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# Exogenous glycosaminoglycans induce complete inversion of retinal ganglion cell bodies and their axons within the retinal neuroepithelium

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ABSTRACT **Prior to forming an axon, retinal ganglion cells retain a primitive radial configuration while maintaining ventricular and vitreal endfeet attachments. During their subsequent differentiation, ganglion cells polarize their cell body and axon only along the vitreal surface. When the ventricular surfaces of intact retinas in organ culture were exposed to free chondroitin sulfate (CS) in solution, both the cell body and nerve fiber layers were repolarized to the opposite side of the neuroepithelium. However, the basal lamina remained in its usual position. Thus, the ability to initiate an axon is not restricted to the vitreal endfoot region of differentiating neurons, and in addition, the radial position at which the axon emerges can be mediated by the location and concentration of the extracellular CS milieu.**

The factors responsible for the proper establishment of stratified architectures typical of all layered structures in the mammalian brain are unknown. During vertebrate retinal histogenesis, the polarization of ganglion cell bodies and their axons is a critical determinant for the proper location of the ganglion cell and optic fiber layers that form a template for the development of subsequent neuronal strata (1). Retinal ganglion cells are the first neuronal subtypes committed to leave the cell cycle, and shortly after terminal mitosis they express a neuron-specific β-tubulin isoform. Marked by a monoclonal antibody (mAb) to β-tubulin, TUJ1 (2), young retinal neurons, just prior to elaborating an axon, retain a radial configuration and are anchored to the inner and outer surfaces of the retinal neuroepithelium by both ventricular and vitreal endfeet (also see refs. 3–8). Axons are generated solely in the marginal zone (nearest the lens) as buds from the vitreal endfeet. Importantly, the vitreal surface is the only one that allows growing axons passage out of the eye. During axonogenesis the ganglion cell somata also migrate in a vitreal direction. We have reported previously that prior to and during axonogenesis, the vitreal endfeet are immersed in a matrix containing a chondroitin sulfate (CS) proteoglycan (CSPG) (3). As development proceeds, the CSPG epitope clears centrifugally from the optic fissure as waves of ganglion cells differentiate synchronously within, and send axons away from, the receding CSPG matrix. Because of the timing and distribution of their expression, the carbohydrate moieties of this native CSPG could play a role in regulating the establishment of soma and nerve fiber layer polarity in the retina. To gain insight into the function of the CS glycosaminoglycan (GAG) chains during retinal differentiation, free chain CS GAGs were added to the bathing medium of intact cultured retinas. After various times in culture the retinas were assayed for evidence that changes in the location of the CS milieu could either induce an ectopic axon from the ventricular surfaces of primitive neurons or repolarize their somata.

