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Chondroitin Sulfate Proteoglycan Specific to Retinal Horizontal Neurons

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ABSTRACT

Proteoglycans (PGs) are a diverse group of highly glycosylated macromolecules that are implicated in the development and maintenance of neuronal circuitry. With its highly ordered, layered structure, the retina ideally serves to define the synthesis, processing, and distribution of these molecules within a specific cellular subpopulation. In retinal sections, monoclonal antibody (MAb) 6A2 immunostained a horizontal cell-specific antigen. Antigen 6A2 was expressed within abundant processes in the outer plexiform layer and in rare neurites that extend across the inner nuclear layer to the inner plexiform layer. Ultrastructurally, the antigen was localized to cisternae within horizontal cell somata, along tubulovesicular structures in dendrites, and in the perisynaptic space encircling presynaptic terminals of the cone photoreceptor triad. These findings suggest that this PG is synthesized within the horizontal cells, transported to the terminals, and released into the extracellular spaces just proximal to the synapse. Based on the focal stain in the adjacent photoreceptor cell, it is possible that antigen is pinocytosed by this cell and is concentrated at the ribbon synapse. In Western immunoblots of retinal homogenates, MAb 6A2 recognized a heterogeneous chondroitin sulfate (CS) PG (CSPG) of approximately 400–500 kDa. After sequential enzymatic removal of CS glycosaminoglycans, a major broad band of 300–500 kDa was identified by MAb 1B5, which detects CSPGs that bear uronic acid linked to unsulfated N-acetylgalactosamine as the initial disaccharide in the CS chain. Localization of this PG around presynaptic terminals of the horizontal neuron and at the ribbon synapse suggests that it may play a modulatory and sustaining role at the synapse. J. Comp. Neurol. 390:268–277, 1998.

Indexing terms: ultrastructure; glycosaminoglycan; immunocytochemistry; triad; synapse

Horizontal cells are retinal interneurons that modulate information transfer from photoreceptor pedicles to bipolar cell dendrites in the outer plexiform layer (OPL). There are three morphologically distinct types of horizontal neurons in the human retina; HI, HII, and HIII cells (Kolb et al., 1992). In mammals, horizontal cells are located near the margin of the inner nuclear layer (INL) adjacent to the OPL, where nerve terminals form a dense, highly ordered network.

Horizontal cells have a complex and extensive dendritic tree that makes synaptic contact with either rod or cone cell terminals (Wässle and Boycott, 1991). These terminals include the ribbon synapse, where, typically, the synaptic complex is a triad composed of two horizontal cell lateral processes and a central, postsynaptic, bipolar cell terminal (Dowling and Boycott, 1966; Blanks et al., 1974; Linberg and Fisher, 1988). A rod cell may have a single triad at its terminal, whereas each cone cell pedicle may have multiple synaptic triads.

Horizontal cells are distinguished from other neurons in the central nervous system (CNS) by the coexpression of two intermediate filament proteins, neurofilament (Peichl and González-Soriano, 1993) and vimentin (Dräger, 1983; Dahl and Bignami, 1991). Vimentin is transiently expressed in other neurons only during development, whereas there is sustained expression of vimentin in cells of mesenchymal origin and in some glia.

The specificity of horizontal cells may also be determined by cell surface and extracellular matrix proteins

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including proteoglycans (PGs). These complex molecules play a role in the formation of functional neuronal circuitry by mediating cell migration, axonal pathfinding, and synaptogenesis (Crossin et al., 1989; Morriss-Kay and Tuckett, 1989; Geisert et al., 1992; Maeda et al., 1992). Although PGs have been identified in the retina, to our knowledge, none is preferentially expressed in the horizontal cell (Porrello et al., 1986; Johnson and Hageman, 1987; Geisert et al., 1992).

Recently, we described a chondroitin sulfate (CS) PG (CSPG), somataglycan-S, which is localized to neurons of the human and murine spinocerebellar systems. This PG is immunoreactive with monoclonal antibody (MAb) 6A2 and is detectable on Western immunoblots as a broad band of approximately 600 kDa. Ultrastructurally, the antigen lies intersynaptically along the surface membranes of somata and proximal dendrites and extends into the extracellular space surrounding putative inputs (Williams et al., 1990, 1994).

In this study, we identified a PG that is immunoreactive with MAb 6A2 and is localized to horizontal neurons. Double labelling of these neurons with MAb 6A2 and anti-vimentin antibody verified their identity, their relation to the photoreceptor and bipolar cells, and, especially, their relation to the triad ribbon synapse. This retinal preparation allows visualization of the cytoplasmic and extracellular localization of this antigen in horizontal neuron somata and neurites. Partial biochemical characterization confirmed the corresponding antigen to be a CSPG.

MATERIALS AND METHODS

Materials

Tissue Tek OCT embedding medium was obtained from Miles, Inc. (Naperville, IL). Mounting media was obtained from BioGenix Laboratories (San Ramon, CA). Aminoethylcarbazole, protease inhibitors, and all buffer chemicals were obtained from Sigma (St. Louis, MO). Protease-free bovine serum albumin was obtained from Boehringer Mannheim (Indianapolis, IN). Affi-gel 10 beads, 2-β-mercaptoethanol and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad (Richmond, CA). Sephacryl HR-300 gel was obtained from Pharmacia (Piscataway, NJ). Streptavidin biotinylated horseradish peroxidase complex, the ECL reagent kit, and Rainbow standards were obtained from Amersham (Arlington Heights, IL). Protease inhibitors were purchased from Sigma, except for Pefabloc, which was obtained from Boehringer Mannheim. Chondroitinase ABC (ChABC) was obtained from Seikagaku America (Rockville, MD). Centricon filtration units were obtained from Amicon (Beverley, MA).

Antibodies

Preparation of MAb 6A2, an IgM antibody, has been described previously (Fujita et al., 1982). The 6A2 hybridoma line was generously provided by Dr. Seymour Benzer (California Institute of Technology, Pasadena CA). Anti-vimentin antibody was obtained from Boehringer Mannheim, and MAb 1B5 was purchased from Seikagaku America Inc. (Rockville, MD). Vectastain ABC kit, biotinylated μ-chain-specific, anti-mouse IgM antibody and biotinylated IgG heavy- and light-chain-specific anti-rabbit antibody and Vector SG peroxidase substrate kit were obtained from Vector Laboratories (Burlingame, CA). Anti-mouse IgG biotinylated species-specific whole antibodies and streptavidin biotinylated horseradish peroxidase complex were obtained from Amersham.

Tissue preparation

Bovine eyes were collected from the Shamrock Meat Co. (Los Angeles, CA). The corneas were dissected with scissors, and the eyeballs were quartered with a razor blade. Care was taken to preserve as much vitreous as possible to prevent the retina from folding. Each quarter was snap-frozen in liquid nitrogen-chilled isopentane. Serial sections (10 μm) were cut and mounted onto gelatin-coated slides. Because the MAb 6A2 epitope is sensitive to prolonged aldehyde fixation, tissue was useful only if it was fixed immediately prior to immunostaining. Sections were immersed in either ice-cold acetone for 5 minutes or in picric acid fixative (50 mM Tris containing 0.9% NaC1, 4% paraformaldehyde, 0.25% glutaraldehyde, and 10% picric acid, pH 9.0) for 10 minutes. Sections were washed twice in phosphate-buffered saline (PBS), pH 7.4, for 5 minutes each, then immersed in PBS containing 0.25% Triton X-100 for 5 minutes, and finally washed in PBS twice for 5 minutes each.

