facilitate neuronal plasticity.

The perineuronal nets (PNNs)² are dense extracellular matrix structures that surround the soma and dendrites of many neurons in the mature CNS, in particular the GABAergic interneuron in the cerebral cortex. PNNs are composed of chondroitin sulfate proteoglycans (CSPGs), link proteins, hyaluronan, and tenascins (2, 3). PNNs appear relatively late during development, at the end stage of refinement and consolidation of the neuronal circuitry (4–6), coinciding with closure of the critical period for plasticity (7, 8). PNNs have a structure similar to cartilage. They contain aggrecan, neurocan, versican, and brevican, which are CSPGs from the lectican family, and phosphacan, a surface-bound CSPG. The lecticans interact with hyaluronan via their N-terminal link modules, and the interactions are further stabilized by one or both of the link proteins, cartilage link protein (Crtl1), and brain link protein 2 (Bral2) (3, 4, 9). The C terminus of the CSPGs also binds to tenascin-R, producing a stable ternary structure. The degree of cross-linking makes the PNN core components resistant to solubilization, which requires 6 M urea (10). The CSPGs are extensively

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 GM093627 (to L. H. W.). This work was also supported by The Christopher and Dana Reeve Foundation (to J. W. F. and L. H. W.), Wings for Life (to J. C. F. K.), European Union FP7 Programme AxRegen, and Grants-in-aid for Scientific Research on Innovative Areas 24110501 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) (to K. S.).

¹ To whom correspondence should be addressed: John van Geest Centre for Brain Repair, University of Cambridge, Forvie Site, Robinson Way, Cambridge CB2 0PY, UK. Tel.: 44-1223-331191; Fax: 44-1223-331174; E-mail: jcfk2@cam.ac.uk.

² The abbreviations used are: PNN, perineuronal net; CS, chondroitin sulfate; ChABC, chondroitinase ABC; Sema 3A, semaphorin 3A; CS-E, chondroitin sulfate type E; HS, heparan sulfate; GAG, glycosaminoglycan; PG, proteoglycan; Crtl1, cartilage link protein 1; AP, alkaline phosphatase; DRG, dorsal root ganglion; CSPG, chondroitin sulfate proteoglycan; WFA, *W. floribunda* agglutinin; Hep, heparin; IP, immunoprecipitation.

glycanated by chondroitin sulfate (CS) glycosaminoglycan (GAG) chains. The GAG composition with respect to sulfation differs between the PNN-associated GAGs and the GAGs derived from the more soluble PGs in the CNS (10). Because the binding properties of GAGs depend on their pattern of sulfation, this suggests that 2–3% of total brain CS-GAG chains associated with the PNNs may have different binding affinities than those in the free-floating matrix.

The interest in the PNNs stems from the evidence that they are responsible for the restriction of plasticity in the CNS that occurs at the end of the critical periods. Degradation of CS by enzymatic treatment using chondroitinase ABC (ChABC) has demonstrated remarkable enhancement in plasticity in the visual cortex (8) and in the injured CNS (11–13) and prolongation of memory (14). The same enhancement of plasticity and memory is also observed in animals that lack *Crtl1* in the CNS and therefore have attenuated PNNs, indicating that ChABC promotes plasticity through digestion of CS-GAGs in PNNs (9).

Although it is established that PNNs restrict plasticity, the mechanism in which they do so is unknown. A possible mechanism involves semaphorin3A (Sema3A), which binds to neuropilins on axons and synapses to affect axon growth and synaptic change (15–17). Recent studies also suggest that Sema3A mediates its effect by regulating local protein synthesis in axons (18, 19). Sema3A is a guidance signal involved in several axon guidance and neuronal migratory events during development (20–22). However, the expression of Sema3A persists at a considerable level in parts of the CNS into adulthood (23). Its presence affects synapse dynamics (15, 17) and may influence plasticity in the mature CNS (22). An interaction between CS and Sema3A has been previously reported *in vitro* in neuronal cell culture (25) and also by co-localization studies of CSPG and Sema3A during development *in vivo* (26). More recently, we have shown that Sema3A is concentrated around PNNs in the mature CNS and that it co-localizes with several components of the PNNs (1). Moreover, counteracting the effects of Sema3A using decoy receptor bodies for neuropilin-1 partly restores ocular dominance plasticity in the adult CNS, indicating that Sema3A is a PNN effector (27). Sema3A is bound there through CS-GAGs in the PNNs because *in vivo* ChABC digestion removes the Sema3A staining (1). Although ChABC may also digest hyaluronan, to a much lesser extent, the binding of Sema3A is specific to CS-GAGs. This is supported by the observation that an administration of *Streptomyces* hyaluronidase, which is specific to hyaluronan digestion, into adult rat brains did not solubilize the perineuronal Sema3A staining.

In this study, we further characterize the interaction between Sema3A and various CS isoforms. We have studied the potential of enhancing plasticity and regeneration by interfering with the interaction using an anti-CS-E antibody, GD3G7 (28). We report that Sema3A binds to CS-E units that contain disulfated E disaccharides and in those extracted from the PNNs. This interaction serves to bind Sema3A to the PNN GAGs, and the combination of Sema3A and GAG is inhibitory to axonal growth.

EXPERIMENTAL PROCEDURES

Recombinant Sema3A—Sema3A constructs used in the experiments were described previously (25, 29). Recombinant

proteins of alkaline phosphatase (AP) fused to chicken collapsin-1 (Sema3A-AP) or green fluorescent protein (GFP) fused to rat semaphorin3A (Sema3A-GFP), as depicted in Fig. 1, were expressed in HEK 293T cells cultured in DMEM supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μ g/ml; all from Invitrogen). Recombinant Sema3A proteins were derived from media or cell lysate of the transfected HEK cells. Transfections of plasmids were performed by Amaxa (Lonza, Switzerland), according to the manufacturer's instructions, followed by a collection of conditioned media and cell lysates after 24 h. Media were concentrated 5–10 times using Microcon (3 kDa molecular mass cutoff) filter devices (Millipore, MA). Detection of the expressed Sema3A-AP or -GFP was done by slot blot or Western blot analysis. Sema3A-Fc used in the microarray assay was purchased from R&D Systems.

Tissue Extraction from Mouse Brains—Fractionation of brain tissue was performed using the procedure in Refs. 10, 30 with minor modifications. Adult mice were sacrificed and decapitated. The brains were removed and stored at -70°C . On the day of extraction, one brain was thawed in buffer 1 ($1\times$ TBS, pH 7.0, with Complete Mini protease inhibitors) (Roche Diagnostics) and homogenized on ice using a tight-fitting Potter-Elvehjem homogenizer. The homogenate was then centrifuged at $20,000\times g$ for 30 min at 4°C to separate nonsoluble material (pellet) from the soluble material (supernatant). The homogenization and subsequent centrifugation were repeated twice, and the supernatants were pooled and termed extract 1. The subsequent extractions with buffer 2 (buffer 1 + 0.5% Triton X-100), buffer 3 (buffer 2 + 1 M sodium chloride (NaCl)), and buffer 4 (buffer 3 + 6 M urea) were carried out by the same procedure with a minor modification. Instead of using the homogenizer, a pipette was used to gently resuspend the material in the pellet to facilitate extraction as follows: extract 1 contains soluble molecules; extracts 2 and 3 contain membrane-associated molecules, and extract 4 contains dense matrix-associated molecules. Protein measurements of the extracts were carried out using the Total Protein kit, Micro Lowry, Peterson's Modification (Sigma), or BCA protein assay (Pierce/Thermo Scientific).

Isolation and Purification of PGs and GAGs from Rat Brains—PGs and GAGs were isolated from extracts 1–4 of adult rat brains (five brains per extraction) with slight modifications of the procedure described in Refs. 10, 30. Extracts 1–4 were dialyzed against low salt ion-exchange buffer (50 mM Tris, pH 7.5, 2 M urea, 0.2 M NaCl), filtered, and subjected to DEAE ion-exchange chromatography (GE Healthcare). Bound PGs were eluted using high salt ion-exchange buffer (low salt buffer + 1 M NaCl). The high salt PG eluate was dialyzed against $1\times$ PBS, pH 7.0, and divided into two parts as follows: one for PG isolation using ethanol precipitation (95% ethanol, 1.3% potassium acetate, CH_3COOK) or subjected to further GAG purification as described previously (10, 30). The final GAG extracts were termed according to the initial brain extraction as follows: soluble GAGs (originating from extract 1), detergent GAGs (extracts 2 and 3), and PNN GAGs (extract 4). The GAG concentration in the extracts was determined by Blyscan assay (Biocolor, UK).

Semaphorin3A Interacts with CS-E in PNNs

Enzymatic Release of Sema3A from Rat Brain—To test for the release of Sema3A from the PNNs, adult rat brain was homogenized in appropriate enzyme buffers and divided into enzyme and nonenzyme incubations. The samples were digested with 100 milliunits of ChABC (EC 4.2.2.4), chondro-4-sulfatase (EC 3.1.6.9), or chondro-6-sulfatase (EC 3.1.6.10) in 0.1 M NH₄Ac, pH 8.0 (all enzymes were isolated from *Proteus vulgaris* and were purchased from Seikagaku Corp., Japan). The reactions were incubated at 37 °C overnight. Hyaluronan digestion was done by adding 100 turbidity reducing units/ml hyaluronidase (EC 4.2.2.1) from *Streptomyces hyalurolyticus* (Seikagaku Corp., Tokyo, Japan) in 0.15 M NaCl, 0.02 M acetate buffer, pH 6.0. Heparan sulfate (HS) digestion was done using 25 milliunits each of heparitinase I (EC 4.2.2.7) and III (EC 4.2.2.8) from *Flavobacterium heparinum* in 0.05 M sodium acetate, 5 mM calcium acetate buffer. Both enzymes were purchased from Sigma. Sema3A released from the brain homogenate was determined by Western blotting of the supernatant after centrifugation (as in the extraction procedure) and presented as the ratio of the band intensity between the Sema3A in the enzyme-digested sample, and normalized it to the nonenzyme-treated sample.

