A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth

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Summary

In the developing retina, retinal ganglion cell (RGC) axons elongate toward the optic fissure, even though no obvious directional restrictions exist. Previous studies indicate that axon–matrix interactions are important for retinal ganglion cell axon elongation, but the factors that direct elongation are unknown. Chondroitin sulfate proteoglycan (CS-PG), a component of the extracellular matrix, repels elongating dorsal root ganglion (DRG) axons in vitro and is present in vivo in the roof plate of the spinal cord, a structure that acts as a barrier to DRG axons during development. In this study, we examined whether CS-PG may regulate the pattern of retinal ganglion cell outgrowth in the developing retina.

Immunocytochemical analysis showed that CS-PG was present in the innermost layers of the developing rat retina. The expression of CS-PG moved peripherally with retinal development, always remaining at the outer edge of the front of the developing axons. CS-PG was no longer detectable with immunocytochemical techniques when RGC axon elongation in the retina is complete.

Results of studies in vitro showed that CS-PG, isolated from bovine nasal cartilage and chick limb, was inhibitory to elongating RGC axons and that RGC growth cones were more sensitive to CS-PG than were DRG neurites tested at the same concentrations of CS-PG. The behavior of retinal growth cones as they encounter CS-PG was characterized using time-lapse video microscopy. Filopodia of the RGC growth cones extended to and sampled the CS-PG repeatedly. With time, the growth cones turned to avoid outgrowth on the CS-PG and grew only on laminin.

While numerous studies have shown the presence of positive factors within the retina that may guide developing RGC axons, this is the first demonstration of an inhibitory or repelling molecule in the retina that may regulate axon elongation. Taken together, these data suggest that the direction of RGC outgrowth in the retina may be regulated by the proper ratio of growth-promoting molecules, such as laminin, to growth-inhibiting molecules, like CS-PG, present in the correct pattern and concentrations along the retinal ganglion cell pathway.

Key words: chondroitin sulfate proteoglycan, dorsal root ganglion, retina.

Introduction

A crucial step during the differentiation of retinal ganglion cells (RGCs) is their elaboration of axons exclusively toward the optic fissure, where they coalesce to form the optic nerve. This process occurs in sequence beginning with the first differentiated RGCs at the central retina and proceeds peripherally in all directions (Halfter et al. 1983; Ramon y Cajal, 1892). Understanding the mechanism(s) that control the sequential generation of neurons and the direction of RGC outgrowth is an especially intriguing problem, given the nature of the neuroepithelial field from which the RGCs arise. No obvious directional restrictions exist within the environment of the developing RGC, except for the channel arrangement of the neuroepithelial processes, and a proper orientation of these channels has not been discovered that could successfully orient growth in one direction.

Postulated mechanisms that might control the direction of RGC outgrowth have been examined using a variety of techniques, such as three-dimensional reconstruction of serial sections (Silver and Sidman, 1980), silver-stained retinal whole mounts (Goldberg and Coulombre, 1972; Halfter et al. 1985), mechanical disruption of retinal ganglion cells and the basal lamina (Goldberg, 1977), scanning electron microscopy (Suburo et al. 1979) and enzyme treatment of retinal whole mounts (Halfter and Deiss, 1984). Further, the retina has been analyzed to discover molecules that may promote neurite outgrowth (Cohen et al. 1987; Halfter, 1989; Rogers et al. 1983), and to search for molecular gradients within the retina and tectum to account for the direction of axonal outgrowth (Bonhoeffer and
The results of these studies and others have provided clues as to the factors that permit RGC elongation along the outer margins of the brain. A central finding of these investigations is that axon–matrix interactions, specifically those between the neurons and extracellular matrix (ECM) in the vicinity of the neuroepithelial endfeet of the inner-limiting membrane are important (Goldberg, 1977; Halfter et al., 1983; Rogers et al., 1983; Silver and Robb, 1979; Silver and Sidman, 1980).

However, a gradient that might explain the stereotyped pattern of axonal outgrowth has not been found within the marginal region.

The ECM contains a large number of molecules that have a variety of effects on cell–cell and cell–substratum interactions. Laminin, a component of the basal lamina and neuroepithelial cell surface, has profound stimulatory effects on the growth of retinal neurites (Cohen et al., 1987; Cohen et al., 1986; Halfter, 1989; Halfter, 1989; Liesi and Silver, 1988; Rogers et al., 1983; Smalheiser et al., 1984). Laminin is developmentally regulated and is present in locations where it may promote the elongation of axons along the marginal zone within the developing retina. However, in spite of the fact that laminin is an excellent substratum for the growth of RGC axons, and that it is present along the RGC pathway, the patterning of this glycoprotein alone does not appear to contain the information necessary to direct RGC axonal growth (Halfter, 1987). Neural cell adhesion molecule (NCAM) has also been described along glial elements on the presumptive visual pathway (Silver and Rutishauser, 1984). However, NCAM's broad distribution indicates that it is not likely to be a critical molecule in providing directional information for the elongating RGC axons. It seems feasible then that other molecules are present that either alone, or in combination with laminin or NCAM, may direct the trajectory of RGC axons.