Immunocytochemistry

Tissue sections were immunostained, as reported previously, by using the avidin-biotin immunoperoxidase method (Williams et al., 1990). For double-staining of sections, tissues were labelled with MAb 6A2 and anti-vimentin and were washed with PBS after the initial chromagen reaction (aminoethylcarbazole substrate for antigen 6A2 and 4-chloro-1-naphthol for vimentin). The staining procedure was then repeated on each section by using the reciprocal primary antibody, and sections were coverslipped with agarose mounting media. Stained slides were observed by transmitted tungsten light and were photographed by using either a Zeiss photomicroscope (Thornwood, NY) or the EDGE high-definition microscope (The Edge Scientific Instrument Corporation, Los Angeles, CA) for stereo pair images. The resulting kodachromes were scanned into Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) with a Nikon Coolscan (Tokyo, Japan), and the images were adjusted for contrast and color.

Electron microscopy

Bovine eyes were coronally hemisectioned and placed in 0.1% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid in 0.1 M PBS, pH 7.4, at room temperature. After 20 minutes of fixation, the retinas were carefully separated from the choroid with forceps. The isolated retinal tissue was placed for an additional 2 hours in the same fixative at room temperature and was then rinsed twice with 10% normal goat serum in PBS. Elongated strips of retina were then cut, rolled, and covered with liquified agar that had been cooled almost to the point of solidification. The agar and retinal tissue were further cooled to 4°C, and 50-μm sections were cut on a Vibratome. The cut sections were placed into culture dish wells containing 10% normal goat serum in PBS for 1 hour, transferred to wells containing 0.1% saponin in PBS (SPBS) for an additional hour, and then exposed to primary antibody (MAb 6A2; 1:1 supernatant) in 0.1% SPBS overnight, followed by a 30-minute rinse in PBS. The tissue was incubated in biotinylated anti-mouse IgM for 90 minutes, followed by another PBS wash for 30 minutes. After a 5-minute fixation in 1% glutaraldehyde and 2%

paraformaldehyde (in 0.1 M PBS only) and a subsequent 30-minute PBS wash, the tissue was exposed to ABC complex for 1 hour. Following another PBS wash (30 minutes), the staining was developed with 3,3′-diaminobenzidine (DAB), and the samples were then fixed again with 1% glutaraldehyde and 4% paraformaldehyde (in PBS). A 30-minute PBS wash was followed by exposure of tissue to osmium tetroxide (2%) for 30 minutes, dehydration with ethanol and propylene oxide, and embedding in Epon/propyleneoxide 1:1 for 30 minutes. After subsequent incubation in Epon overnight and polymerizion at 60°C, sections were cut and viewed with a Philips 301 electron microscope (Mahwah, NJ). Photographic negatives of the fields of interest were scanned into Adobe Photoshop with a Hewlett-Packard HP Scan Jet 11cx/T scanner (Corvallis, OR), and the images were adjusted for contrast.

Purification of somataglycan-S

A previously described protocol for the purification of somataglycan-S from brain was used (Williams et al., 1994). Bovine retina (3 g) was homogenized in 10 mM sodium phosphate buffer, pH 7.4, containing 0.85 M sucrose, 1 mM EDTA, 100 mM NaC1, 40 mM aminocaproic acid, 4 mM N-ethylmaleimide. and 1 ml/liter of protease inhibitor mix (PIM; 0.5 mM antipain, 0.5 mg/ml leupeptin, 2.3 mg/ml Pefabloc, 0.1 mg/ml pepstatin, and 5 mg/ml aprotinin). The homogenate was centrifuged at $\times 30{,}000g$ for 90 minutes, and the supernatant (Slb) was separated from the pellet (Plb). Slb fractions were further purified on a Sephracryl-HR 300 gel-filtration column, and fractions were analyzed by polyacrylamide gel electrophoresis (PAGE; Laemmli, 1970) and Western immunoblots (Towbin et al., 1979). The pooled fractions of each protein peak (8–10 ml total volume) were applied to a MAb 6A2 immunoaffinity chromatography column, and bound material was eluted with 50 mM diethylamine, pH 11.5. For final analyses, the immunoblots were incubated as described previously (Williams et al., 1994).

Analysis of glycosaminoglycans and oligosaccharides

Because some samples eluted from the affinity column also contained small amounts of MAb 6A2 IgM and the protease inhibitors, we assessed MAb 6A2 immunoreactivity on immunoblots and adjusted sample concentrations for optimal visualization. In each set of experiments, the absolute amounts of undigested control and ChABC-digested antigen 6A2 samples applied to the gel were identical, although the volumes varied to include the added enzyme.

The high-molecular-weight, immunoaffinity-purified antigen 6A2 from the S1b fraction was suspended in 50 mM Tris acetate, pH 7.8, with 5 mM EDTA, 40 mM aminocaproic acid, 4 mM N-ethylmaleimide, and PIM and was then incubated at 37°C for 18 hours with ChABC added at a final concentration of 0.01 units/20 µl sample. Control and ChABC samples were incubated at 37°C overnight, mixed with SDS sample buffer, and analyzed by SDS-PAGE and Western immunoblots. For visualization of the high-molecular-weight glycosylated species, the Alcian-blue ammoniacal silver stain (Al-Ag) was used (Krueger and Schwartz, 1987).

Immunoblots of the digests were incubated either with MAb 6A2 or MAb 1B5, which after ChABC cleavage binds to the stubs bearing unsaturated uronic residues linked to unsulfated N-acetylgalactosamine, or with MAbs 2C6 and 3B3, which bind to unsaturated or saturated uronic acid residues linked to N-acetylgalactosamine 4-sulfate and 6-sulfate, respectively. The developed immunoblots were scanned into Adobe Photoshop with the HP ScanJet 11cx/T scanner and were adjusted for contrast.

RESULTS

Horizontal cell specificity of MAb 6A2

MAb 6A2 specifically labelled the cell bodies and processes of retinal horizontal cell neurons. By light microscopy, transverse sections revealed a dense layer of MAb 6A2 immunoreactivity concentrated in sublamina 2 of the OPL (OPL-S2), where horizontal and bipolar cells make synaptic contact with rod spherules and cone pedicles (Koontz and Hendrickson, 1993). Figure 1A shows that the horizontal cell bodies were just visible at the interface of the INL and the OPL. In some retinal preparations, faint, diffuse staining was also observed at the inner plexiform layer (IPL)-INL interface. Occasional, fine processes extended across the INL to the IPL.