ELISA—The ELISA was modified from Ref. 28. Biotinylated GAGs, 0.5 or 2.0 μg per well, were immobilized onto streptavidin-coated plates of 384 or 96 wells (Pierce/Thermo Scientific). CS variants and HS were purchased from Seikagaku Corp. (Japan), and CS-A was isolated from whale cartilage; CS-B was from pig skin; CS-C and CS-D were from shark cartilage, CS-E was from squid cartilage; HS was from bovine kidney, and heparin was from porcine intestine. The basic disaccharide units from the different CS variants are shown in Fig. 1B. Although the biotinylated heparin was purchased from Sigma, the brain-derived GAGs were extracted and purified as described above. Biotinylation of GAGs was performed by EDC and biotin-LC-hydrazide conjugation (Pierce/Thermo Scientific). After GAGs were immobilized on the plates, the plates were blocked in 1% BSA and subjected to the binding of recombinant Sema3A-AP (or -GFP) in 1:5 to 1:8 dilutions. Detection of Sema3A-AP was done by direct measurement of absorbance at 405 nm using *p*-nitrophenyl phosphate (Sigma). To detect binding of Sema3A-GFP, the wells were incubated with 1:2,000 rabbit anti-GFP antibody (Abcam), or 1:2,000 rabbit anti-Sema3A antibody (Abcam), and subsequently with 1:10,000 AP-conjugated anti-rabbit antibody (Invitrogen). Detection was done by measuring the absorbance at 405 nm using *p*-nitrophenyl phosphate. The indirect ELISA was performed in a similar fashion, and the recombinant Sema3A-AP was incubated with the competing GAGs or blocking antibody at room temperature for 30 min before being transferred to the wells that were all coated with biotinylated heparin. While the phage display antibody GD3G7 is specific to CS-E (28), the control antibody MPB49V does not bind to any CSs.

Carbohydrate Microarray Assays—Microarrays containing natural GAGs enriched in the CS-A, CS-C, CS-D, and CS-E motifs (Seikagaku Corp.), dermatan sulfate (also called CS-B; Sigma), hyaluronic acid (HA; Sigma), heparin (Hep; Neoparin, Alameda, CA), HS (Sigma), or chondroitin sulfate (CS; Sigma) were printed on poly-D-lysine-coated glass surfaces as

described previously (31, 32). Arrays were blocked with 10% FBS in 1× PBS with gentle rocking at room temperature for 1 h, followed by a brief rinse with 1× PBS. Sema3A-Fc (R&D Systems, Minneapolis, MN) was reconstituted in 1% BSA in 1× PBS, added to the slides (100 μl at a concentration of 2 μM per slide, and incubated at room temperature for 3 h. The slides were briefly rinsed three times with 1× PBS and then incubated with a 1:5,000 Cy3-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h in the dark with gentle rocking. The microarrays were then washed (three times of 1× PBS and two times with de-ionized water), dried under a stream of air, and scanned at 532 nm using a GenePix 5000a scanner. Fluorescence quantification was performed using GenePix 6.0 software (Molecular Devices, Sunnyvale, CA). Experiments were performed in triplicate, and the data represent the average of 10 spots per concentration averaged from the three experiments (±S.E., error bars).

Western Blot and Slot Blot Assay—12 μg of protein from each brain extract was subjected to SDS-PAGE and Western blotting using the NuPAGE system (Invitrogen). Primary antibodies used were against Sema3A (1:2,000, Abcam) and Crtl1 (1:2,000, R&D Systems). Secondary antibodies used were anti-rabbit-HRP (1:50,000, GE Healthcare) and anti-goat-HRP (1:3000, Vector Laboratories). Detection of Western blots was done using ECL+ (GE Healthcare). In the dot blot, 0.3, 1, and 3 μg of CS-E and CS-B in 1× PBS, or 1, 5, and 10 μg of bovine aggrecan (Sigma) in 1× PBS were applied to nitrocellulose membrane (GE Healthcare). Anti-CS-E antibody GD3G7 was added in 1:400 dilution to the membrane. This antibody contains a VSV-G tag, which was used for detection. After washes, antibody against VSV-G tag P5D4 (1:20,000, Abcam) was added. The blot was subsequently probed with anti-mouse HRP antibody (1:50,000, GE Healthcare). Detection was done using ECL+ (GE Healthcare).

Lectin Histochemistry—Fresh frozen sagittal sections from adult rat brain (15 μm) were first labeled with biotinylated *Wisteria floribunda* agglutinin (WFA, 5 μg/ml; Sigma) and then with Alexa Fluor-488-streptavidin (1:2,000; Invitrogen). The sections were subjected to 20-min washes of buffers as described for the extraction of GAGs from brain tissue. Post-wash fixation was done in 3% paraformaldehyde. Histochemical staining was performed as described previously (10). Fluorescence imaging was captured with conventional fluorescence microscope.

Co-immunoprecipitation—PNN PGs isolated from extract 4 as described above were incubated with or without recombinant Sema3A-GFP lysate, also described above. 30 μl of Sema3A-GFP lysate was added to 300 μl of PNN PG solution in the presence of 1:300 rabbit anti-GFP antibody (Abcam). After 1 h of incubation, 30 μl of protein-A magnetic beads (Invitrogen) were added. Washes and magnetic separation were performed according to manufacturers' protocol. To analyze the CS-dependent Sema3A-GFP retention of CSPGs, magnetic beads were treated with ChABC (as before) followed by Western blot analysis (as described above). CSPGs retained by Sema3A-GFP and released by ChABC digestion was detected using 1:2,000 of mouse monoclonal 2-B-6 antibody (Seikagaku

Corp.), which is specific against the digested glycan stump after ChABC digestion of CS GAG chains.

Dorsal Root Ganglia (DRG) Outgrowth Assay—DRGs were dissected from Sprague-Dawley rats (~3 months). Each ganglion was then cut into 4–6 smaller explants. To determine the inhibitory effect of Sema3A, 100 μ l of a mixture of Sema3A (1:10, 100, and 1,000) and 1 μ g/ml laminin was coated on the glass coverslips for 1 h at room temperature. To test for the efficiency of GD3G7 or various GAG-degrading enzymes in blocking Sema3A from binding to the PNN-GAGs, isolated PNN-GAGs were added to the coverslips together with laminin for 30 min and then rinsed briefly with sterile 1 \times PBS. GD3G7, MPB49V, and various GAG lyases were then added to the coverslips and incubated for 30 min at room temperature. The coverslips were rinsed three times with sterile 1 \times PBS before the addition of 1:10 Sema3A for 30 min at room temperature. The coverslips were rinsed again before the ready-cut explants were plated on the coverslips. The explants were cultured in DMEM supplemented with insulin/transferrin/selenium (1 \times), penicillin/streptomycin/fungizone (1 \times), and 10 ng/ml nerve growth factor for 2 days at 37 $^{\circ}$ C, 5% CO₂. To quantify the axonal outgrowth, DRG explants were immunostained for β III tubulin for their neurites. The 10 longest neurites from each explant were measured and averaged using the Leica Application Suite (Leica Microsystems). The average neurite length was then calculated from all the explants on each coverslip to give a final measurement for each condition. In some experiments, the number of neurites per explant was quantified, and the explants were grouped accordingly into three different classes (3 or less neurites, 4–19 neurites, 20 or more neurites).

RESULTS

Our previous work has demonstrated that Sema3A is concentrated on the PNNs and is removed by ChABC treatment implying that it is bound to the GAG chains of CSPGs (1). To study if Sema3A binds specifically to individual CS isoforms, we have characterized the binding of Sema3A to different defined CS and HS structures and to glycans extracted from the adult CNS.

Sema3A Interacts with CS GAGs—To investigate the binding of Sema3A to immobilized CS preparations with known sulfation patterns, we used two methods, ELISA (both a direct and a competitive ELISA) and GAG microarray. To minimize the possible interference of different tags on Sema3A binding, we used three variants of recombinant Sema3A to consolidate the results (Fig. 1). Sema3A-AP is a fusion protein with an N-terminal AP sequence fused to chick Sema3A (also called collapsin 1), which lacks its intrinsic signal sequence (29). Sema3A-GFP is modified by an insertion of GFP between the Sema3A signal sequence and the downstream domains, as described previously (25). Sema3A Fc is a purified Sema3A protein fused with a His tag at the N terminus and a human IgG peptide at the C terminus. Initially, we screened for direct binding of Sema3A to various CS GAGs, as well as HS and heparin (Fig. 2A). Sema3A interacted specifically with CS-E (enriched in 4,6-disulfated disaccharide units) and displayed very strong binding to heparin. To minimize possible interference or masking of binding sites by biotin, we confirmed these results using a competitive

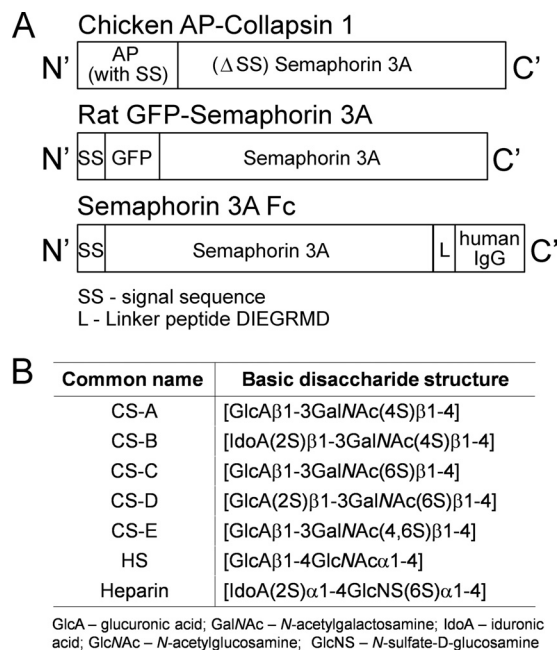


FIGURE 1. **A**, schematic diagram of the differently tagged Sema3A fusion proteins used in this study. For chicken AP-collapsin 1, the AP was fused to the N-terminal part of chicken collapsin 1 lacking a signal sequence (SS) (29). For rat GFP-semaphorin 3A, the GFP was inserted after the N-terminal signal sequence in the rat Sema3A sequence (24). Sema3A-Fc is procured from the R&D Systems and contains a His tag at the N terminus and a C-terminal IgG sequence. **B**, chemical structures of the various GAGs used in this study.