One group of ECM molecules that may control axon outgrowth in the retina are the proteoglycans. These abundant molecules in the ECM of the developing brain are characterized by a diverse and complex chemical composition, which, in turn, results in a potentially wide functional heterogeneity (Herdon and Lander, 1990). Variability in both the protein and the carbohydrate components of proteoglycans contributes to this diversity (Gallagher, 1989). Further complexity results from the interactions of proteoglycans with other molecules, such as the glycoproteins laminin (Chiu et al., 1986), fibronectin (Haugen et al., 1990), collagen (Rosenberg et al., 1985; Ruoslahti, 1989), cytotactin (Hoffman et al., 1988), hyaluronectin (Delpech and Halavent, 1981), NCAM (Cole et al., 1985) and trophic factors (Walicke, 1988), to name a few. Depending on their location, type and molecular interactions, proteoglycans may either promote (Chiu et al., 1986) or inhibit axonal elongation (Oohira et al., 1991; Perris and Johansson, 1990; Snow et al., 1990a and b).

Chondroitin sulfate proteoglycan (CS-PG) displays a variety of effects on cell adhesion and outgrowth. A large, cartilage CS-PG inhibits chick embryonic fibroblast adhesion to serum- or collagen-coated substrata (Knox and Wells, 1979) and to fibronectin (Rich et al., 1981). A large hyaluronan-aggregating CS-PG is inhibitory to neural crest cell migration on fibronectin, when added to the culture medium (Perris and Johansson, 1990). Neurite outgrowth from chick dorsal root ganglia (DRG) is inhibited by CS-PG, as well as heparin and hyaluronic acid, in a three-dimensional culture gel that contains fibronectin (Carbonetto et al., 1983). Recent studies identified for the first time a KS-PG-containing region, the roof plate of the spinal cord, which acts as a boundary to DRG and possibly commissural axons (Snow et al., 1990a). The roof plate also expresses CS-PG (Perris et al., 1991). Studies in vitro tested the behavior of chick DRG neurons on nitrocellulose substrata coated with a chondroitin sulfate/keratan sulfate proteoglycan (CS/KS-PG) mixed with laminin.

These studies showed that CS/KS-PG inhibits neurite outgrowth in a concentration-dependent manner and that, in this system, the CS and KS moieties of the PG, not the protein core, are responsible for the inhibitory effect (Snow et al., 1990b).

Previous information about matrix molecules and neurite behavior suggest that the direction a neurite grows may be based on a summation of neurite outgrowth-promoting and outgrowth-inhibiting clues in the environment. Given that growth-promoting molecules are present within the RGC pathway (e.g. laminin and NCAM) and that certain proteoglycans can inhibit the advance of growing neurites, we hypothesized that RGCs may use laminin and/or NCAM as a growth-promoting substratum, while relying on other molecules such as proteoglycans, to determine where growth is 'off-limits'. If present at restricted locations along the edges of the optic pathway, inhibitory proteoglycans could impart directionality to RGC axon elongation.

The aim of this study was to determine (1) whether sulfated proteoglycans were present in the developing retina with the proper spatial and temporal expression to enable them to influence the direction of RGC axon outgrowth, (2) whether a CS-PG is inhibitory to the advance of RGC neurites in culture, as has been shown for other cell types, and (3) what the possible mechanisms of RGC outgrowth inhibition by CS-PG might be. The approach was to use enzymatic digestion of CS-PG, as well as video microscopy to observe RGC growth cones as they interact with CS-PG. The results of this study indicate that CS-PG may have an important influence on the directionality of elongating RGC axons during retinal development.

Materials and methods

Immunocytochemistry of tissue sections

Primary antibodies used in this study include: CS-56 (Avnur and Geiger, 1985), a monoclonal antibody that recognizes undigested chondroitin sulfate chains; 3-B-3 (Couchman et al., 1984) which recognizes chondroitin-6-sulfate 'stubs';...
following digestion with chondroitinase ABC; 4-D-1 and 8-C-2 to keratan sulfate proteoglycan (Perris et al. 1991) kindly provided by B. Caterson; RT97, an anti-neurofilament antibody, and antibody 33, which recognizes heparan sulfate proteoglycan, both obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biology, University of Iowa, Iowa City, IA under contract NO1-HD-6-2915 from the NICHD; and TuJ1, a monoclonal antibody directed to a neuron-specific isotype of beta-tubulin (Moody et al. 1989). The monoclonal antibody TuJ1 was generously provided by Dr Frankfurter (University of Virginia, Charlottesville). A rabbit affinity-purified polyclonal antibody directed to fibronectin was kindly provided by J. McCarthy and L. Furcht, University of Minnesota.

Embryonic day 13–17 rats were dissected and decapitated. Heads were immersed in 4 % paraformaldehyde for 2 h at 4°C then cryoprotected with 30 % sucrose in 0.1 M Dulbecco’s phosphate buffered saline (D-PBS; Gibco) overnight. Cryostat transverse sections (7–10 µm) through the retina were collected on gelatin-subbed slides.