## METHODS

**Organ Cultures.** Timed pregnant Sprague–Dawley rats were obtained from a commercial vendor (Zivic–Miller). Embryos delivered by Cesarean section on embryonic day 13.0 (E13.0) were placed in ice cold Dulbecco's modified Eagle's medium/Ham's F-12 medium, 1:1 (vol/vol) (DMEM/F-12). Eyes were removed with the aid of a dissection microscope using bright-field optics. The eyes were then transferred to ice-cold calcium/magnesium-free medium and further dissected. The sclera and extraocular muscles along with the pigmented epithelium were removed. The lens along with the vitreous body was not removed. Whole retinal preparations from left and right eyes were divided into control and experimental groups and were maintained in 48-well clusters in DMEM/F-12 culture medium supplemented with glucose and 10% (vol/vol) fetal calf serum under a 5% $CO_2$/95% air atmosphere at 37°C. In the experimental group, GAGs were added directly to the medium. Retinas that adhered to the bottom of the dish were not harvested. The CS used for these experiments was purified from shark cartilage and consisted of alternating copoly(β-glucuronic acid-[1–3]-*N*-acetyl-β-galactosamine-6-sulfate-[1–4]) ($n$ = 40 retinas). Prior to their addition to the medium, to denature any contaminating proteins, the GAGs were heated to 70°C for 20 min. In addition, keratan sulfate purified from bovine cornea ($n$ = 40 retinas) and hyaluronic acid purified from bovine vitreous humor were used ($n$ = 40 retinas). Retinas remained healthy in 10 mg/ml concentrations of CS. This was determined by comparing mitotic indices (numbers of mitotic figures per section) in treated ($n$ = four retinas, an average of 15 mitoses per section) and control retinas ($n$ = 6 retinas, an average of 17 mitoses per section) from serial 1- to 2-μm plastic sections stained with toluidine blue. An additional group of retinas were cultured for 48 hr starting at embryonic day 13.0 with CS ($n$ = 20) and without CS ($n$ = 20). Total retinal protein from these retinas was run through 7% polyacrylamide, transferred to nitrocellulose, blotted with mAb TUJ1, and scanned with a Shimadzu dual-wavelength thin-layer chromatographic scanner. These experiments revealed that the amount of β-tubulin in retinas treated with CS, and therefore the number of differentiating retinal ganglion cells, was comparable to that in control retinas. Initial indications of repolarization were detected at 2 and 5 mg/ml, but because these smaller concentrations of CS failed to sufficiently penetrate the ventricular surface of the neuroepithelium (as determined visually by the degree to which these different concentrations of the biotinylated GAG penetrated the neuroepithelium), the actual concentration of CS that eventually

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Abbreviations: CS, chondroitin sulfate; GAG, glycosaminoglycan; CSPG, CS proteoglycan; mAb, monoclonal antibody.

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penetrated the neuroepithelium and contacted the ventricular endfeet was probably well below the 2, 5, and 10 mg/ml concentration in the media. In the CS-treated retinas, the native vitreal CSPG was still present and receded centrifugally as previously reported (3). Thus, it is likely that relatively higher concentrations of CS were needed on the ventricular surface to counteract the native vitreal CSPG/CS.

**GAG Labeling.** Prior to their addition to the medium, GAGs were labeled with biotin-LC-hydrazide (Pierce). The GAG of interest was dissolved in labeling solution (100 mM sodium acetate 0.02% sodium azide, pH 5.5). To produce aldehyde groups, 30 mM sodium periodate was added, and the solution was incubated in the dark for 30 min at room temperature. To stop the sodium periodate reaction, sodium sulfite was added and incubated for 5 min at room temperature. To form biotinylated GAGs, 5 mM biotin-LC-hydrazide was then added to the reaction tube and incubated for 1 hr at room temp. To remove the unreacted biotin, the product was centrifuged at 1000 × *g* for 20 min in Centricon-30 microconcentrators (Amicon), diluted in labeling solution, and centrifuged three more times.

**Detection of GAGs and Neurons.** In an effort to remove the unbound GAG–biotin complex from within the retinal neuroepithelium, the medium was changed and replaced with DMEM/F-12 three times at 30-min intervals. Retinas were then rinsed in DMEM/F-12 every 5 min four more times, fixed in 4% paraformaldehyde, and sectioned with a vibratome. Sections were mounted on gelatin/chrome alum-coated slides, incubated in horseradish peroxidase (HRP)-conjugated streptavidin, and treated with 3,3′-diaminobenzidine. To view neurons, alternate sections were incubated with mAb TUJ1 (1:1000 dilution phosphate-buffered saline containing Triton), rinsed, and incubated in fluorescein-conjugated goat anti-mouse IgG.

**Mitotic Index.** To obtain mitotic counts, retinas were fixed in Rager's fixative overnight at 4°C, postfixed in 2% $OsO_4$, routinely processed for plastic sectioning and embedded in Spurr's resin. Sagittal sections (1–2 μm) were taken serially through the entire retina and stained with toluidine blue. Mitotic counts were taken from serial drawings every 5 μm with the aid of a camera lucida.