ELISA. Biotinylated heparin was immobilized to the plates, and untagged CS GAGs were assayed for their ability to block the strong interaction of Sema3A with the bound heparin (Fig. 2B). Similar to the direct binding experiment, the results demonstrated that in the presence of CS-E, less Sema3A interacted with the immobilized heparin in the wells. This suggests that Sema3A interacts with CS-E, which competes with Sema3A binding to heparin (Fig. 2B, 5th and 7th bars). Competitive binding was also seen with CS-B (Fig. 2B, 2nd bar) and to a lesser extent with CS-A (4-sulfated) and HS (1st and 6th bars). CS-C (6-sulfated) and -D (rich in 2,6-disulfated units) did not affect Sema3A binding to heparin (Fig. 2B, 3rd and 4th bar). This shows that the binding of Sema3A to GAGs is specific to their sulfation pattern rather than on the overall number of sulfate groups on the GAGs.

To further characterize the interactions with CS-B and -E, we tested the binding of Sema3A with increasing concentrations of CS-B, -C, and -E in a competitive ELISA. Both CS-B and -E competed strongly with heparin binding at all the concentrations tested, but there was a minimal effect with CS-C (Fig. 2C). The binding interactions of CS-B and -E were indistinguishable. We also employed a different recombinant variant of Sema3A to discount the effects of the AP tag on the binding. Repeating the experiments using Sema3A-GFP-containing lysate reproduced the same binding patterns (data not shown). To verify that this binding is due to a specific interaction of the expressed Sema3A but not other contaminating proteins present in the partially purified Sema3A, we compared the binding affinity to heparin using cell lysate recovered from Sema3A-GFP-expressing cells and non-Sema3A-expressing cells (Fig. 2D). Although the lysate from Sema3A-GFP-expressing cells

Semaphorin3A Interacts with CS-E in PNNs

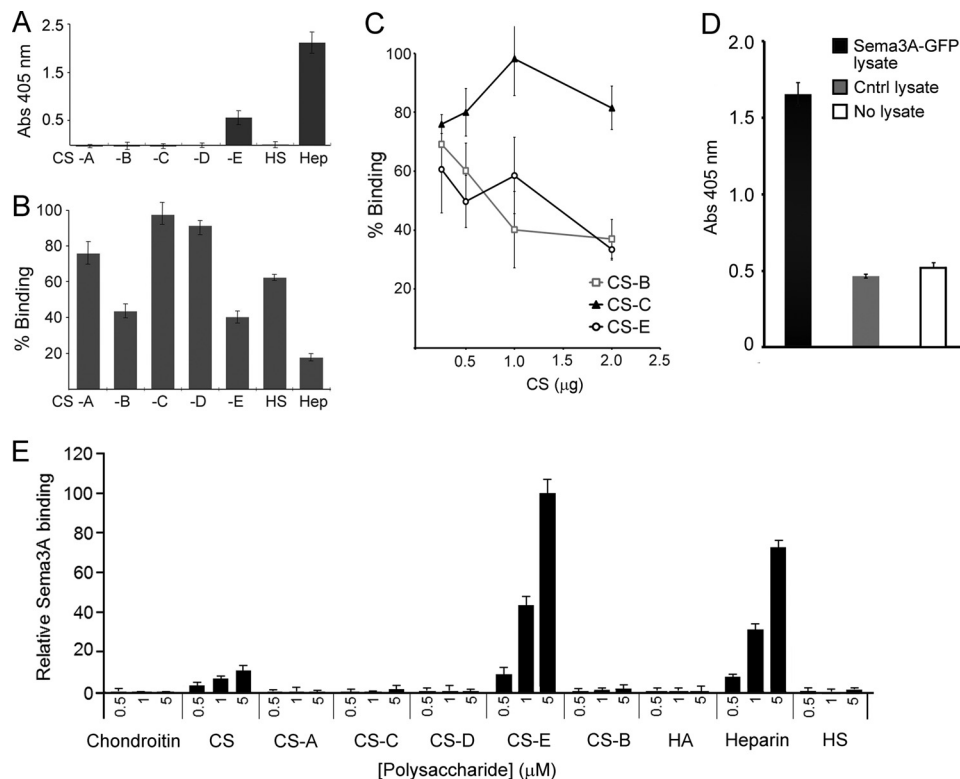


FIGURE 2. Sema3A demonstrates preferential binding to CS-E and CS-B. Recombinant Sema3A-AP was used in direct (A) and competitive (B and C) ELISA toward various CS GAGs. A, although Sema3A-AP interacts very specifically with heparin and CS-E, it does not bind to other CSs. The binding affinity was determined by AP activity measured at absorbance at 405 nm, $n = 4$, mean \pm S.D. B, Sema3A-AP interaction with heparin in competition with various CS GAGs, HS, and heparin. The binding of Sema3A to heparin was inhibited after incubation with CS-B, CS-E, and heparin. The binding was determined by AP activity measured at 405 nm. Competitive inhibition presented as percentage of binding in relation to reading in Sema3A-AP only sample (equals 100% binding), $n = 4$, mean \pm S.D. C, measurements of dose-dependent inhibition of various concentrations of CS-B, CS-C, and CS-E in blocking Sema3A-AP interaction toward heparin in a competitive ELISA. Both CS-B and CS-E inhibit the binding of Sema3A to heparin, whereas CS-C does not. Determination of Sema3A-AP binding and presentation as in B, $n = 3$, mean \pm S.D. D, control experiment measuring binding to heparin using lysate from Sema3A-GFP-transfected cells, lysate from non-transfected (Cntrl lysate), and no lysate. Note that Sema3A-GFP cell lysate interacted very strongly to the immobilized heparin, whereas the control lysate gave similar signal level as the negative control “no lysate.” This suggests that the binding of Sema3A-GFP to heparin is due to the expressed Sema3A in the lysate but not other protein contaminants. Binding affinity was measured and presented as absorbance at 405 nm, $n = 3$, mean \pm S.D. E, Sema3A-Fc binds selectively to CS-E-enriched polysaccharides on GAG microarrays. Microarrays containing different concentrations (0.5, 1, and 5 μ M) of the indicated GAG polysaccharides were incubated with Sema3A-Fc, followed by a Cy3-conjugated anti-mouse IgG secondary antibody, and analyzed using a Gene-Pix 5000A scanner. Data represent the mean \pm S.E. from three experiments ($n = 10$ per condition). CS-A is 4-sulfated, CS-B is 2,4-disulfated, CS-C is 6-sulfated, CS-D is 2,6-disulfated, and CS-E is 4,6-disulfated.

bound strongly to the heparin (Fig. 2D, 1st bar), the binding of the control cell lysate was as weak as the negative “no lysate” control (2nd and 3rd bars).

To further consolidate this specific interaction of Sema3A to CS-E and heparin, we also employed a carbohydrate microarray to examine the binding of Sema3A to various CS GAGs enriched in specific sulfation motifs, as well as CS-B, HA, heparin, and HS (Fig. 2E). This method allows a direct comparison of protein binding across diverse GAG classes enriched in specific sulfation motifs. Sema3A exhibited a strong, concentration-dependent binding to CS-E and heparin, with weaker binding to CS, a GAG preparation that contains multiple sulfation motifs. Little or no binding to CS-A, CS-C, or CS-D was observed, highlighting the specificity of Sema3A for the CS-E sulfation motif.

Sema3A Interacts with GAGs from the PNNs via Its Interaction with CS-E—We then characterized the interactions of Sema3A with the GAGs isolated from adult rat brains. In previous work, we have developed a sequential extraction method to separate PNN GAGs from the soluble or membrane-attached GAGs. These sequential fractions contain GAGs with

different sulfation patterns suggesting different binding properties (10). The GAGs recovered from the sequential extraction were biotinylated and immobilized on streptavidin-coated plates. Sema3A demonstrated a strong and specific binding affinity toward the PNN GAGs but showed little binding to the soluble and membrane-bound GAGs (Fig. 3A). We have previously demonstrated that the PNN GAG fraction contains ~70% CS and ~30% HS (9). To examine if the binding is due to the presence of CS or HS, we treated the GAG solution with ChABC, heparitinases I and III, or a combination of the enzymes in a competitive ELISA. The enzymes were inactivated by incubation at 70 °C, after which we tested for the Sema3A binding (Fig. 3B). Digestion by either ChABC or heparitinases alone gave only a modest reduction in the ability of the PNN GAGs to compete for Sema3A binding to immobilized heparin (Fig. 3B, 2nd to 4th bars; $p < 0.05$). However, when both types of enzymes were used together, Sema3A binding was abolished (bar 5). This suggests that both CS and HS GAGs in the PNNs may contribute to the binding of Sema3A.

The structure of the CS GAGs derived from PNNs contains ~2% of the CS-E units and 0.8% of the CS-B (2,4-disulfated)

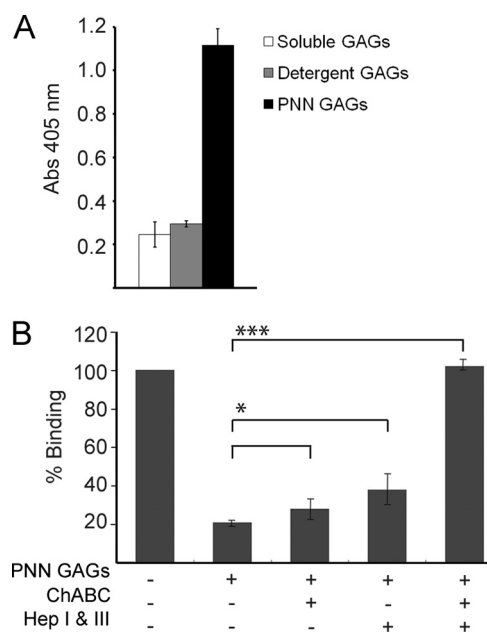


FIGURE 3. Sema3A binding to GAGs extracted from adult rat brain demonstrates high binding affinity to PNN GAGs. Recombinant Sema3A-AP binding to brain GAGs in direct (A) and competitive ELISA (B). A, Sema3A-AP binds with high affinity to PNN-GAGs but not to the soluble GAGs or detergent GAGs in a direct ELISA. Binding affinity was determined by AP activity measured and presented as absorbance at 405 nm, $n = 3$, mean \pm S.D. B, PNN-GAGs (with and without ChABC or Hep I and III treatment) was preincubated with the Sema3A-AP before adding onto heparin-coated wells in an indirect ELISA. The binding of Sema3A alone is defined as 100% (1st bar). Incubation with PNN GAGs decreases the binding to ~20% (2nd bar). Pretreatment of the PNN GAGs with ChABC (3rd bar) or Hep I and III (4th bar) only partially removes the inhibition. However, co-treatment of both ChABC and heparitinases restores the Sema3A-AP binding to the immobilized heparin. Binding affinity determined by AP activity measured as absorbance at 405 nm and presented as a percentage of binding relative to reading in Sema3A-AP only sample (equals 100%), $n = 4$, mean \pm S.D., two-tailed paired t test: *, $p < 0.05$, ***, $p < 0.001$.