At higher magnification, antivimentin staining is visible as a layer overlapping the area of MAb 6A2 staining. In stereo pairs (Fig. 1B), the horizontal cell cytoplasm contained vimentin bordered by a dense band of MAb 6A2 immunostaining in OPL-S2. Transversely oriented glial processes passing through the outer nuclear layer (ONL) also expressed vimentin.

Ultrastructural localization of antigen 6A2

Localization of antigen 6A2 to horizontal cells and their processes was confirmed ultrastructurally (Figs. 2, 3). The sensitivity of the MAb 6A2 epitope to glutaraldehyde necessitated additional fixation of tissues after immunocytochemistry, resulting in somewhat decreased resolution and some tissue disruption.

MAb 6A2 immunoreactivity was identified focally in the horizontal cell cytoplasm (Fig. 2A). Control sections with no primary antibody were free of label (Fig. 2B). At higher magnification (Fig. 2C), the staining was concentrated along membrane-enclosed channels, which were somewhat dilated as a result of the tissue processing, with some resembling Golgi-associated structures (not shown). Horizontal cell processes were internally labelled focally along tubulovesicular structures (Fig. 2D).

MAb 6A2 immunostaining was focally intense throughout the most external aspect of the OPL, among synaptic triads of photoreceptor cells (Fig. 3A). There, the staining was most intense in the extracellular space surrounding the horizontal cell lateral processes. Lateral and bipolar cell synaptic terminals were unstained. Some stain was also present in the photoreceptor cell immediately subjacent to the synaptic space (Fig. 3A,C). Photoreceptor membrane-enclosed vesicles and channels were free of staining, as was the remainder of the photoreceptor cell. Although the majority of the synaptic triads were stained, there were also numerous adjacent unlabelled triads nearby (not shown). In control sections with no exposure to MAb 6A2, this perisynaptic space was free of stain, although ribbon synapses were somewhat electron dense (Fig. 3B).

Biochemistry of antigen 6A2

The purification protocol is indicated schematically in Figure 4A. Each fraction obtained after centrifugation was

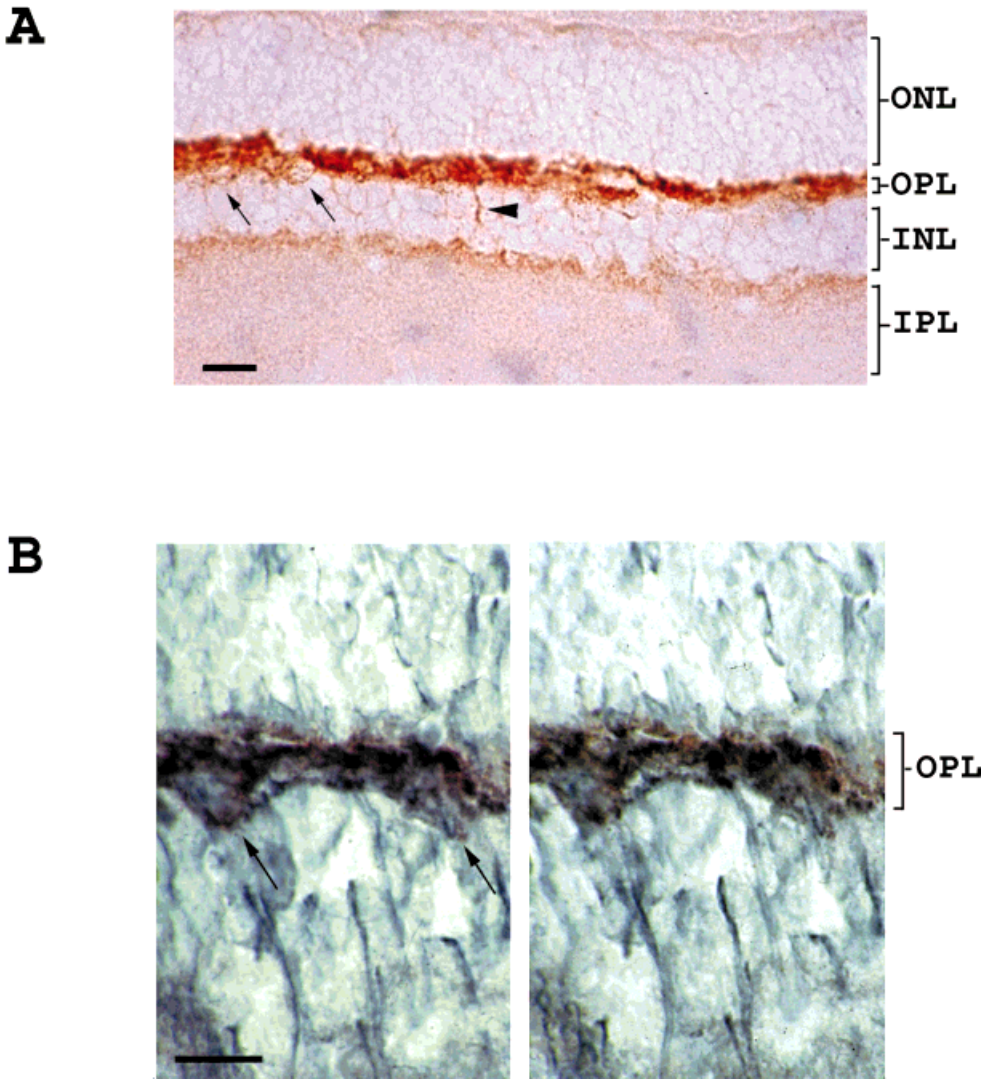


Fig. 1. Retina immunocytochemistry. **A:** Monoclonal antibody (MAb) 6A2 stains focally and densely along the outer plexiform layer (OPL) S-2. Staining extends along the outer margin of the inner nuclear layer (INL). Horizontal cells somata (arrows) lie just at the junction of the outer nuclear layer (ONL) and the OPL. A process (arrowhead) that extends from a horizontal cell traverses the INL. There is faint immunostaining along the outer margin of the IPL. **B:** At higher magnification, a stereo pair of bovine retina cryostat sections stained with both MAb 6A2 (brown) and antivimentin (blue) reveals dual reactivity, possibly associated with the somata, and focal dense staining by MAb 6A2 of the OPL-S2. Scale bars = 50 μm.

analyzed on Al-Ag-stained polyacrylamide gels and companion immunoblots with MAb 6A2 (Fig. 4B). The Al-Ag stain, which recognizes glycosaminoglycans (GAGs), detected a high-molecular-weight (approximately 500 kDa), broad band in the homogenate (lane 1) and in the soluble (Slb) fraction (lane 1). On Western immunoblots, MAb 6A2 was immunoreactive with these same fractions (lanes 2). There was only scant immunoreactivity in the S2a detergent-soluble fraction.

For further enrichment and purification of the glycoconjugate, we chose the Slb fraction, because it provided readily abundant amounts of soluble material. Affinity chromatography with MAb 6A2 yielded enrichment of the antigen from the pooled void volume. A high-molecular-weight, broad band of between 400 kDa and 500 kDa was detected (Fig. 4B, S1c, lane 2). To ensure that no other species immunoreactive with MAb 6A2 were present in the other fractions, samples from each gel filtration peak were pooled, separately applied, and eluted from the MAb 6A2 affinity column. Only the high-molecular-weight void volume fraction was enriched with antigen 6A2 after immunoaffinity purification (not shown).