Neural retina of white Leghorn chicken embryos (Gallus gallus) were staged (Hamburger and Hamilton, 1951) and dissected in D-PBS or Hanks Balanced Salt Solution (HBSS; Gibco). The retinas were immersed overnight (9–15 h) in a periodate-lysine-paraformaldehyde fixative (McCleland and Nakane, 1974), rinsed in D-PBS, embedded in acrylamide (Johnson and Blanks, 1984), and cryosectioned or were prepared as above for rat retina. 10 µm sections were collected on gelatin-coated glass slides and stained. The sections were stained using either the immunoperoxidase or immunofluorescence technique (described below).

Rat and chicken sections were incubated in 0.1 M D-PBS containing 3 % normal goat serum (NGS; ICN Immunobiologicals) and 0.05 % Triton X-100 (Fisher Scientific Co.), pH 7.3 for 30 min at room temperature. They were then incubated overnight in primary antibodies, washed 3 times for 1 h each in D-PBS or HBSS, incubated overnight in secondary antibodies conjugated to peroxidase, rhodamine or to biotin, and washed extensively in HBSS. The biotin-conjugated sections were reacted with either Texas Red conjugated to avidin (Amersham, Arlington Heights, IL) or the ABCVectastain Elite (Vector Laboratories, Burlingame, CA). The slides were coverslipped in N-propyl gallate to reduce photobleaching of the fluorochrome and observed with a Leitz Orthoplan 2 fluorescent microscope or an Optiphot (Nikon). Photographs were taken with Kodak Tech-Pan (ASA 50–80) or Kodak TMAX film (ASA 400).

Preparation of the tissue culture substratum

Two types of tissue culture substrata were used to analyze the behavior of elongating RGC axons, depending upon the assay.

(1) Directionality assay

Tissue culture dishes (60x15 mm) were coated with 0.5 ml of a solution of nitrocellulose (2 cm x 10 cm; Schleicher and Schuell, Type BABS) dissolved in methanol (25 ml) and allowed to dry in a laminar flow hood (Lamnaw and Lemmon, 1987). One half of the dish was coated with a mixture of a chondroitin sulfate proteoglycan (CS-PG; 0.01–1.0 mg ml⁻¹) derived from bovine nasal cartilage (kindly provided by L. Culp) or chick limb (kindly provided by A. Caplan and D. Carrino) and 10 µg ml⁻¹ laminin (Gibco) or purified from EHS tumor (Palm et al. 1985). Note: throughout this report, wherever ‘CS-PG’ or ‘proteoglycan’ is stated, it will refer to this mixture of CS-PG and laminin, since laminin was always added to the proteoglycan to supply a growth-promoting molecule. CS-PG was applied in a smooth layer across half of the dish with a bent glass Pasteur pipette. Subsequently, the entire dish was coated with 10–100 µg ml⁻¹ laminin to coat the opposite half of the dish with a growth-promoting substratum. The proteoglycan mixture included rhodamine isothiocyanate (RTIC; 5–10 %) in some cases to allow for localization of the proteoglycan during analysis and photography. The border between CS-PG and laminin, along the center of the dish, was sharp and straight. Media (DMEM/F12 with 5 % fetal calf serum, 5 % chick embryo extract, or chick serum, and 1 % penicillin/streptomycin) was added to the dish immediately following application of the substratum molecules. Each dish was then stored in an incubator to prevent photobleaching of the fluorochrome while the retinal explants were prepared, i.e. 1–2 h (see below).

(2) Video microscopy analysis

Glass coverslips (24x30 mm) were cleaned with sulfuric acid (20 %) and 0.1 M sodium hydroxide, rinsed in distilled water, followed by D-PBS and dried. The coverslips were attached over holes drilled in 50-mm Petri dishes (60x10 mm; Falcon Labware, Oxnard, CA) with a mixture of bee’s wax, lanolin and petroleum (1:1:1). Dishes with coverslips were UV-sterilized for 1 h and treated as sterile from this point on. Half of the coverslip was coated with 0.6–1 mg ml⁻¹ CS-PG overnight at 4°C. The proteoglycan was removed and the coverslip washed with Voller’s carbonate buffer. The coverslip was coated with 10–100 µg ml⁻¹ laminin overnight or for 3 h at 37°C. Each dish was then stored in an incubator to prevent photobleaching of the fluorochrome while the retinal explants were prepared, i.e. 1–2 h (see below).