(10). Despite the low proportions of these disulfated forms, the preceding results suggest that they may be responsible for the specific binding of Sema3A to the PNN structure. To specifically target CS-E interactions, we utilized a specific blocking antibody, GD3G7. This phage display antibody recognizes CS-E-containing epitopes in the developing brain (28). We first confirmed the specificity of the antibody in a slot blot assay against CS-B, CS-E and bovine aggrecan (Fig. 4A). We found that GD3G7 binds specifically to CS-E but not CS-B, and binds to bovine aggrecan. Aggrecan is a CSPG which bears multiple CS-GAG chains, the GD3G7 antibody is probably recognizing the CS-E units present in aggrecan's GAG chains. We then investigated the antibody's ability to block the interaction of Sema3A with CS-E and with PNN GAGs. Addition of GD3G7 reduced the binding of Sema3A to immobilized CS-E by 68% (Fig. 4B). The GD3G7 antibody also blocked Sema3A from binding to brain-derived PNN GAGs by 65% (Fig. 4C) and in a concentration dependent manner (Fig. 4D). In comparison to the control phage displayed MPB49V antibody, GD3G7 was more effective in blocking Sema3A binding to PNN GAGs over a range of concentrations.

Release of Endogenous Sema3A from Rat Brain by Biochemical and Enzymatic Extraction—Our previous work has shown that Sema3A localizes to the PNN structures in the brain (1).

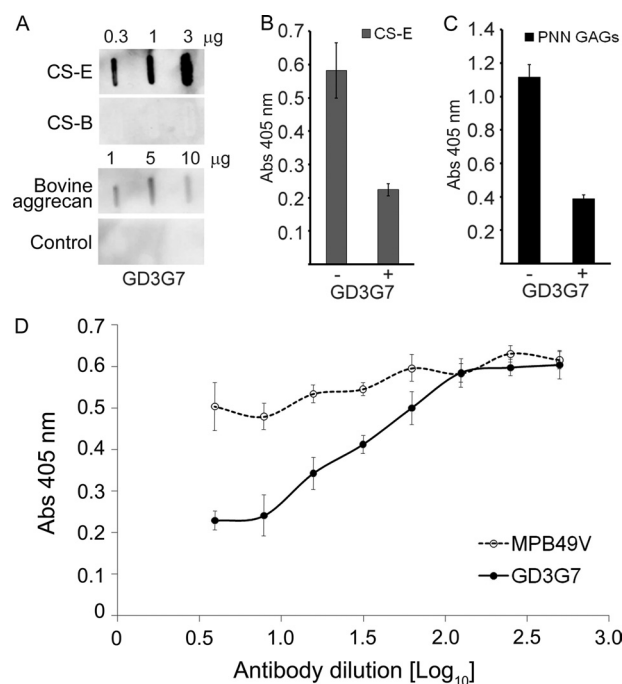


FIGURE 4. Sema3A binding to PNN GAGs is inhibited by blocking the CS-E structure. A, slot blot assay using anti CS-E phage display antibody GD3G7 (26) toward CS-E, CS-B, and bovine aggrecan. The result demonstrates that although the GD3G7 antibody specifically recognizes CS-E and detects a CS-E-like structure in aggrecan, it does not bind to CS-B. B and C, ELISA of Sema3A-AP interaction toward CS-E and PNN GAGs, with and without the addition of anti-CS-E antibody GD3G7. GD3G7 effectively blocks the binding of Sema3A-AP to both CS-E and PNN GAGs by ~60%. Binding affinity was determined by AP activity measured and presented as absorbance at 405 nm, $n = 3$, mean \pm S.D. D, a direct ELISA of Sema3A-AP interaction toward PNN GAGs as in C using various concentrations of GD3G7 and a nonspecific control phage display antibody MPB49V. It shows a concentration-dependent blocking of GD3G7 toward Sema3A-AP binding to the PNN-GAGs. $n = 3$, mean \pm S.D.

Although the PNNs are distributed in several areas in the rat brain, in the cerebral cortex, PNNs are mainly associated with GABAergic interneurons (33, 34). Sema3A co-localization with PNNs was particularly strong in layers IV and V in the cerebral cortex and was mainly associated with parvalbumin-positive interneurons (1). To characterize Sema3A binding to the PNNs, we performed our sequential extraction procedure on adult rat cortical sections, monitoring the release of Crt11 and Sema3A at each step (Fig. 5). As in our previous work (10), the PNNs and PNN GAGs remained in place through the extraction in $1\times$ TBS, 0.5% Triton X-100, and 1 M NaCl (Fig. 5A), as demonstrated by WFA staining. Intact PNNs was observed after the $1\times$ TBS wash. After the washes with Triton X-100 and 1 M NaCl, the staining in the neurites was weakened (*arrowheads* in Fig. 5A), but the intensity on the soma remained the same (*arrows* in Fig. 5A). We could not perform WFA staining in the cortical sections after 6 M urea wash due to the poor tissue integrity. However, to investigate if Sema3A is released after PNNs were dissolved in 6 M urea buffer, we continued to monitor the release of Sema3A and Crt11 (a key molecule in consolidating PNNs) using Western blot. Only with the wash in 6 M urea did the CNS tissues release the majority of the Crt11 protein, suggesting the dissolution of the PNNs (Fig. 5B, left panel). The binding of Sema3A to the PNNs is weaker than that of the Crt11. The majority of Sema3A was released by the addition of

Semaphorin3A Interacts with CS-E in PNNs

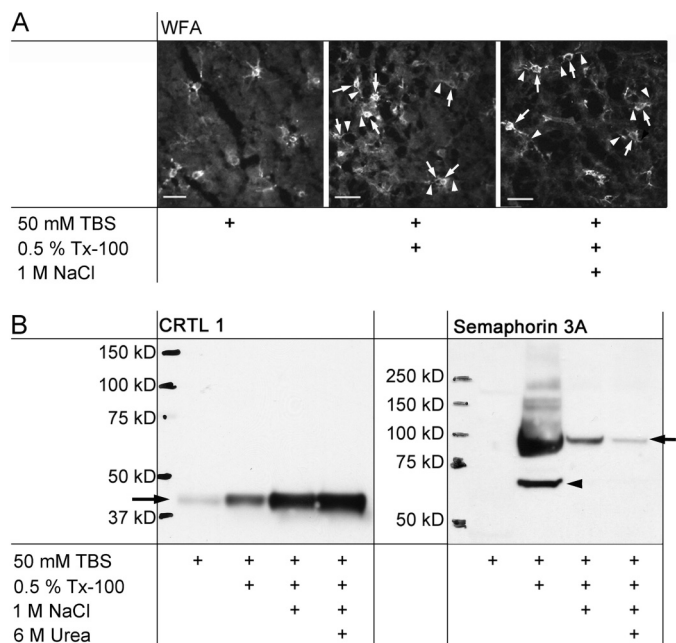


FIGURE 5. Solubilization of PNNs, CRTL1, and Sema3A with different buffers from adult rodent brains. *A*, adult rat cortical sections were sequentially washed with buffers of increasing denaturing power and stained with biotinylated WFA PNNs. Treatment with Triton X-100 (*Tx-100*) and 1 M NaCl leads to a decrease in the WFA staining in the neurites (*arrowheads*) but not on the soma (*arrows*). Scale bar equals 50 μ m. *B*, Western blot of the buffers collected after the sequential extraction from mouse brain homogenates. Although the majority of CRTL 1 is released with 6 M urea buffer, most of the Sema3A is released with Triton X-100 buffer, to a much lesser extent in the 1 M NaCl or 6 M urea buffers.

detergent, 0.5% Triton X-100 (Fig. 5, *right panel*). Both the full-length (~95 kDa) and N terminus of processed Sema3A (~65 kDa) could be observed in this extract. However, it is important to note that the Sema3A remains bound at physiological conditions (50 mM TBS) and is retained at the cell surfaces as described in several *in vitro* studies (25, 35).