Next, antigen 6A2 was tested for the GAG chain CS by incubation with ChABC (Fig. 4C). Western blots with MAb 6A2 revealed a more polydisperse species ranging from 300 kD to 500 kD (Fig. 4C, lane 2), although there appeared to be relatively little change in migration after ChABC digestion compared with the untreated sample (Fig. 4C, lane 1). Strong immunoreactivity with MAb 1B5 (Fig. 4C, lane 2) confirmed the cleavage of CS side chains. These results contrast with the absence of MAb 1B5 immunoreactivity of the nondigested sample in lane 1 (Fig. 4C). More specifically, reactivity with MAb 1B5 in Figure 4C, lane 2 indicates that the initial disaccharide in the CS

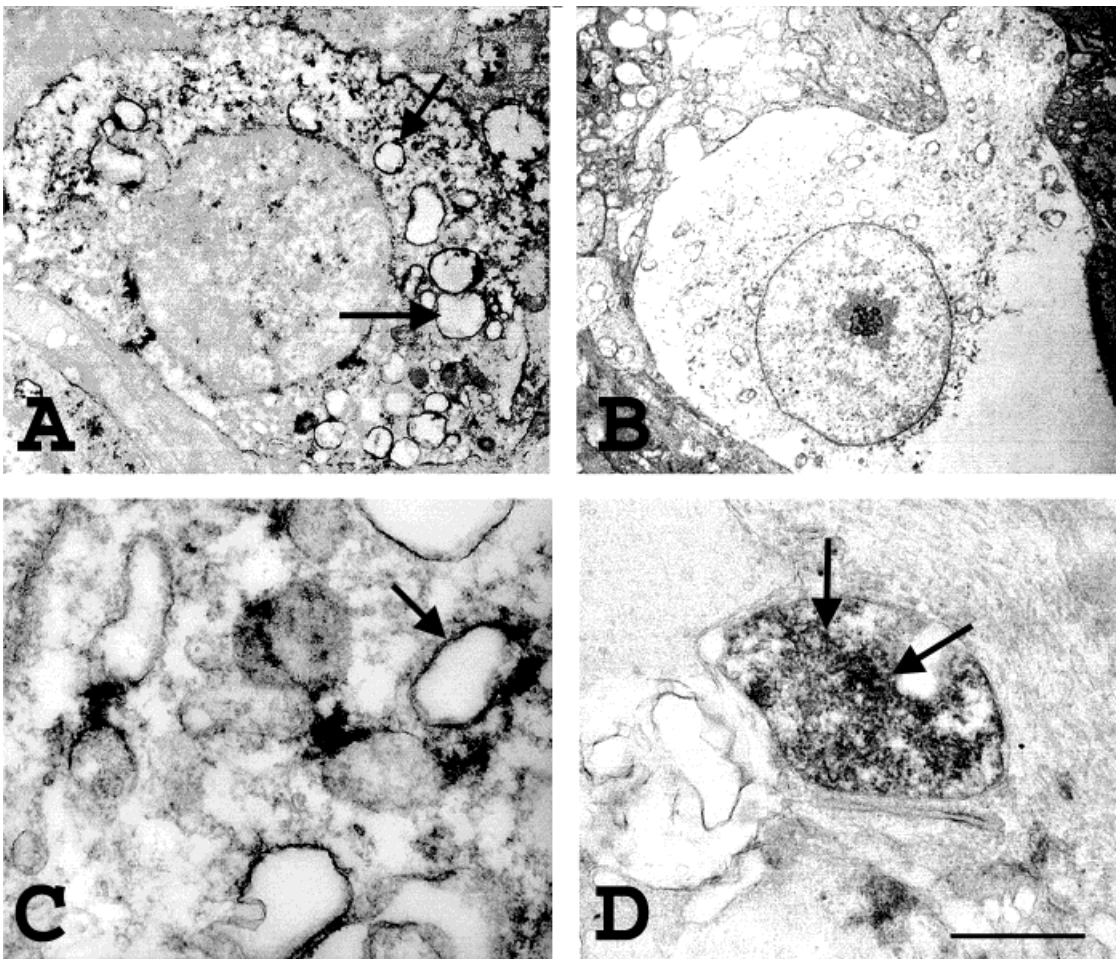


Fig. 2. Ultrastructural localization of MAb 6A2 in horizontal cells: **A:** MAb 6A2 focally stains the cytoplasm, including distended cisternae (arrows), of a horizontal cell. **B:** In the absence of primary antibody, the horizontal cell is unstained. **C:** In a higher magnification of A, the staining is seen concentrated along membrane-enclosed profiles (arrow) that resemble distended, smooth endoplasmic reticulum or Golgi-associated cisternae and vesicles. **D:** Processes within the OPL show dense immunostaining around tubulovesicular structures. Scale bar = 7 μm in A,B, 1.75 μm in C,D.

chain, which remained after ChABC digestion, is unsulfated N-acetylgalactosamine linked to uronic acid (Couchman et al., 1984). In addition to a 300–500 kDa smear (Fig. 4, lane 2), a 250-kD band was generated that was weakly reactive with MAb 6A2 but strongly immunoreactive with MAb 1B5. Immunoblots of additional samples digested with ChABC were not reactive with MAbs 2B6 and 3B3, suggesting that 4′ and 6′ sulfated disaccharides are not present on the digested PG (not shown).

DISCUSSION

The cellular and molecular diversity of the CNS is well represented in the mammalian retina. Our results identify a novel horizontal cell marker; a CSPG specifically localized within the somal cytoplasm and neurites and the extracellular space surrounding presynaptic terminals. The PG may also be pinocytosed by the photoreceptor and concentrated at the ribbon synapse.

Colocalization of antigen 6A2 with the cytoplasmic staining of vimentin confirms that the labelled neurons are horizontal cells (Dräger, 1983; Dahl and Bignami, 1991). MAb 6A2 also labelled the OPL in human and mouse retinas (unpublished observations) indicating that antigen 6A2 is highly conserved.

With the predictable polarity and short span of horizontal cell processes viewable within a single section, anterograde transport of antigen 6A2 via these dendrites from the soma to the presynaptic terminals is a likely possibility.

Similar to the modulatory role of spinocerebellar system neurons in the CNS via recurrent collateral inhibition (Oscarsson, 1973), horizontal cells are inhibitory neurons exerting their action through γ-aminobutyric acid (GABA; Brecha 1992; Greferath et al., 1994; Vardi et al., 1994) and modulating information transfer from the photoreceptors to the bipolar cells. The specific localization of retinal antigen 6A2 to the horizontal neuron but not to other GABAergic neurons of the retina suggests that it is expressed in a subpopulation of inhibitory neurons that specialize in negative feedback modulation.