Preparation of retina strip explants

Neural retinae from embryonic day 6 (E6) chicks were dissected in Ca²⁺/Mg²⁺-free buffer (CMF), (Halfter et al. 1983). In brief, for each dissected eye, the surrounding tissue, lens and pigment epithelium were removed. The retinae were spread flat onto a black nitrocellulose membrane filter (Sartorius SM 1306) ganglion cell layer up. Prior to use, the filter was soaked in 0.2 % Concanavalin A (Con A) in CMF overnight at 4°C to facilitate attachment. To remove excess Con A before mounting the retinae, the filters were blotted onto dry filter paper. Further gentle blotting was done to attach the retina to the filter securely. Following the final blotting and while still moist, the retina and membrane filter were cut into 350 µm strips perpendicular to the optic fissure, i.e. in a nasotemporal direction, using a Brinkman tissue chopper.

Retina strip tissue culture protocol

Tissue culture dishes containing prepared substrata were removed from the incubator and washed one time with medium. Medium was added to cover the bottom of the dish. For the directionality assay, the retina strips were transferred individually, ganglion cell layer down, to the dishes in the following manner. One strip was placed on the proteoglycan half of the dish, parallel to, and close to, the border between the two substrata with the growth orientation toward the border. Chick retinal ganglion cells preferentially elongate toward the optic fissure (Halfter et al. 1983). Similarly, another retina strip was laid down on the laminin side of the dish with its growth orientation facing the border between the two substrata. The technique is illustrated in Fig. 1. Small metal bars were placed across the ends of the strips (a region
of the filter to which retinal explants do not extend; see Fig. 1) to hold the explant securely to the bottom of the dish for attachment. Media was then added to 4 ml to cover the explants and metal bars fully. The explants were incubated for 36–48 h in a 37°C, 5% CO₂/95% air incubator.

For video microscopy analysis and for enzyme assays (see below), retinal strips were placed only on the laminin side of the coverslips with the preferred orientation of axonal outgrowth toward the border between the two substrata.

Analyses of retina strip cultures

After 12–24 h, the culture dishes were photographed on a Nikon inverted Diaphot microscope, then preserved with 4% paraformaldehyde/0.01% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) and coverslipped with Mowial (Morton Thiokol, Inc.) to prevent photobleaching of the fluorochrome. Some cultures were photographed with a Leitz Orthoplan 2 microscope. Cultures were photographed with epifluorescence optics to locate the border between the CS-PG and the laminin sides of the dish. Using a Variolume, the retinal neurites could be observed with transmitted light while fluorescence from the RTTC, denoting the position of the CS-PG-containing areas, was also visible.

Video microscopy

24 h after explanting the retinae, RGC growth cones were analyzed at the border between laminin and CS-PG using an inverted microscope (IM-35; Carl Zeiss, Thornwood, NY) with a Dage-MTI Model 65 Newvicon videocamera attached to a Panasonic TQ-2026F optical disc time-lapse video recorder. Phase-contrast images were enhanced with a Quantex QX-7 Image Processor using the software program ICOS. Other images were recorded with a silicon-intensified target (SIT) camera (65 MK II MTI; Dage-MTI, Michigan City, IN).

Chondroitinase ABC digestion of CS-PG chains from the protein core in vitro

Chondroitinase ABC (chondroitin ABC lyase; chondroitin ABC eliminase) catalyzes the removal of A'-acetylhexosaminide linkages in chondroitin-4- and chondroitin-6-sulfate, dermatan sulfate, chondroitin and hyaluronic acid, resulting in protein-enriched core molecules with modified linkage oligosaccharides. The enzyme does not act on keratan sulfate, heparin or heparan sulfate.

Retina strip explants were cultured for 24 h to achieve abundant neurite outgrowth. Chondroitinase ABC (0.1 Units ml⁻¹) or keratanase (0.1 U ml⁻¹) diluted in appropriate buffers was added to the media, and the dishes returned to the incubator. The cultures were analyzed at 7 h and 27 h after enzyme addition. Prior to addition of enzyme and at the 7 h time point, the cultures were photographed. After 27 h, the cultures were fixed with 4% paraformaldehyde/0.01% glutaraldehyde, coverslipped with Mowial and photographed again. Controls received heat-denatured enzyme (15 min at 70°C) or a non-specific enzyme (heparitinase, 0.1 U ml⁻¹).

Immunocytochemistry of retinal strips

Using the immunocytochemical protocol described above, some retinal strip cultures were labeled with antibodies CS-56 and 3B-3 to CS-PG, antibodies 4-D-1 and 8-C-2 to keratan sulfate proteoglycan, antibody 33 to HS-PG, antibody RA4 to the 44×10³ Mr cytoplasmic protein in RGCs and antifibronectin (see M and M, page 1474 for details about antibodies).

Results

The goal of this study was to investigate the potential role of sulfated proteoglycans, in particular, chondroitin sulfate proteoglycan (CS-PG), in regulating the direction of RGC axonal elongation in the developing retina. To this end, CS-PG was localized in the retina during the initial stages of RGC axon outgrowth and the effects of CS-PG on neurite outgrowth were assessed.