We next investigated the nature of the interaction between endogenous Sema3A and PNNs using high salt and GAG lyase digestions to modify the GAGs, while measuring the release of Sema3A from brain tissue. Treatment with 1 M NaCl released Sema3A from rat brains, indicating that the binding is partly dependent on ionic interactions, as with other GAG-protein interactions (Fig. 6*A*). To further analyze the nature of GAGs involved in the interaction, brain homogenates were treated with enzymes specifically degrading hyaluronan, CS, or HS (Fig. 6*B*). ChABC treatment increased the release of Sema3A into the soluble fraction, whereas degradation of other GAGs did not (Fig. 6, *B* and *C*). Although ChABC to some extent acts on hyaluronan, the fact that hyaluronidase digestion did not release Sema3A implies that CS GAGs is the major binding partner in the brain extracellular matrix. The *in vitro* binding assay described in Fig. 2 indicates that Sema3A binds to CS GAGs containing di-sulfated disaccharides (CS-E and CS-B). This is consistent with our finding on sulfatase digestion here. Using chondro-4- or -6-sulfatase, we demonstrated that the removal of 6-sulfate from CS GAGs released Sema3A to the same extent as ChABC (Fig. 6, *B*, *middle panel*, and *C*, *2nd* and *3rd bar*). CS-E chains containing 4,6-di-*O*-sulfated E-units are digested by chondro-6-sulfatase, and it appears that the

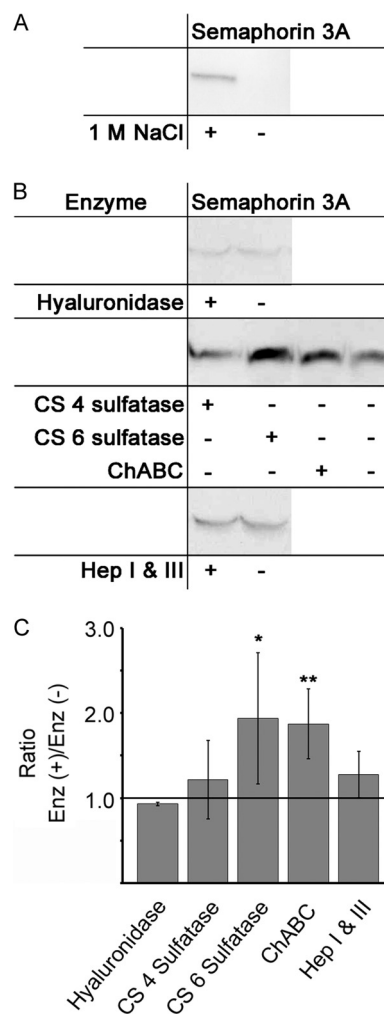


FIGURE 6. Release of Sema3A from brain tissue after enzymatic degradation of GAG chains. The released Sema3A was detected by Western blot of the extracted soluble fractions after enzyme treatment. *A*, Sema3A is released with high salt (1 M NaCl) wash. *B*, whereas digestion with ChABC and chondro-6-sulfatase causes an increase in the release of Sema3A from the brain tissue, digestion of hyaluronan using hyaluronidase, chondro-4-sulfatase on CS-A, and Hep I and III does not increase the release Sema3A (*C*) or quantification of the signal in the Western blots in *B*. Relative release is presented as a ratio of Sema3A signal from the enzyme-treated brain homogenate against the non-enzyme-treated samples, $n = 2-4$, mean \pm S.D., two-tailed paired *t* test: *, $p = 0.054$; **, $p < 0.01$.

chondro-6-sulfatase removes the 6-*O*-sulfate groups from the 4,6-disulfated GalNAc in the CS-E units, creating the 4-*O*-sulfated GalNAc to which Sema3A does not bind. It has been reported that chondro-6-sulfatase removes a 6-*O*-sulfate group from both GalNAc-6-sulfate and GalNAc-4,6-di-*O*-sulfate units (36). The chondro-4-sulfatase, which has been reported to remove 4-*O*-sulfate groups from GalNAc-4-*O*-sulfate even from the internal positions under harsh conditions (37) and does not act on GalNAc-4,6-di-*O*-sulfate (-E unit) (38), did not release Sema3A. The release data are consistent with Sema3A binding to CS-E in the PNNs.

Sema3A Interacts with a Large CSPG in the PNNs—The PNN matrix consists of several PGs carrying CS GAG chains. To investigate if the CS-E that binds Sema3A resides on a specific PG, we performed a co-IP of Sema3A after incubation with PNN PGs. Sema3A-GFP lysate was incubated with the PNN

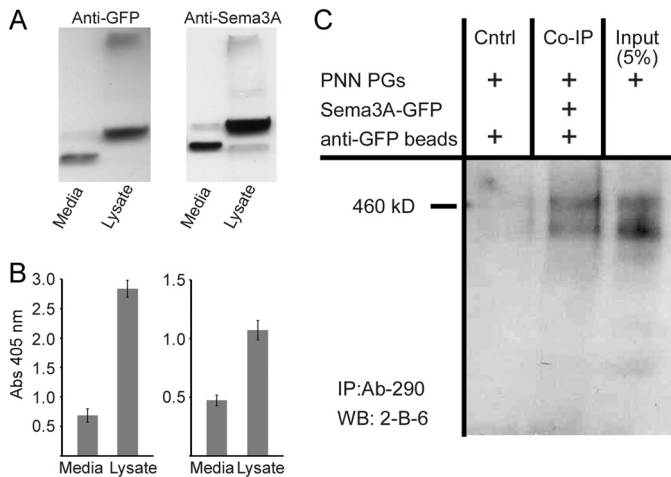


FIGURE 7. Co-immunoprecipitation (co-IP) of brain CSPG(s) with Semaphorin3A-GFP. *A* and *B*, full-length Semaphorin3A-GFP recovered from cell lysate binds to heparin. Recombinant Semaphorin3A-GFP in media and cell lysate were analyzed by Western blotting (WB) (*A*) and used in direct ELISA (*B*). *A*, Western blotting of collected cell media and lysate from Semaphorin3A-GFP-transfected cells. N-terminal (65 kDa) and full-length recombinant Semaphorin3A-GFP (95 kDa) were detected by anti-GFP or anti-Semaphorin3A antibody. This suggests that the recombinant Semaphorin3A-GFP is cleaved by normal cellular processing giving a fragment corresponding to the wild type 65 kDa N-terminal fragment (25). *B*, direct interaction with Semaphorin3A-GFP in media and lysate toward heparin. Because of extensive cleavage of the secreted Semaphorin3A-GFP, we applied conditioned media as well as lysate in the ELISA. Binding to heparin-coated wells was detected when using the lysate, containing mainly full-length Semaphorin3A-GFP. Binding affinity was measured and presented as absorbance at 405 nm, $n = 3$, mean \pm S.D. *C*, PNN PGs isolated from the adult rat brain was incubated with and without Semaphorin3A-GFP lysate (1st and 2nd lanes), followed by IP against GFP using anti-GFP antibody on protein A magnetic beads. The beads were then treated with ChABC, and the supernatant was analyzed using SDS-PAGE and Western blot assay. 2-B-6 antibody was used to detect the stub on the released CSPG(s) after the enzyme treatment. Two bands slightly smaller than 460 kDa observed in the Semaphorin3A-treated samples suggest that Semaphorin3A-GFP is binding to one (or more) high molecular weight CSPG(s) in the brain homogenates. The size of the band suggests that the CSPGs could be aggrecan. 5% of the input was added as a control after chondroitinase treatment (3rd lane).

PGs isolated from extract 4 (the PNN fraction), followed by IP against the GFP part of Semaphorin3A using anti-GFP antibody. Semaphorin3A-GFP lysate was used in this experiment due to a proteolytic cleavage of Semaphorin3A-GFP by the normal cellular process, and full-length Semaphorin3A-GFP can only be recovered in cell lysate instead of culture media (Fig. 7, *A* and *B*). After incubation with the anti-GFP antibody, the Semaphorin3A-GFP-PNN-PG complex was pulled down by protein A magnetic beads. CSPG was then released by ChABC treatment and analyzed by Western blot (Fig. 7*C*). We were able to detect a large core protein with the use of an antibody (clone 2-B-6) recognizing the stubs of the CS GAG chains that are left behind following ChABC treatment (39). The size of the CSPG protein core that was pulled down corresponds to aggrecan. However, by blotting with antibodies directed against epitopes in the protein part of aggrecan, we were not able to detect the band. The discrepancy could be due to a difference in sensitivity. As the aggrecan has multiple attachment sites for GAG chains (up to ~ 100), the ChABC-treated protein core probably contains multiple "stub" epitopes that the 2-B-6 antibody can bind, although the epitopes in the protein core are fewer and with variable affinity.

PNN GAGs and Semaphorin 3A Inhibits Adult DRG Outgrowth—Both Semaphorin3A and CSPGs are associated with axon growth inhibition in the adult nervous system. It is probable

that parts of PNNs' capacity to reduce plasticity are based on the inhibition from CSPGs in restricting axon growth, which is potentiated by Semaphorin3A binding. To determine the biological function of our biochemical findings, we assessed the growth inhibitory properties of PNN GAGs, Semaphorin3A and the two together in an *in vitro* assay using DRG neurons. To investigate substrate-mediated effects, we first coated the coverslips with laminin and subsequently with Semaphorin3A and/or PNN-GAGs, and DRG explants were then cultured on these substrate-coated coverslips, and the neurite extension was assayed. DRG explants cultured on 25 $\mu\text{g/ml}$ PNN-GAGs extended both shorter ($\sim 50\%$ shorter) and fewer neurites compared with laminin alone (Fig. 8, *A* and *B*). The addition of Semaphorin3A on PNN-GAGs further suppressed the neurite outgrowth from the DRGs. A dose-dependent inhibition was observed when increasing concentrations of Semaphorin3A (at 1:100, 50, and 10 dilution) were used (Fig. 8, *A* and *B*). A combination of Semaphorin3A and PNN-GAGs led to an 80% decrease in the neurite length when compared with laminin alone, a further 30% lower than when PNN-GAGs/laminin was used. Similarly, this Semaphorin3A (1:50)/PNN-GAGs combination decreased the number of neurites extending from the explants (Fig. 8*B*). The data imply that there is an additive effect of Semaphorin3A and PNN-GAGs in inhibiting neurite initiation (Fig. 8*B*) as well as outgrowth (Fig. 8*A*).

We have previously shown that CS-E is enriched in the PNN-GAGs (10), and to investigate the role of CS-E in this inhibition, we cultured the DRGs in the presence of anti-CS-E antibody. We chose to culture the DRG explants in 1:50 Semaphorin3A with 15 $\mu\text{g/ml}$ PNN GAGs in subsequent experiments based on the above result. Again, we observed a greater inhibition with Semaphorin3A and GAGs together than with either alone (Fig. 8, *C*, and 3rd, 4th, and 5th bars in *D*). Enzymatic removal of CS-GAGs using ChABC completely abolished the inhibition from PNN-GAGs/Semaphorin3A, whereas Hep I and III treatment partially reversed the inhibition (Fig. 8, bar 5 and 6). To investigate if CS-E is involved in this neurite outgrowth inhibition, we measured neurite growth on the combination of Semaphorin3A and PNN-GAGs in the presence of the anti-CS-E antibody GD3G7. Whereas the addition of anti-CS-E antibody partially neutralized the additive inhibition from Semaphorin3A/PNN-GAGs, the control antibody did not relieve the inhibition (Fig. 8, 7th and 8th bar).