Localization of this antigen in cisternal structures, including Golgi-like channels of horizontal cells, was confirmed ultrastructurally. The strongly immunopositive tubulovesicular structures inside the horizontal cell processes contribute to the dense staining in the OPL. Distally,

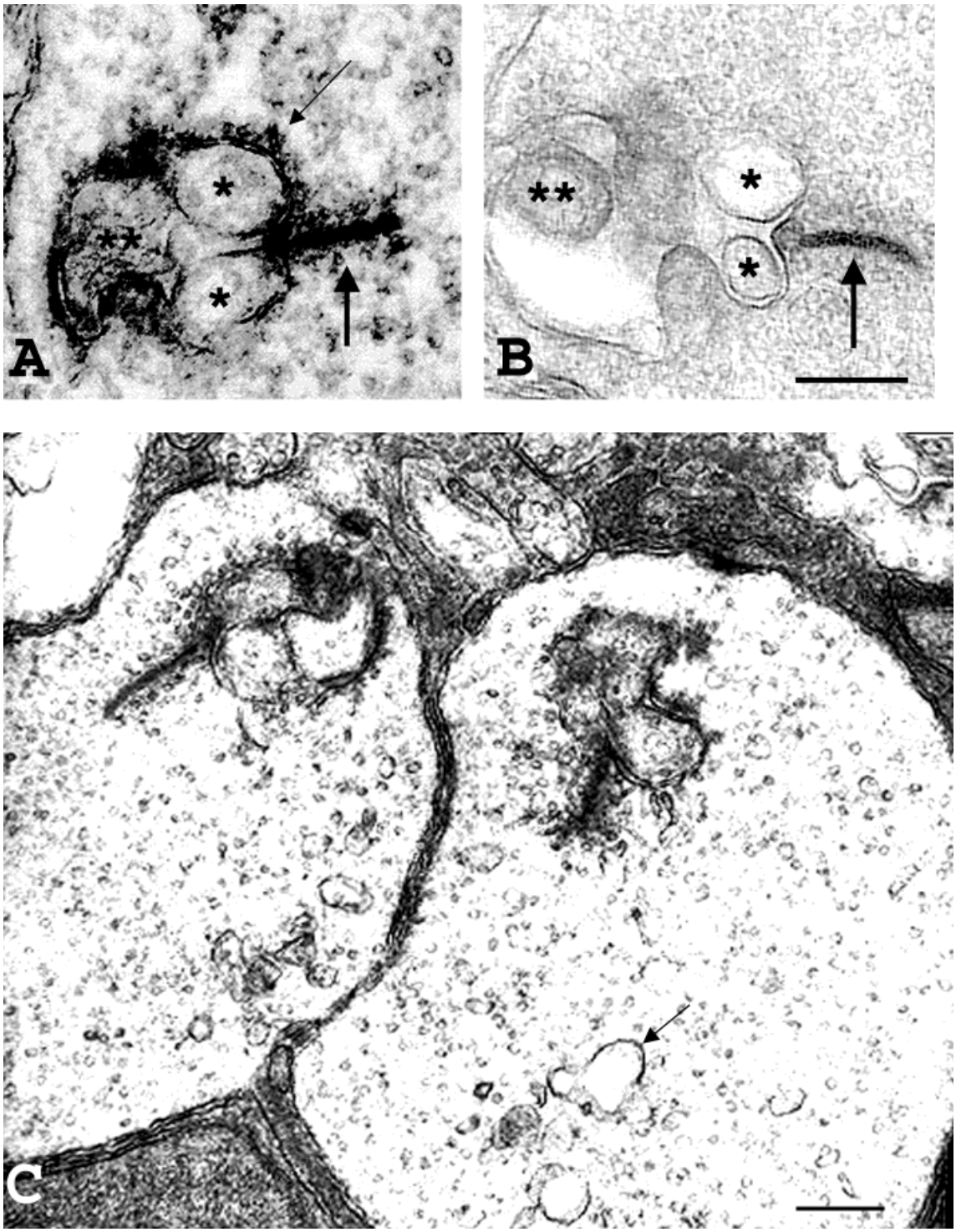
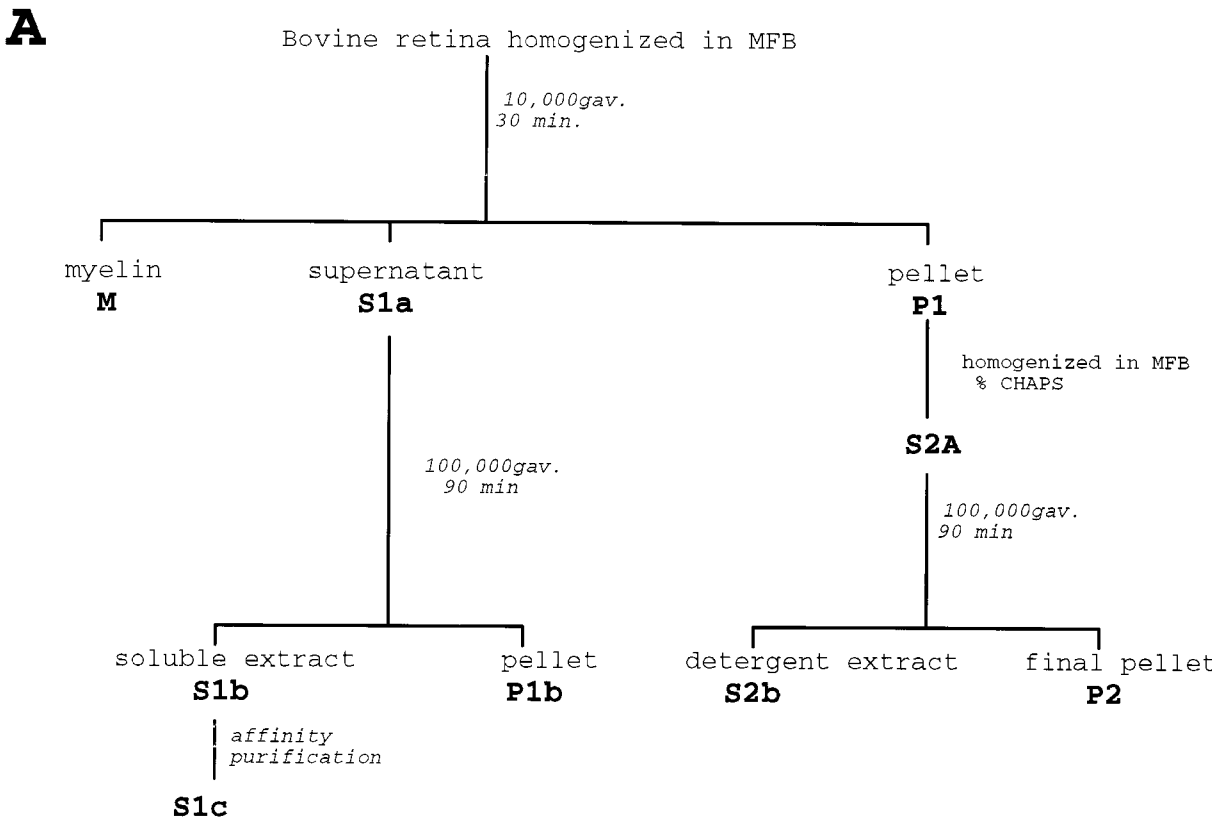
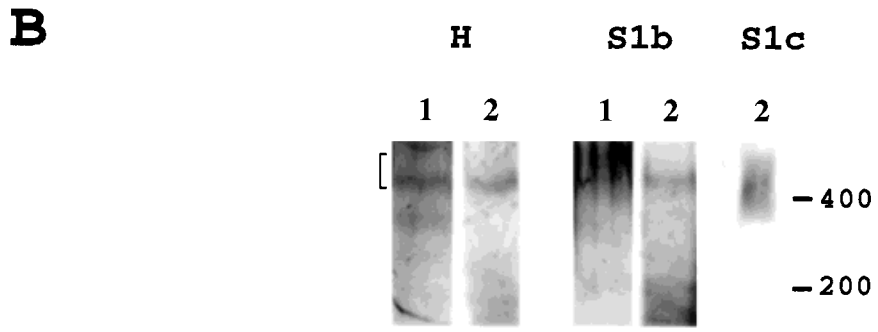