Chondroitin sulfate proteoglycan expression in the retina is developmentally regulated

Chondroitin sulfate proteoglycan is expressed in both the rat and chicken retina during development. In rat, on embryonic day 14 (E14), differentiating RGCs are located in the central retina, as detected by an antibody to neuron-specific β-tubulin (Fig. 1A). CS-PG, which is expressed preferentially in the vitreal-most layers of the retina, flanks the β-tubulin-positive RGCs (Fig. 1B and C). With continued development, CS-PG moves peripherally, and remains at the outer edges of the differentiating RGCs, i.e. peripheral to the β-tubulin.
Fig. 2. Chondroitin sulfate proteoglycan expression in chicken retina. (A) Retinal ganglion cells, labeled with TuJ1, lie in the center of stage 21 chicken retina. Dorsal is at the top of the figure. (B) The expression of CS-56 is greatest in the ventral retina (v) peripheral to the position of differentiated RGCs shown in view A. CS-PG is also present as punctate deposits in the central retina (c). Only sparse labeling is present in the dorsal retina (d). CS-PG is present in the vitreous and surrounding mesenchymal tissue (also shown in view D). (C) In stage 23 chicken retina, TuJ1-positive RGCs extend further peripherally than in stage 21 (ventral is to the left). (D) CS-PG is expressed only at the peripheral-most area of the ventral retina where retinal ganglion cells are absent (arrows). Compare views C and D by aligning the flexures of the retina. (Scale bar=90 μm.)

staining. By E17, CS-PG is no longer detectable in the retina with immunocytochemical techniques. Comparison of the staining patterns of TuJ1 with CS56, then, demonstrates that there is an inverse relationship between the position of the differentiating RGCs and the region of the retina expressing CS-PG.

CS-PG immunoreactivity in chicken retina, although similar to rat in ventral regions, differs in its location and staining pattern centrally and dorsally. At stage 15–16, when no neurons are evident with TuJ1 labeling, CS-56 labeling exists at approximately the same intensity throughout the inner layer of the retina (not shown). At stage 21, labeling with TUJ1 localizes differentiated RGCs to the center portion of the retina (Fig. 2A). Neural retina ventral to the TuJ1-positive region is devoid of RGCs and expresses CS-PG (Fig. 2B), like the pattern seen in rat retina. However, central retina continues to express CS-56 immunoreactivity, but the intensity of staining is decreased in comparison to pre-neuronal stages. Dorsal retina, where there are no neurons at this stage, shows greatly reduced CS56 immunoreactivity. At stage 23, TuJ1
labeling indicates that axons have extended from RGCs further toward the periphery of the retina (Fig. 2C). Comparison of TuJ1 labeling with CS56 immunoreactivity on this day of development shows that CS-PG is expressed just peripheral to the TuJ1 staining, i.e. peripheral to the RGCs in the ventral retina (Fig. 2D).

Thus, unlike rat retina, CS-PG remains in the central retina of chicken, but at lower levels than in the periphery. Also, although no RGCs can be detected in the dorsal retina, CS-PG is present in fine granules. Thus, the precise inverse correlation between the location of the RGCs and the expression of CS-PG found in rat retina pertains only to the ventral portion of the chicken retina.

**A chondroitin sulfate-containing proteoglycan inhibits retinal ganglion cell axon outgrowth in vitro**

Cartilage chondroitin sulfate proteoglycan (CS-PG) consists mainly of CS chains bound to a protein core with a small number of keratan sulfate chains (Heinegard and Paulsson, 1984). The results of a previous study showed that this proteoglycan inhibits the elongation of chick dorsal root ganglion neurites in vitro (Snow et al. 1990b). We tested in vitro whether CS-PG may also inhibit RGC neurite elongation.

Neurites from retinal strip explants grew well on 10 μg ml⁻¹ laminin bound to either nitrocellulose-coated plastic or sulfuric acid/sodium hydroxide-washed glass coverslips. Outgrowth began around 18 h in culture and was extensive by 24–36 h. However, when retinal neurites encountered CS-PG (Note: CS-PG wherever stated in the text indicates a CSPG + laminin mixture; see Materials and methods for explanation) in a culture assay consisting of alternating stripes of laminin and CS-PG, they stopped abruptly or turned at the border of the CS-PG-containing lane and grew only on the laminin, not on the CS-PG (Fig. 3). Interestingly, a ten-fold increase in the concentration of laminin in the CS-PG/laminin mixture did not result in RGC neurites crossing the border, as occurred with chick DRG cultures (Snow et al. 1990b). The tips of the lamelipodia were located about 1–2 μm from the edge of the CS-PG lanes (Fig. 3, arrow).

**Video microscopy of RGC outgrowth**

Time-lapse video microscopy showed that the filopodia of the RGC growth cones are long and thin and extend to and sample the CS-PG repeatedly. Fig. 4 shows a series of photographs from video microscopic recordings showing the approach of RGC growth cones toward a CS-PG stripe (Figs 4A and B), sampling of the CS-PG and retraction of filopodia (Figs 4C and D). The process of sampling and retracting continues as the growth cone gradually turns and grows on the laminin lane in an alternate direction. As long as a growth cone is at the border between laminin and the proteoglycan,
Directionality of retinal ganglion cell outgrowth

filopodia will sample the proteoglycan but will not grow onto it. Fig. 4E shows the growth cones as they change their direction of migration to avoid CS-PG. When contact is made with neighboring growth cones, pushing and pulling occurs between them with one growth cone or group of growth cones dominating to determine the direction of growth (Figs 4F and G). The same region shown in Figs 4A–E are shown 17 h later without (Fig. 4H) and with (Fig. 4I) epifluorescence optics to indicate the position of the CS-PG stripe.