DISCUSSION

This study was inspired, based on extensive data from us and others, by the importance of PNNs in controlling CNS plasticity and the effectiveness of ChABC in restoring plasticity and recovery after removing the PNNs in the damaged nervous system. The mechanism by which PNNs control plasticity is not known, but our observation of Semaphorin3A binding to the PNNs suggests that presentation of Semaphorin3A by PNNs to synapses and axonal sprouts could be a key role of CSPGs in the PNNs.

Semaphorin3A Binds to CS-E in PNNs—Semaphorin3A is a potent regulator of neurite growth (22) and cell migration (40) in the developing nervous system, and it has strong effects on synapse dynamics (15–17). Cell surface retention of Semaphorin3A has been observed on Neuro-2a cells, from which it is released by ChABC treatment or an addition of CS-B or heparin; this implicated

Semaphorin3A Interacts with CS-E in PNNs

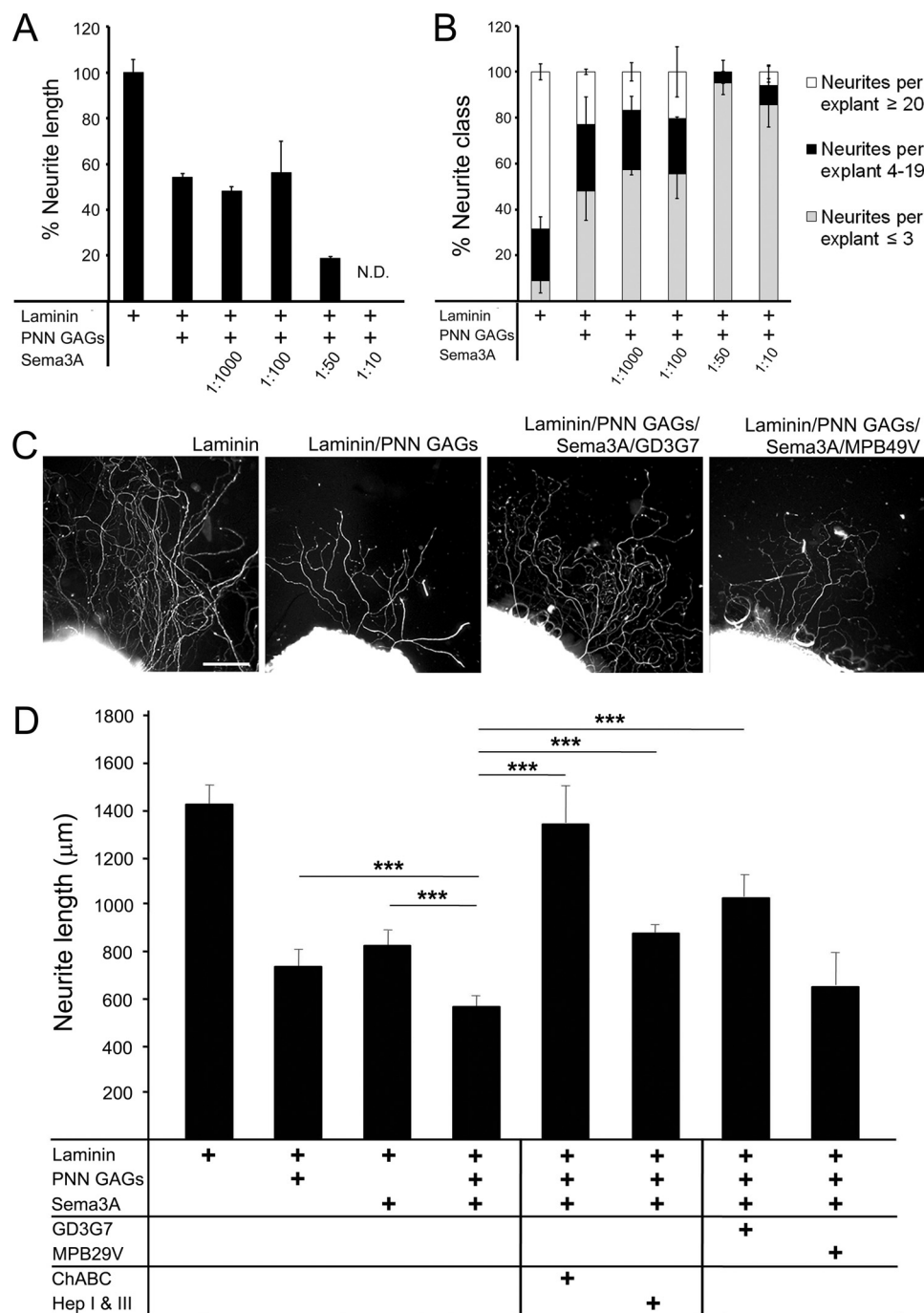


FIGURE 8. Combination of Semaphorin3A and PNN-GAGs confers a stronger inhibition on DRG culture that is alleviated with anti-CS-E antibody. Substrate inhibition of DRG-neurite outgrowth by PNN GAGs and Semaphorin3A. Adult rat DRG explants were cultured on substrate containing laminin, Semaphorin3A, and/or PNN GAGs and subsequently stained with β III tubulin for the neurites. Outgrowth measurement is presented as an average of 10 neurites per explant, 2–10 explants per condition from two separate experiments. *A*, measurement of neurite outgrowth of DRG explants on 25 μ g/ml PNN GAGs in combination with increasing amounts of Semaphorin3A. DRG explants cultured on PNN-GAGs showed a reduction in neurite length when compared with laminin control. Further reduction was observed when the explants were cultured with increasing concentration of Semaphorin3A (1:50 and 1:10) suggesting Semaphorin3A confers a stronger inhibition upon PNN GAGs on neurite inhibition. Increasing amounts of Semaphorin3A demonstrated a dose-dependent inhibition. Data are presented as mean \pm S.E. *B*, classification of neurite outgrowth ability of the DRG explants according to the number of neurites per explant. Explants cultured on PNN GAGs showed a decrease in the percentage of explants bearing >20 neurites. This inhibition was even stronger when the explants were cultured in the presence of both PNN GAGs and Semaphorin3A. The majority of explants project less than three neurites. The data are presented as mean \pm S.E. for each class. *C*, images of the DRG explants cultured on “laminin,” “laminin plus PNN GAGs,” “laminin, PNN GAGs, Semaphorin3A, and control antibody (MPB49V),” and “laminin, PNN GAGs, Semaphorin3A, and anti-CS-E antibody” (GD3G7). Scale bar equals 200 μ m. *D*, quantification of neurite outgrowth of the DRG explants culture with the presence of anti-CS-E antibody. Explants cultured on PNN GAGs or Semaphorin3A project significantly shorter neurites than the laminin control (1st to 3rd bars). A combination of PNN GAGs and Semaphorin3A leads to a stronger inhibition on the outgrowth (4th bar), and this inhibition is ameliorated when the PNN GAGs were treated with ChABC (5th bar), Hep I and III (to a lesser extent, bar 6) and anti-CS-E GD3G7 antibody. Treatment with control MPB49V antibody does not remove the inhibition from the substrates. $n = 60$ –100, mean \pm S.D., two-tailed paired t test, ***, $p < 0.0001$.

GAG interactions in retaining Semaphorin3A at the cell surface (25). Our recent work in rat brains demonstrated that Semaphorin3A is retained at the surface of PNN-bearing neurons and is released by ChABC injection into the brain. This indicates that Semaphorin3A is bound to CS-GAGs in the PNNs (1). In this study, we have continued to characterize the nature of the GAGs, which are responsible for the binding of Semaphorin3A to the PNNs. The Semaphorin3A in brain tissue can be released by treatment with 1 M NaCl or detergent buffer, indicating that it is attached to glycans by moderate affinity charge interaction. To characterize the binding, we first examined binding to glycans of known sulfation pattern. Similar to our previous study, Semaphorin3A bound to heparin and CS-GAGs (25). However, not all forms of CS-GAGs bind to Semaphorin3A. CS-E and -B show strong interactions in our ELISA, whereas CS-A, -C, and -D do not. With GAG microarrays, the results are very similar, although less binding to CS-B was observed, presumably due to differences in the percentage of 2,4-disulfated CS in the CS-B derived from pig skin compared with pig intestine. Moreover, the fact that CS-D disulfated disaccharide units do not bind Semaphorin3A is significant because it indicates that the specific sulfation pattern defines the binding site on glycans rather than the mere overall charge of the molecule. Organization of the disaccharides into local binding domains with specific affinity toward interacting proteins has been described (41–43). In general, GAG-binding proteins discriminate to various degrees between the different GAG structures. Whereas the anti-coagulant effect of binding of anti-thrombin 3 to heparin requires a specific organization of sulfate groups, less specificity exists for some other protein-GAG interactions (44), and several growth factors have been shown to interact with variable structures and types of GAGs (45). In previous work, we analyzed the composition of the different sulfate structures in the PNN fraction by disaccharide analysis (10), showing that CS-B and CS-E disaccharides are only responsible for 0.8 and 2% of the total PNN GAGs. Despite this small percentage, our results indicate that CS-containing E units in the PNNs are the main target for Semaphorin3A binding. The exact composition of the GAG structure responsible for Semaphorin3A interaction cannot be deduced from the present data. Indeed, it is likely that a set of various GAG sequences that include CS-E, and possibly CS-B, are able to interact with Semaphorin3A. Hence, we cannot totally exclude the contribution from other CS-disaccharides in this interaction.

We have also assayed for the binding of Semaphorin3A to PNN GAGs, isolated by a sequential purification method from adult brain (10). Semaphorin3A showed a strong binding to the PNN fraction, but almost no binding to the GAGs extracted in the soluble or detergent-soluble fractions, which come from the diffuse matrix throughout the CNS. This finding is consistent with our hypothesis that the different sulfation composition in the different fractions may confer different binding properties to the glycans. Our results demonstrate that a CS-E disaccharide-containing structure (found in PNNs) is the main contributor to the binding and that this binding could be blocked by an anti-CS-E antibody. To further test the conclusion that Semaphorin3A binds to CS-containing E units, we digested brain tissue GAGs with two sulfatases, measuring Semaphorin3A release from the tissue. Digestion with chondro-6-sulfatase, which removes all 6-O-sulfate

groups in the GAG chains and converts 4,6-disulfated E units into 4-O-sulfated A units, released as much Semaphorin3A as ChABC. The 4-sulfatase, which releases 4-O-sulfate groups from internal GalNAc-4-O-sulfate, but not from CS-E (37), did not release Semaphorin3A. Overall, our results support the idea that the majority of Semaphorin3A is retained on the PNNs through binding to CSs containing E disaccharide units. It would be interesting to investigate the context of CS-E residues in the GAG chains and also where these putative binding sites are located along the CS chain.