Fig. 3. Ultrastructural localization of MAb 6A2 at horizontal cell photoreceptor cell synapses. **A:** Within the OPL, a synaptic triad shows MAb 6A2 staining in the extracellular space (small arrow) surrounding the horizontal cell lateral processes (single asterisks) and a bipolar cell input (double asterisks), all of which are unstained. **B:** Control sample with no MAb 6A2 primary antibody shows the preterminal perisynaptic space. The ribbon synapse of the photoreceptor cell is strongly electron dense in A (large arrow), B (arrow), and C. **C:** Two photoreceptor terminals show concentration of stain extending to the ribbon synapse but absence of stain elsewhere in the photoreceptor cell cytoplasm, including within vesicles (arrow). Scale bars = 1.75 μm.

the site of release of antigen 6A2 into the extracellular space of the photoreceptor cell synaptic triad could not be definitely confirmed. In keeping with our previous observations of somataglycan-S in the spinal cord, the 6A2 antigen is not found in the synaptic cleft or within the lateral processes of the triad at ribbon synapses (Williams et al.,





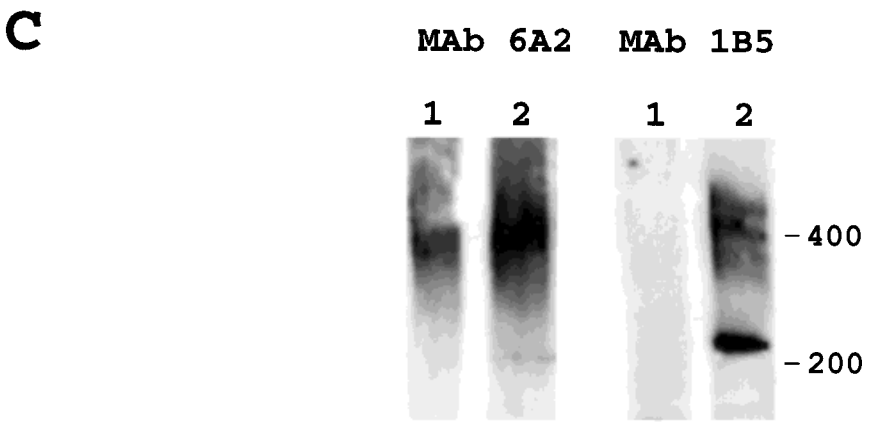


Fig. 4. Purification and partial characterization of retinal antigen 6A2: **A:** Purification scheme for high molecular weight antigen. **B:** After electrophoresis on 3–15% gradient polyacrylamide gel, homogenate (H) and supernatant (Slb) fraction were either stained with the Alcian-blue ammoniacal silver stain (Al-Ag) stain (**lanes 1**) or transferred to nitrocellulose and probed with MAb 6A2 (**lanes 2**). The high-molecular-weight proteoglycan band (400–500 kDa) was detected with the Al-Ag stain in the homogenate and Slb. MAb 6A2 immunoreactivity colocalized with this band in the homogenate and Slb fractions. After MAb 6A2 affinity purification of the Slb fraction, a high-molecular-weight species ranging from 400 kD to 500 kD was identified (S1c; lane 2). **C:** MAb 6A2 shows the affinity-purified antigen 6A2 in **lane 1** as a broader band in **lane 2** after chondroitinase ABC (ChABC) digestion. Antigen 6A2 shows no reactivity with MAb 1B5 prior to lyase digestion (lane 1). Cleavage of chondroitin sulfate GAGs is confirmed by staining of this band in lane 2 by MAb 1B5. A strong band at 250 kD also appears in this lane.

1990, 1994); it is concentrated extracellularly in the perisynaptic space proximal to the terminal. Immunoreactive material is present in the photoreceptor cytoplasm in close proximity to the terminal membrane and concentrated along the ribbon synapse. Because there is no ultrastructural evidence for the 6A2 antigen being manufactured by the photoreceptor cell, it is possible that it is being endocytosed and concentrated at the ribbon synapse. Uptake into photoreceptor cells has been demonstrated previously by Evans et al. (1981) and is enhanced by elevated potassium concentration.

Focal accumulation of antigen 6A2 encircling presynaptic GABAergic processes and the lateral process at the photoreceptor ribbon synapse suggests functions in the development and maintenance of stable GABAergic inputs. This localization may serve to concentrate vital factors at the synapse. PGs are ligands for many other molecules and have been demonstrated to bind to growth factors (Ruoslahti and Yamaguchi, 1991; Yayon et al., 1991; Lopez-Casillas et al., 1993; Aviezer et al., 1994; Spivak-Kroizman et al., 1994; Schlessinger et al., 1995), and our data (LeMay et al., 1995) suggest that members of the neurotrophin family, NGF, NT-3, and NT4/5, bind to somataglycan-S obtained from human motor cortex. The PG may be providing growth factor support for the ribbon synapse at which it is concentrated or may have a role in neurotransmitter transport.

The subcellular localization of the horizontal cell PG resembles that of several other PGs in the mammalian CNS. Ultrastructurally, PGs identified by VC1.1, 473, and CAT-301 antibodies, like somataglycan-S and the retinal PG, surround the cell membrane but are absent from pre- and postsynaptic terminals and clefts (Hockfield and McKay, 1983; Naegele et al., 1988; Watanabe et al., 1989). Two MAbs, 374 and VC1-1, are immunoreactive in the retina. MAb VC1.1, which binds to the HNK-1 epitope, labels the IPL and the OPL with equal intensity (Arimatsu et al., 1987), whereas MAb 374 staining of the OPL was stronger than that of the IPL (Fujita and Kudo, 1992). Another PG, the synaptic vesicle, transmembrane, keratan sulfate proteoglycan, synaptoglycan, which is detected by MAb SV2 (Scranton et al., 1993), is found in all synaptic types in the IPL and the OPL (Okada et al., 1994). Synaptoglycan is a novel type of transmembrane transporter and may mediate the influx of neurotransmitters (Feany et al., 1992) or other vesicle constituents into synaptic vesicles (Bajjalieh et al., 1994).