Chondroitinase ABC treatment restores RGC neurite outgrowth
Since chondroitin sulfate but not keratan sulfate was localized to the retina, the focus of this series of experiments was to determine the importance of the CS portion of the CS/KS-PG molecule. To determine the effect of CS-PG on RGC outgrowth, we added chondroitinase ABC (protease-free and with protease inhibitors added) to the media of chick retina (E6–7) cultures in which retinal strip explants were grown on laminin stripes that alternated with stripes of CS-PG (Snow et al. 1990b). Prior to the addition of enzyme, retinal neurites elongated on laminin, but did not elongate onto CS-PG (Fig. 5A). After establishment of distinct boundaries of axon growth at the interface between stripes of laminin and stripes of CS-PG, chondroitinase ABC was added to the media at a concentration of 250 μg ml⁻¹ (0.1 U ml⁻¹). At 7 h after the addition of enzyme to the culture media, some neurites had grown onto the CS-PG stripes (Fig. 5B). One day later (27 h), many neurites had crossed onto the CS-PG stripes (Fig. 5C). Interestingly, some neurites remained at the border of the stripe and did not grow onto the CS-PG regardless of the amount of time following chondroitinase addition (Fig. 5C; arrows).

Direction of outgrowth of retinal neurites in vitro
The direction that RGC axons extend from the cell body may be a function of the molecules that growth
cones contact during initial outgrowth versus those available to them as they extend. In order to test this hypothesis, we coated halves of culture dishes with laminin and the other halves with CS-PG in a variety of concentrations. Retinal explants were then placed on each side of the border between the two molecules with the orientation of outgrowth directed toward the border. This paradigm is illustrated in Fig. 6A. This assay allowed us to test how a growth cone would respond if it initiated an axon on laminin and then encountered CS-PG in various ratios, versus how it would behave if forced to initiate an axon in a CS-PG environment.

This assay supported our previous finding in that retinal neurites growing on laminin were repelled by CS-PG (Fig. 6B, left). Unlike chicken DRG neurites, however, chicken retinal neurite outgrowth could not be elicited from explants placed directly onto the CS-PG substratum (Fig. 6B, right) over a range of concentrations from 1 mg ml\(^{-1}\) to 1 \(\mu\)g ml\(^{-1}\) CS-PG.

**Immunocytochemistry of retinal strip explants**

To determine which cells of the retina produce CS-PG, the retinal strip explants of some cultures were labeled with antibodies against CS-PG (CS56 and 3B3), keratan sulfate (4-D-1 and 8-D-9), heparan sulfate proteoglycan (33–2), RGC antigen (RA4) and fibronectin. Labeling with monoclonal antibody RA4 showed immunoreactivity along the axons and growth cones of all of the processes extended from the explant (Fig. 7A). It is known that this antibody shows little to no staining of RGC soma after 24 h in culture and this was verified in cultures of dissociated retina (not shown). Labeling with antibodies to CS-PG and HS-PG showed that cells within the explant expressed CS-PG (Fig. 7B) as well as HS-PG (not shown). Keratan sulfate was not expressed by retinal cells with the antibodies used. Although it appears that the cells in Fig. 7B are aligned along the edge of the explant, this may be an edge effect in staining due to curvature of the explant with fixation. In dissociated cell culture, CS-PG-positive cells are dispersed throughout the culture. In some explants, cells that are not RGCs (i.e. do not express immunoreactivity with RA4), migrate out onto the laminin-coated substratum, but remain close to the explant (not shown). These cells stain positively for CS-PG. Although these cells had a fibroblast-like morphology, they did not express fibronectin, as detected by a rabbit polyclonal antibody to fibronectin that is routinely used in our laboratory.

From these data, from reports that suggest that certain glial cells synthesize proteoglycans in vivo and in vitro (Glimelius et al. 1978; Gallo and Bertolotto, 1990; Snow et al. 1990a; Haugen et al. submitted) and from data suggesting that glial cells have not yet differen-

![Fig. 5. Chondroitinase ABC removes chondroitin sulfate chains from the protein core and allows retinal ganglion cell neurites to grow over the CS-PG stripes. (A) Before the addition of 0.1 U ml\(^{-1}\) of Chondroitinase ABC, neurites are inhibited from crossing onto the CS-PG-containing stripe. (B) After 7 h following enzyme addition, some neurites have begun to elongate onto the stripe region. (C) One day later, abundant, long neurites infiltrate the once-inhibitory stripe region. Following the neurites along their trajectory, it is evident that some neurites still do not cross onto the stripe, even after 27 h following the addition of enzyme (arrows; 144×).](image-url)
Directionality of retinal ganglion cell outgrowth