We used three different Semaphorin3As, namely Semaphorin3A-AP, Semaphorin3A-GFP, and Semaphorin3A-Fc, in our study to eliminate the possibility that the reported binding results are due to any possible interference from the tag proteins. We also verify that the observed binding was due to the expressed Semaphorin3A but not the presence of other proteins present in the media/lysate (Figs. 2D and 7B). Moreover, although Semaphorin3A-AP and Semaphorin3A-GFP are collected and partially purified from culture media or cell lysate, the Semaphorin3A-Fc is of >90% purity according to the supplier's information.

Binding to HSPGs—PNNs contain both CSPGs and HSPGs. The majority of PNN GAGs are of CS-type (~70%), and the rest is mainly HS and possibly also some keratan sulfate (10). We therefore asked whether some of the Semaphorin3A binding is due to the presence of HSPGs. We treated the PNN GAGs with ChABC or heparitinases before assaying for the Semaphorin3A binding (Fig. 3B). Neither enzyme alone abolished Semaphorin3A binding, but a combination of the two led to a complete abolition of binding, indicating that both types of GAG in the PNNs are capable of interacting with Semaphorin3A. However, only ChABC and chondro-6-sulfatase released Semaphorin3A from brain tissue, and heparitinases were ineffective, suggesting that the majority of Semaphorin3A binding is to CS-GAG (Fig. 6). This is supported by our recent work showing that ChABC injection into adult rat brain completely abolishes the Semaphorin3A staining around PNNs (1). Taken together, Semaphorin3A is capable of interacting with various GAGs, but our results indicate that the main contribution to the interaction with PNNs is via the CS-E disaccharide units in the CSs.

Effects of Semaphorin3A-PNN GAG Binding—We explored the response of adult DRG neurons to PNN GAGs in combination with Semaphorin3A (Fig. 8). The growth of DRG neurites was inhibited in the presence of either PNN-GAGs or Semaphorin3A alone, as demonstrated in several *in vitro* systems using soluble Semaphorin3A (25, 46). The addition of Semaphorin3A to PNN GAGs confers further inhibition on the neurite outgrowth, both on neurite length and number of neurites per explant. Our findings are in line with other studies investigating the relationship between CSs and Semaphorin3A. Zimmer *et al.* (26) has shown that combining CSPG and soluble Semaphorin3A-Fc leads to a 10% increase in the repellent activity in an *in vitro* stripe migration assay of cortical interneurons during development. This suggests a combined effect of CSs and Semaphorin3A. However, the interaction with CS-GAGs was not further characterized. In this work, we demonstrated that the binding of Semaphorin3A to CSs is likely to be mediated via the CS-E units and that the addition of anti-CS-E antibody GD3G7 in the DRG culture relieved the outgrowth inhibition from the addition of Semaphorin3A (Fig. 8, B and C). The possible additive

Semaphorin3A Interacts with CS-E in PNNs

effect of Sema3A with PNN-GAGs could be attributed by the independent signaling pathways these two families of molecules are eliciting. Whereas Sema3A signals through the neuropilin-1 (NP-1)/plexin receptor complex (47–50), the inhibition from CSs in the PNN-GAGs can be triggered via the recently identified receptors, such as protein-tyrosine phosphatase- σ , leukocyte common-related phosphatase, Nogo receptors, or contactin pathways (51–54). It is possible that the simultaneous presentation of both Sema3A and CSs on the neuronal surface confer stronger inhibitory properties to the PNNs, which will trigger both inhibitory signaling pathways in the incoming growth cones and therefore exclude new connections from PNN-surrounded neurons in the adult CNS.

The role of Sema3A in the adult CNS is not understood. Sema3A expression is sustained after birth, but at a lower level in several areas of the CNS (23). However, the receptor complex NP-1/plexin for Sema3A are both widely expressed in the adult CNS (47–50). The effect of Sema3A has been suggested to be tuned by heparin, facilitating stronger binding to NP-1 (25). Indeed, Sema5A, another member from the Sema family, acts as both positive and negative guidance cue in neuronal outgrowth depending on its interaction with HS or CS (55). Our observation that Sema3A is presented on PNNs, coupled with the established role of PNNs in controlling plasticity, suggests that PNNs may exert their effect partly through Sema3A. The PNNs may utilize the Sema3A in controlling neurite growth and synapse dynamics. This hypothesis has been tested by expressing NP-1-fc in adult visual cortex, leading to reactivation of ocular dominance plasticity. However, it is unlikely that all the effects of PNNs are mediated through this mechanism. Recent reports have implicated the role of PNNs in the modulation of synapses through AMPA receptor mobility, which is unlikely to involve Sema3A (56, 57). Moreover, several CSPG receptors, such as protein-tyrosine phosphatase- σ , contactin, and Nogo receptor 3, are also widely expressed in the CNS (51–54). They may mediate growth inhibition by directly binding to the CS GAGs (27).³ However, the specific interaction of Sema3A to CS-E units suggests that an anti CS-E antibody or other CS-E blocker may aid in restoring plasticity and promoting recovery of the CNS after injury.

Acknowledgments—We thank Dr. Gerdy B. ten Dam for advice on the purification and use of the antibodies GD3G7 and MPB49V. We also thank Dr. Antoshechkin and the Caltech Millard and Muriel Jacobs Genetics and Genomics Laboratory for assistance with printing of the polysaccharide microarrays.

REFERENCES

1. Vo, T., Carulli, D., Ehlert, E. M., Kwok, J. C., Dick, G., Mecollari, V., Moloney, E. B., Neufeld, G., de Winter, F., Fawcett, J. W., and Verhaagen, J. (2013) The chemorepulsive axon guidance protein semaphorin 3A is a constituent of perineuronal nets in the adult rodent brain. *Mol. Cell. Neurosci.* **56C**, 186–200
2. Matthews, R. T., Kelly, G. M., Zerillo, C. A., Gray, G., Tiemeyer, M., and Hockfield, S. (2002) Aggrecan glycoforms contribute to the molecular

³ Boggio, E. M., Ehlert, E. M., Moloney, E. M., Mecollari, V., Fawcett, J. W., Verhaagen, J., and Pizzorusso, T., poster presented at the FENS Abstr. Session 98.07, Poster B39-3526, Barcelona, Spain (July 14–18, 2012).

- heterogeneity of perineuronal nets. *J. Neurosci.* **22**, 7536–7547
3. Yamaguchi, Y. (2000) Lecticans: organizers of the brain extracellular matrix. *Cell. Mol. Life Sci.* **57**, 276–289
4. Carulli, D., Rhodes, K. E., and Fawcett, J. W. (2007) Upregulation of aggrecan, link protein 1, and hyaluronan synthases during formation of perineuronal nets in the rat cerebellum. *J. Comp. Neurol.* **501**, 83–94
5. Celio, M. R., and Blümcke, I. (1994) Perineuronal nets—a specialized form of extracellular matrix in the adult nervous system. *Brain Res. Rev.* **19**, 128–145
6. Vitellaro-Zuccarello, L., Meroni, A., Amadeo, A., and De Biasi, S. (2001) Chondroitin sulfate proteoglycans in the rat thalamus: expression during postnatal development and correlation with calcium-binding proteins in adults. *Cell Tissue Res.* **306**, 15–26
7. Galtrey, C. M., and Fawcett, J. W. (2007) The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Res. Rev.* **54**, 1–18
8. Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J. W., and Maffei, L. (2002) Reactivation of ocular dominance plasticity in the adult visual cortex. *Science* **298**, 1248–1251
9. Carulli, D., Pizzorusso, T., Kwok, J. C., Putignano, E., Poli, A., Forostyak, S., Andrews, M. R., Deepa, S. S., Glant, T. T., and Fawcett, J. W. (2010) Animals lacking link protein have attenuated perineuronal nets and persistent plasticity. *Brain* **133**, 2331–2347
10. Deepa, S. S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., Sugahara, K., and Fawcett, J. W. (2006) Composition of perineuronal net extracellular matrix in rat brain: a different disaccharide composition for the net-associated proteoglycans. *J. Biol. Chem.* **281**, 17789–17800
11. Galtrey, C. M., Asher, R. A., Nothias, F., and Fawcett, J. W. (2007) Promoting plasticity in the spinal cord with chondroitinase improves functional recovery after peripheral nerve repair. *Brain* **130**, 926–939
12. Harris, N. G., Nogueira, M. S., Verley, D. R., and Sutton, R. L. (2013) Chondroitinase enhances cortical map plasticity and increases functionally active sprouting axons after brain injury. *J. Neurotrauma* **30**, 1257–1269
13. Wang, D., Ichiyama, R. M., Zhao, R., Andrews, M. R., and Fawcett, J. W. (2011) Chondroitinase combined with rehabilitation promotes recovery of forelimb function in rats with chronic spinal cord injury. *J. Neurosci.* **31**, 9332–9344
14. Romberg, C., Yang, S., Melani, R., Andrews, M. R., Horner, A. E., Spillanti, M. G., Bussey, T. J., Fawcett, J. W., Pizzorusso, T., and Saksida, L. M. (2013) Depletion of perineuronal nets enhances recognition memory and long term depression in the perirhinal cortex. *J. Neurosci.* **33**, 7057–7065
15. Bouzioukh, F., Daoudal, G., Falk, J., Debanne, D., Rougon, G., and Castellani, V. (2006) Semaphorin3A regulates synaptic function of differentiated hippocampal neurons. *Eur. J. Neurosci.* **23**, 2247–2254
16. Sahay, A., Kim, C. H., Sepkuty, J. P., Cho, E., Huganir, R. L., Ginty, D. D., and Kolodkin, A. L. (2005) Secreted semaphorins modulate synaptic transmission in the adult hippocampus. *J. Neurosci.* **25**, 3613–3620
17. Tran, T. S., Rubio, M. E., Clem, R. L., Johnson, D., Case, L., Tessier-Lavigne, M., Huganir, R. L., Ginty, D. D., and Kolodkin, A. L. (2009) Secreted semaphorins control spine distribution and morphogenesis in the postnatal CNS. *Nature* **462**, 1065–1069
18. Manns, R. P., Cook, G. M., Holt, C. E., and Keynes, R. J. (2012) Differing semaphorin 3A concentrations trigger distinct signaling mechanisms in growth cone collapse. *J. Neurosci.* **32**, 8554–8559
19. Li, C., Bassell, G. J., and Sasaki, Y. (2009) Fragile X mental retardation protein is involved in protein synthesis-dependent collapse of growth cones induced by semaphorin-3A. *Front. Neural Circuits* **10.3389/neuro.04.011.2009**
20. Behar, O., Golden, J. A., Mashimo, H., Schoen, F. J., and Fishman, M. C. (1996) Semaphorin III is needed for normal patterning and growth of nerves, bones, and heart. *Nature* **383**, 525–528
21. Schwarting, G. A., Kostek, C., Ahmad, N., Dibble, C., Pays, L., and Püschel, A. W. (2000) Semaphorin 3A is required for guidance of olfactory axons in mice. *J. Neurosci.* **20**, 7691–7697
22. Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., and Yagi, T. (1997) Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* **19**, 519–530