Our biochemical data are complementary to the ultrastructural localization of antigen 6A2, in that the retinal PG is present both in soluble and membrane-associated compartments. A highly soluble form of the retinal antigen was extracted from Slb fractions without detergents or strong dissociating conditions. However, the pellet remaining after detergent extraction (P2) also contained immunoreactive antigen, which was demonstrated as an extended smear on immunoblots. Our previous studies using Triton X-100 partitioning of mouse brain somataglycan did not reveal a transmembrane form (Williams et al., 1994). It is possible that hydrophilic GAG chains hinder partitioning of the PG into the detergent phase and the soluble and pellet fraction PGs are identical. In support of this, after isolation of the CAT-301 PG from cat brain, Zaremba et al. (1989) detected antigens in the soluble and pellet fraction that were physically and biochemically indistinguishable. Axonal transport studies of the goldfish visual system indicate that a significant proportion of soluble molecules conveyed by rapid axonal transport from the Golgi complex to the cell membrane in secretory vesicles are PGs (Rippelino and Elam, 1988). However, because an equivalent amount of very similar GAGs can be isolated from the pellet fraction it is possible that some vesicles are resistant to detergent extraction and remain intact after homogenization.

Because the purification procedure did not include strong dissociating reagents, the MAb 6A2 PG may be copurified with its ligands. The 250-kDa species detected by MAb 1B5 after ChABC digestion of the sample may be a ligand or, speculatively, a homophilically binding glycoform. Lyase digestion of affinity-purified antigen 6A2 revealed considerable heterogeneity in the carbohydrate substitutions of this PG. Binding of MAb 1B5 to the retinal PG after ChABC digestion confirmed that it is a CSPG bearing uronic acid linked to unsulfated N-acetylgalactosamine as the initial disaccharide in the GAG chain (Couchman et al., 1984; Caterson et al., 1985). Within each broad band detected by MAbs 6A2 or 1B5, several sharply focused bands were also resolved. These species may be alternatively spliced proteins with similar glycosylation or glycoforms of one or more core proteins. Preliminary studies suggest the presence of keratan sulfate GAGs (not shown). These results confirm biochemical similarity between the retinal PG and the brain PG, somataglycan-S, which also bears the MAb 6A2 epitope.

Other PGs with a cellular localization similar to antigen 6A2 have been biochemically characterized. The CAT-301, S-103 L, and BNC-PG epitopes are present on core proteins that are similar, if not identical, to aggrecan, the large CSPG of cartilage (Fryer et al., 1992; Krueger et al., 1992; Asher et al., 1995). MAb 5D5 recognizes versican, the large hyaluronate-binding fibroblast PG, which is localized to glia as well as neuronal surfaces (Bignami et al., 1993). MAb 6B4 identifies a glycoform of phosphacan, the truncated extracellular variant of the receptor-like protein tyrosine phosphatase (RPTPβ; Maurel et al., 1994, 1995). The core protein of the PG recognized by MAb 6A2 has not yet been determined, in part due to its complexity and the restricted quantities present in the CNS. The localization of somataglycan in the CNS to the neurons of a functional system, the spinocerebellar system, suggests that the MAb 6A2 epitope defines the PG as a unique glycoform. Isolation and molecular characterization of the core protein(s) of antigen 6A2 will be necessary to resolve the identity of this complex molecule.

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LITERATURE CITED

Arimatsu, Y., J.R. Naegele, and C.J. Barnstable (1987) Molecular markers of neuronal subpopulations in layers 4, 5 and 6 of cat primary visual cortex. J. Neurosci. *7:*1250–1263.

Asher, R.A., R.J. Scheibe, H.D. Keiser, and A. Bignami (1995) On the existence of a cartilage-like proteoglycan and link proteins in the central nervous system. Glia *13:*294–308.

Aviezer, D., D. Hecht, M. Safran, M. Eisinger, G. David, and A. Yayon (1994) Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis and angiogenesis. Cell *79:*1005–1013.

Bajjalieh, S.M., G.D. Frantz, J.M. Weimann, S.K. McConnell, and R.H. Scheller (1994) Differential expression of synaptic vesicle protein 2 (SV2) isoforms. J. Neurosci. *14:*5223–5235.

Blanks, J.C., A.M. Adinolfi, and R.N. Lolley (1974) Synaptogenesis in the photoreceptor terminal of the mouse retina. J. Comp. Neurol. *156:* 81–94.

Bignami, A., G. Perides, and F. Rahemtulla (1993) Versican, a hyaluronate-binding proteoglycan of embryonal precartilaginous mesenchyma, is mainly expressed postnatally in rat brain. J. Neurosci. Res. *34:*97–106.

Brecha, N.C. (1992) Expression of $GABA_A$ receptors in the vertebrate retina. Progr. Brain Res. *90:*3–27.

Caterson, B., J.E. Christner, J.R. Baker, and J.R. Couchman (1985) Production and characterization of monoclonal antibodies directed against connective tissue proteoglycans. Fed. Proc. *44:*386–393.

Couchman, J.R., B. Caterson, J.E. Christner, and J.R. Baker (1984) Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. Nature *307:*650–652.

Crossin, K.L., S. Hoffman, S.S. Tan, and G.M. Edelman (1989) Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. Dev. Biol. *136:*381–392.

Dahl, D., and A. Bignami (1991) Neurofilament phosphorylation in the axonless horizontal cells of rat retina. Brain Res. *553:*163–166.

Dowling, J.E., and B.B. Boycott (1966) Organization of the primate retina: Electron microscopy. Proc. R. Soc. London B. Biol. Sci. *166:*80–111.

Dräger, U.C. (1983) Coexistence of neurofilaments and vimentin in a neurone of adult mouse retina. Nature *303:*169–172.

Evans, J.A., L. Liscum, D.C. Hood, and E. Holtzman (1981) Uptake of horseradish peroxidase by presynaptic terminals of bipolar cells and photoreceptors of the frog retina. J. Histochem. Cytochem. *29:*511–518.

Feany, M.B., S. Lee, R.H. Edwards, and K.M. Buckley (1992) The synaptic vesicle protein SV2 is a novel type of transmembrane transporter. Cell *70:*861–867.

Fryer, H.J.L., G.M. Kelly, L. Molinaro, and S. Hockfield (1992) The high molecular weight CAT-301 chondroitin sulfate proteoglycan from brain is related to the large aggregating proteoglycan from cartilage, aggrecan. J. Biol. Chem. *267(14):*9874–9883.

Fujita, S.C., and J. Kudo (1992) A novel member to the family of perineuronal antigens associated with subpopulations of central neurons in the rat. Exp. Brain Res. *88:*345–354.

Fujita, S.C., S.L. Zipursky, S. Benzer, A. Ferrus, and S.L. Shotwell (1982) Monoclonal antibodies against the *Drosophila* nervous system. Proc. Natl. Acad. Sci. USA *79:*7929–7933.