Retina explant

Fig. 6. (A) Schematic diagram showing the paradigm used to test unidirectionality in the retina. Laminin (LN) was bound in a range of concentrations on the left while CS-PG (PG) was bound in a range of concentrations mixed with laminin on the right side of the dish. Retinal strips were explanted to the dish oriented so that their direction of preferred outgrowth, toward the optic fissure in vivo, is facing the boundary between the two substrata (Drazba and Lemmon, 1990). (B) A phase-contrast photograph of the explants growing toward the border between the laminin-only side of the dish (left) and the CS-PG/LN side. Neurites growing on laminin stopped or turned at the CS-PG border while neurites on the CS-PG/LN substratum did not initiate neurites, regardless of the concentration of CS-PG, (187X).

Discussion

Retinal ganglion cell axons grow in a stereotyped pattern toward the optic fissure during development, even though no obvious directional restrictions exist. This study has attempted to identify factors that may regulate the direction of retinal axon elongation.

Immunostaining of the neural retina at the earliest stages of axonal outgrowth showed that CS-PG has an appropriate spatial and temporal expression to interact with elongating RGC axons. The results of in vitro studies have illustrated the inhibitory effects of CS-PG on initial outgrowth and elongation of neurites from retinal explants. This effect is seen with very low amounts of CS-PG and when the CS-PG is mixed with the growth-promoting glycoprotein, laminin. Enzyme degradation of CS chains from CS-PG indicated that these glycosaminoglycan chains are the components responsible for inhibition of retinal neurite outgrowth.

Coordination of growth-promoting and growth-inhibiting influences in the retina

The restricted and developmentally regulated expression of laminin (Cohen et al. 1987), and other adhesion molecules in the retina where RGC axons grow, combined with the restricted expression of CS-PG where axons do not grow, suggest that the direction of RGC axon elongation could be determined by the net effects of growth-promoting and growth-inhibiting molecules in the extracellular environment. NCAM, present on neuroepithelial cell endfeet (Silver and Rutishauser, 1984), and laminin, expressed in punctate fashion along the surface of radial cells (Cohen et al. 1987; Liesi and Silver, 1988), may facilitate RGC axons to choose the marginal zone as a preferred growth pathway. Simultaneously, CS-PG, and possibly other growth-inhibiting molecules, may inhibit growth toward the retinal periphery. This effect may only be important for initial axonal outgrowth, i.e. the CS-PG located directly adjacent to the RGCs may set the initial pathway choice for an RGC axon. However, from that point, the axons may then use adhesive cues of the substratum, or geometric constraints to elongate toward the optic fissure.

Interactions between growth-promoting and growth-inhibiting molecules may be complex as is suggested by analysis of the chicken retina. Unlike in rat retina, CS-PG is expressed in central retina of chicken at stages of development when RGC axon initiation and elongation take place. However, chicken retina expresses a variety of adhesion and ECM molecules. Thus, one explanation for the co-localization of CS-PG and neurons in the central chicken retina may be that the inhibitory

tiated at this early stage, we postulate that a possible candidate for CS-PG producing cells of the retina are undifferentiated neuroepithelial cells. However, the absence of specific markers for retinal cell types at this period of development prevents definitive identification of these cells.
Fig. 7. Immunocytochemistry of retinal explants. (A) Retinal ganglion cells extending from the explant stain brightly with antibody RA4 (McLoon and Barnes, 1989). (B) An unidentified cell type within the explant expresses CS-PG. These cells are most obvious at the outer edges of the explants, but can be found within the explant as well. (190×).
effects of CS-PG are masked by a strong positive influence of the adhesive molecules present.

Results of in vitro studies indicate that chicken retinal neurites are more sensitive than DRG neurites to CS-PG. Chick DRG neurites can elongate on 400µg ml⁻¹ CS-PG mixed with 10µg ml⁻¹ laminin (Snow et al. 1990b). In comparison, neurites elongating from chicken retinal explants stop or turn away from a substratum treated with 100µg ml⁻¹ CS-PG mixed with 10µg ml⁻¹ laminin. Thus there are differences in the behavior of these two neuronal types in their response to CS-PG. Studies using ³⁵S-CSPG (Snow and Letourneau, unpublished) indicate that the amounts of CS-PG that bind to the coverslip, if the above concentrations are used, are proportional to the starting solution, i.e. about 1–3% of the CS-PG binds in each dilution. Therefore, differences between the behavior of DRG and retinal growth cones in these assays is a result of the response of the growth cones to CS-PG and not to variability in the amount of CS-PG bound to the substratum.