23. Giger, R. J., Pasterkamp, R. J., Heijnen, S., Holtmaat, A. J., and Verhaagen, J. (1998) Anatomical distribution of the chemorepellent semaphorin III/collapsin-1 in the adult rat and human brain: predominant expression in structures of the olfactory-hippocampal pathway and the motor system. *J. Neurosci. Res.* **52**, 27–42
24. de Wit, J., and Verhaagen, J. (2003) Role of semaphorins in the adult nervous system. *Prog. Neurobiol.* **71**, 249–267
25. De Wit, J., De Winter, F., Klooster, J., and Verhaagen, J. (2005) Semaphorin 3A displays a punctate distribution on the surface of neuronal cells and interacts with proteoglycans in the extracellular matrix. *Mol. Cell. Neurosci.* **29**, 40–55
26. Zimmer, G., Schanuel, S. M., Bürger, S., Weth, F., Steinecke, A., Bolz, J., and Lent, R. (2010) Chondroitin sulfate acts in concert with semaphorin 3A to guide tangential migration of cortical interneurons in the ventral telencephalon. *Cereb. Cortex* **20**, 2411–2422
27. Coles, C. H., Shen, Y., Tenney, A. P., Siebold, C., Sutton, G. C., Lu, W., Gallagher, J. T., Jones, E. Y., Flanagan, J. G., and Aricescu, A. R. (2011) Proteoglycan-specific molecular switch for RPTP σ clustering and neuronal extension. *Science* **332**, 484–488
28. Purushothaman, A., Fukuda, J., Mizumoto, S., ten Dam, G. B., van Kuppevelt, T. H., Kitagawa, H., Mikami, T., and Sugahara, K. (2007) Functions of chondroitin sulfate/dermatan sulfate chains in brain development. Critical roles of E and IE disaccharide units recognized by a single chain antibody GD3G7. *J. Biol. Chem.* **282**, 19442–19452
29. Feiner, L., Koppel, A. M., Kobayashi, H., and Raper, J. A. (1997) Secreted chick semaphorins bind recombinant neuropilin with similar affinities but bind different subsets of neurons *in situ*. *Neuron* **19**, 539–545
30. Kwok, J. C. F., Foscarin, S., and Fawcett, J. W. (2013) in *Neuromethods: Extracellular Matrix* (Powell, E. M., ed) 1st Ed., Springer Science + Business Media, LLC, Humana Press, New York
31. Rogers, C. J., Clark, P. M., Tully, S. E., Abrol, R., Garcia, K. C., Goddard, W. A., 3rd, and Hsieh-Wilson, L. C. (2011) Elucidating glycosaminoglycan-protein-protein interactions using carbohydrate microarray and computational approaches. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 9747–9752
32. Shipp, E. L., and Hsieh-Wilson, L. C. (2007) Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem. Biol.* **14**, 195–208
33. Brückner, G., Härtig, W., Kacza, J., Seeger, J., Welt, K., and Brauer, K. (1996) Extracellular matrix organization in various regions of rat brain grey matter. *J. Neurocytol.* **25**, 333–346
34. Härtig, W., Brauer, K., Bigl, V., and Brückner, G. (1994) Chondroitin sulfate proteoglycan immunoreactivity of lectin-labeled perineuronal nets around parvalbumin-containing neurons. *Brain Res.* **635**, 307–311
35. de Wit, J., Toonen, R. F., and Verhage, M. (2009) Matrix-dependent local retention of secretory vesicle cargo in cortical neurons. *J. Neurosci.* **29**, 23–37
36. Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., and Takeda, K. (1994) Structural studies on the chondroitinase ABC-resistant sulfated tetrasaccharides isolated from various chondroitin sulfate isomers. *Carbohydr. Res.* **255**, 145–163
37. Sugahara, K., and Kojima, T. (1996) Specificity studies of bacterial sulfatases by means of structurally defined sulfated oligosaccharides isolated from shark cartilage chondroitin sulfate D. *Eur. J. Biochem.* **239**, 865–870
38. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* **243**, 1523–1535
39. Sugahara, K., Masuda, M., Harada, T., Yamashina, I., de Waard, P., and Vliegthart, J. F. (1991) Structural studies on sulfated oligosaccharides derived from the carbohydrate-protein linkage region of chondroitin sulfate proteoglycans of whale cartilage. *Eur. J. Biochem.* **202**, 805–811
40. Chen, G., Sima, J., Jin, M., Wang, K. Y., Xue, X. J., Zheng, W., Ding, Y. Q., and Yuan, X. B. (2008) Semaphorin-3A guides radial migration of cortical neurons during development. *Nat. Neurosci.* **11**, 36–44
41. Achur, R. N., Muthusamy, A., Madhunapantula, S. V., Bhavanandan, V. P., Seudieu, C., and Channe Gowda, D. (2004) Chondroitin sulfate proteoglycans of bovine cornea: structural characterization and assessment for the adherence of *Plasmodium falciparum*-infected erythrocytes. *Biochim. Biophys. Acta* **1701**, 109–119
42. Blackhall, F. H., Merry, C. L., Lyon, M., Jayson, G. C., Folkman, J., Javaheerian, K., and Gallagher, J. T. (2003) Binding of endostatin to endothelial heparan sulphate shows a differential requirement for specific sulphates. *Biochem. J.* **375**, 131–139
43. Gama, C. L., Tully, S. E., Sotogaku, N., Clark, P. M., Rawat, M., Vaidehi, N., Goddard, W. A., 3rd, Nishi, A., and Hsieh-Wilson, L. C. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat. Chem. Biol.* **2**, 467–473
44. Kreuger, J., Spillmann, D., Li, J. P., and Lindahl, U. (2006) Interactions between heparan sulfate and proteins: the concept of specificity. *J. Cell Biol.* **174**, 323–327
45. Bao, X., Nishimura, S., Mikami, T., Yamada, S., Itoh, N., and Sugahara, K. (2004) Chondroitin sulfate/dermatan sulfate hybrid chains from embryonic pig brain, which contain a higher proportion of L-iduronic acid than those from adult pig brain, exhibit neuritegenic and growth factor binding activities. *J. Biol. Chem.* **279**, 9765–9776
46. Ben-Zvi, A., Yagil, Z., Hagalili, Y., Klein, H., Lerman, O., and Behar, O. (2006) Semaphorin 3A and neurotrophins: a balance between apoptosis and survival signaling in embryonic DRG neurons. *J. Neurochem.* **96**, 585–597
47. He, Z., and Tessier-Lavigne, M. (1997) Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90**, 739–751
48. Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997) Neuropilin is a semaphorin III receptor. *Cell* **90**, 753–762
49. Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H., and Strittmatter, S. M. (1999) Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99**, 59–69
50. Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M., and Goodman, C. S. (1998) Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell* **95**, 903–916
51. Shen, Y., Tenney, A. P., Busch, S. A., Horn, K. P., Cuascut, F. X., Liu, K., He, Z., Silver, J., and Flanagan, J. G. (2009) PTP σ is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science* **326**, 592–596
52. Fisher, D., Xing, B., Dill, J., Li, H., Hoang, H. H., Zhao, Z., Yang, X. L., Bachoo, R., Cannon, S., Longo, F. M., Sheng, M., Silver, J., and Li, S. (2011) Leukocyte common antigen-related phosphatase is a functional receptor for chondroitin sulfate proteoglycan axon growth inhibitors. *J. Neurosci.* **31**, 14051–14066
53. Mikami, T., Yasunaga, D., and Kitagawa, H. (2009) Contactin-1 is a functional receptor for neuroregulatory chondroitin sulfate-E. *J. Biol. Chem.* **284**, 4494–4499
54. Dickendesher, T. L., Baldwin, K. T., Mironova, Y. A., Koriyama, Y., Raiker, S. J., Askew, K. L., Wood, A., Geoffroy, C. G., Zheng, B., Liepmann, C. D., Katagiri, Y., Benowitz, L. I., Geller, H. M., and Giger, R. J. (2012) NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. *Nat. Neurosci.* **15**, 703–712
55. Kantor, D. B., Chivatakarn, O., Peer, K. L., Oster, S. F., Inatani, M., Hansen, M. J., Flanagan, J. G., Yamaguchi, Y., Sretavan, D. W., Giger, R. J., and Kolodkin, A. L. (2004) Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* **44**, 961–975
56. Frischknecht, R., Heine, M., Perrais, D., Seidenbecher, C. I., Choquet, D., and Gundelfinger, E. D. (2009) Brain extracellular matrix affects AMPA receptor lateral mobility and short-term synaptic plasticity. *Nat. Neurosci.* **12**, 897–904
57. Maroto, M., Fernández-Morales, J. C., Padín, J. F., González, J. C., Hernández-Guijo, J. M., Montell, E., Vergés, J., de Diego, A. M., and García, A. G. (2013) Chondroitin sulfate, a major component of the perineuronal net, elicits inward currents, cell depolarization, and calcium transients by acting on AMPA and kainate receptors of hippocampal neurons. *J. Neurochem.* 10.1111/jnc.12159