Geisert, E.E., R.C. Williams, Jr., and D.J. Bidanset (1992) A CNS specific proteoglycan associated with astrocytes in rat optic nerve. Brain Res. *571:*165–168.

Greferath, U., U. Grunert, F. Muller, and H. Wassle (1994) Localization of $GABA_A$ receptors in the rabbit retina. Cell Tissue Res. *276:*295–307.

Hockfield, S., and R.D.G. McKay (1983) A surface antigen expressed by a subset of neurons in the vertebrate central nervous system. Proc. Natl. Acad. Sci. USA *80:*5758–5761.

Johnson, L.V., and G.S. Hageman (1987) Enzymatic characterization of peanut agglutinin-binding components in the retinal interphotoreceptor matrix. Exp. Eye Res. *44:*553–565.

Kolb, H., K.A. Linberg, and S.K. Fisher (1992) Neurons of the human retina: A Golgi study. J. Comp. Neurol. *318:*147–187.

Koontz, M.A., and A.E. Hendrickson (1993) Comparison of immunolocalization patterns for the synaptic vesicle proteins p65 and synapsin I in macaque monkey retina. Synapse *14:*268–282.

Krueger, R.C., Jr., and N.B. Schwartz (1987) An improved method of sequential Alcian-blue and ammoniacal silver staining of chondroitin sulfate proteoglycan in polyacrylamide gels. Anal. Biochem. *167:*295–300.

Krueger, R.C., Jr., A.K. Hennig, and N.B. Schwartz (1992) Two immunologically and developmentally distinct chondroitin sulfate proteoglycans in embryonic chick brain. J. Biol. Chem. *267:*12149–12161.

Laemmli, U.K. (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature *227:*680– 685.

LeMay, L.G., C. Williams, and C.A. Miller (1995) Somataglycan-S from motor cortex of human brain binds nerve growth factor (NGF) and neurotrophin-4/5 (NT-4/5). J. Neurochem. *64(Suppl):*95A.

Linberg, K.A., and S.K. Fisher (1988) Ultrastructural evidence that horizontal cell axon terminals are presynaptic in the human retina. J. Comp. Neurol. *268:*281–297.

Lopez-Casillas, F., J.L. Wrana, and J. Massague (1993) Betaglycan presents ligand to the TGFβ signaling receptor. Cell *73:*1435–1444.

Maeda, N., F. Matsui, and A. Oohira (1992) A chondroitin sulfate proteoglycan that is developmentally regulation in the cerebellar mossy fiber system. Dev. Bio. *151:*564–574.

Maeda, N., H. Hamanaka, T. Shintani, T. Nishiwaki, and M. Noda (1994) Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan. FEBS Lett. *354:*67–70.

Maeda, N., H. Hamanaka, A. Oohira, and M. Noda (1995) Purification, characterization and developmental expression of a brain-specific chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan. Neuroscience *67(1):*23–35.

Maurel, P., U. Rauch, M. Flad, R.K. Margolis, and R.U. Margolis (1994) Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. Proc. Natl. Acad. Sci. USA *91:*2512–2516.

Morriss-Kay, G., and F. Tuckett (1989) Immunohistochemical localization of chondroitin sulfate proteoglycans and the effects of chondroitinase ABC in 9- to 11-day rat embryos. Development *106:*787–798.

Naegele, J.R., Y. Arimatsu, P. Schwartz, and C.J. Barnstable (1988) Selective staining of a subset of GABAergic neurons in cat visual cortex by monoclonal antibody VC1.1. J. Neurosci. *8:*79–89.

Okada, M., A. Erickson, and A. Hendrickson (1994) Light and electron microscopic analysis of synaptic development in *Macaca* monkey retina as detected by immunocytochemical labeling for the synaptic vesicle protein, SV2. J. Comp. Neurol. *339:*535–558.

Oscarsson, O. (1973) Somatosensory system: Functional organization of spinocerebellar paths. In A. Iggo (ed): Handbook of Sensory Physiology, Vol. II. New York: Springer-Verlag, pp. 339–380.

Peichl, L., and J. González-Soriano (1993) Unexpected presence of neurofilaments in axon-bearing horizontal cells of the mammalian retina. J. Neurosci. *13:*4091–4100.

Porrello, K., D. Yasumura, and M.M. LaVail (1986) The interphotoreceptor matrix in RCS rats: Histochemical analysis and correlation with the rate of retinal degeneration. Exp. Eye Res. *43:*413–429.

Ripellino, J.A., and J.S. Elam (1988) Axonal transport of proteoglycans to the goldfish optic tectum. Neurochem. Res. *13:*479–485.

Ruoslahti, E., and Y. Yamaguchi (1991) Proteoglycans as modulators of growth factor activities. Cell *64:*867–869.

Schlessinger, J., I. Lax, and M. Lemmon (1995) Regulation of growth factor activation by proteoglcyans: What is the role of the low affinity receptors? Cell *83:*357–360.

Scranton, T.W., M. Iwata, and S.S. Carlson (1993) The SV2 protein of synaptic vesicles is a keratan sulfate proteoglycan. J. Neurochem. *61:*29–44.

Spivak-Kroizman, T., M.A. Lemmon, I. Dikic, J.E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, and I. Lax (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation and cell proliferation. Cell *79:*1015–1024.

Thonar, E.M.-M.A., and K.E. Kuettner (1987) Biochemical basis of age-related changes in proteoglycans. In T.N. Wight and R.P. Mecham (eds): Biology of Proteoglycans. New York: Academic Press, pp. 211–246.

Towbin, H., T. Staehlin, and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. Proc. Natl. Acad. Sci. USA *76:*4350–4354.

Vardi, N., D.L. Kaufman, and P. Sterling (1994) Horizontal cells in cat and monkey retina express different isoforms of glutamic acid decarboxylase. Vis. Neurosci. *11:*135–142.

Wässle, H., and B. Boycott (1991) Functional architecture of the mammalian retina. Physiol. Rev. *71:*2.

Watanabe, E., S.C. Fujita, F. Murakami, M. Hayashi, and M. Matsumura (1989) A monoclonal antibody identifies a novel epitope surrounding a subpopulation of the mammalian central neurons. Neuroscience *29:*645–657.

Williams, C., M. Kozlowski, D.R. Hinton, and C.A. Miller (1990) Degeneration of spinocerebellar neurons in amyotrophic lateral sclerosis. Ann. Neurol. *27:*215–225.

Williams, C., D.R. Hinton, and C.A. Miller (1994) Somataglycan-S: A neuronal surface proteoglycan defines the spinocerebellar system. J. Neurochem. *62:*1615–1630.

Yayon, A., M. Klagsbrun, J.D. Esko, P. Leder, and D.M. Ornitz (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell *64:*841–848.

Zaremba, S., A. Guimaraes, R.G. Kalb, and S. Hockfield (1989) Characterization of an activity-dependent, neuronal surface proteoglycan identified with monoclonal antibody CAT-301. Neuron *2:*1207–1219.