The experiments designed to test directional choices of retinal neurites along a defined substratum address a more complex and subtle aspect of the relationship of growth-promoting to growth-inhibiting molecules. The questions that are addressed are (1) whether the initial direction of extension of an RGC axon depends upon the composition of the substratum that the growth cone contacts first as it extends from the cell body (laminin, proteoglycan, or a critical combination of the two), and (2) once the growth cone has contacted that initial substratum molecule or combination of molecules, does that contact influence how the growth cone will react to a second molecule? In contrast to DRG neurites (Snow et al. 1990b), retinal explants placed on CS-PG did not ever extend neurites, even if the concentration of CS-PG was very low (1–10µg ml⁻¹). This lies in contrast to DRG explants that, in some cases, can extend neurites when placed directly on a concentration of 600–1000µg ml⁻¹ CS-PG mixed with 10µg ml⁻¹ laminin (Snow et al. 1990b). Alternatively, neurites from some retinal explants grew onto a low concentration (1–10µg ml⁻¹) of CS-PG mixed with 10–100µg ml⁻¹ laminin when they first initiated on laminin. The fact that RGC axons can grow onto CS-PG from laminin, but cannot initiate an axon directly on CS-PG, may occur because initiation of a neurite may be inhibited more by CS-PG than is extension of a neurite.

The intrinsic sensitivity of RGC growth cones to CS-PG may play a significant role in their response to CS-PG in vivo. The ratio of CS-PG to laminin and/or other growth promoters in the retina at the point of axon initiation must be very low in order for RGCs to begin to elaborate an axon. CS-PG immunoreactivity is high in areas of the retina which flank differentiating RGCs. It is unlikely that they would choose a peripheral direction for outgrowth i.e. toward the CS-PG, but rather would grow in a direction which is opposite to the position of high concentrations of CS-PG, and rich in laminin as well, i.e. generally toward the optic fissure. However, the mechanisms that control the precise guidance of growth to the fissure are yet to be elucidated.

**Chondroitinase ABC digestion of CS-PG**

Digestion of CS chains from CS-PG with chondroitinase ABC indicate that the carbohydrate moiety of this macromolecule is responsible for inhibition of neurite outgrowth, as was shown in previous assays (Snow et al. 1990b), and not the protein core. A percentage of neurites appear not to cross onto CS-PG with enzyme treatment. Several explanations for this result are possible. Previous reports have shown that chondroitinase ABC does not digest KS chains from the protein core (Oike et al. 1980). Endo-β-D-galactosidase digestion followed by keratanase digestion is needed for full removal of KS chains from the core protein (Melrose and Ghosh, 1988). Therefore, keratan sulfate chains, resistant to chondroitinase ABC and still attached to the protein core, may be responsible for the inhibition of the remaining RGC neurites. Alternatively, CS-PG lane crossing may occur as a result of axon branching with only a subpopulation of neurites being capable of branching. Lastly, all neurites may cross the CS-PG lanes following digestion, but since crossing begins at the growth cone, the rest of the neurite may remain lined up along the border between laminin and CS-PG. Further experiments looking at individual neurites are needed to address these possibilities.

**RGC growth cones do not collapse upon contact with CS-PG**

CS-PG does not appear to cause collapse of RGC growth cone morphology, except in a small percentage of cases. The predominant response to CS-PG is continued interaction of the filopodia with CS-PG which ultimately results in turning. Likewise, chick DRG growth cones sample the CS-PG, retract and turn without undergoing collapse to any significant degree (Snow et al. 1990b). This observation may indicate that growth cone collapse caused by cues on unlike neurites (Kapfhammer and Raper, 1987; Raper and Kapfhammer, 1990), tectal membranes (Walter et al. 1987), posterior somite molecule(s) (Davies et al. 1990), and 5-HT (Haydon and McCobb, 1984) may differ from the type of inhibition of neurite outgrowth that is caused by CS-PG.

A recent report shows that the posterior somite contains a ten-fold greater concentration of CS-PG than does anterior somite. Davies et al. (1990) have shown that posterior somite extract induces collapse of chick DRG growth cones. If the repelling molecule is a CS-PG, then it is perplexing how CS-PG causes collapse in the Davies et al. paradigm, but not in this study. Perhaps different CS-PGs can have a variety of effects on growth cone behavior or the response might be dependent upon the particular conditions of the assay in vitro or the environment in vivo.

**Conclusion**

The present study has shown that a chondroitin sulfate
proteoglycan is expressed in the developing retina in a spatial and temporal manner that would allow it to function as a guidance molecule for retinal ganglion cell axons. The in vitro studies show that CS-PG-coated substrata inhibit outgrowth of embryonic chicken retinal neurites even when the proteoglycan is applied at low concentrations or mixed with outgrowth-promoting substances. Further, inhibition of RGC neurite outgrowth is due to the carbohydrate portion of the CS-PG molecule as shown by resumption of growth with enzymatic removal of CS chains from the protein core.

Previous studies have shown the existence of numerous adhesive and growth-promoting molecules in the developing retina, which are thought to contribute to the stereotyped pattern of retinal ganglion cell outgrowth. Here we have described the first example of an inhibitory molecule present in the rat and chicken retina, which, in coordination with other molecules, may dictate the centrally oriented direction of retinal ganglion cell outgrowth.

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