## RESULTS AND DISCUSSION

At E13.0, the retina contained a central core of TUJ1-positive radially oriented ganglion cells that spanned the full thickness of the retina but lacked axons. Retinas with their lenses intact from this stage were cultured as spheres in medium to which various types and concentrations of GAGs were added. The degree to which the GAGs penetrated the ventricular surface of the neuroepithelium was determined by GAG biotinylation. Because the retina and lens were left intact and the organ was cultured as a sealed orb, the CS could only come into contact with the ventricular (outer) surface of the retinal neuroepithelium. Thus, after exposing the cultured eyes to CS for 48 hr, the biotin label (detected with avidin-conjugated peroxidase) was concentrated around the ventricular endfeet (Fig. 1 *Left*). When a separate group of 48-hr CS-treated retinas were subjected to SDS/polyacrylamide gel electrophoresis (PAGE) and immunoblotted with mAb TUJ1, the amount of β-tubulin was comparable to the amount in control preparations (data not shown). In addition, cryostat sections of treated retinas immunostained with TUJ1 showed that the retinal ganglion cells had differentiated on schedule. However, in contrast to control retinas (Fig. 2 *Left* and Fig. 3*A*), all of the retinal ganglion cell axons were found within the novel layer of exogenous CS matrix within the ventricular margin (Fig. 1 *Right*, Fig. 2 *Right*, and Fig. 3 *B–F*). Importantly, when viewed with electron microscopy, the basal

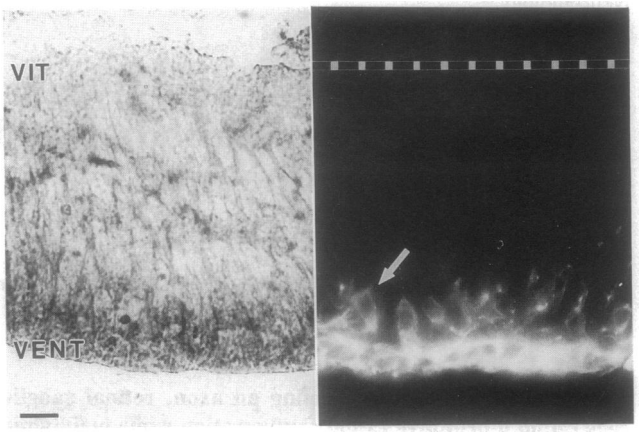


FIG. 1. Repolarized TUJ1-positive nerve fiber and cell body layers occur within the exogenous CS matrix. Whole retinas were cultured before axonogenesis at embryonic day 13.0 for 48 hr in a solution of biotinylated CS (10 mg/ml). Biotinylated CS was detected with horseradish peroxidase-conjugated streptavidin. (*Left*) Labeled CS (black reaction product) was concentrated along the ventricular (VENT) surface of the neuroepithelium. (*Right*) When sections from the same retinas were examined, the TUJ1-positive nerve fiber and cell body layers (arrow) were entirely repolarized within the exogenous CS matrix. The dotted line locates the border of the vitreal (VIT) surface. (Bar = 10 μm.)

lamina was unaltered and only found in its usual position on the pial surface (not shown). The dramatic repolarization of the retinal tissue was concentration dependent and occurred in a stereotyped sequence. First, primitive ganglion cells lost their vitreal (rather than their ventricular) endfoot attachments as the cell bodies moved towards the ventricular surface (Fig. 3*B*). It is also possible that some ectopically differentiating neurons whose somata were already at the ventricular surface (7) at the time of CS addition simply failed to reestablish the vitreal process. Next, ectopic axons emerged along the ventricular margin eventually building a thick nerve fiber layer (Fig. 3 *C–F*). Finally, the remaining radial processes were completely eliminated (Figs. 1 *Right* and 2 *Right*). This series of events gave rise to nerve fiber and cell body layers on the wrong side of the retina that were morphologically indistinguishable from those that form during normal development except that individual axons were not oriented in any particular direction (Fig. 3 *E* and *F* and Fig. 4*B*).

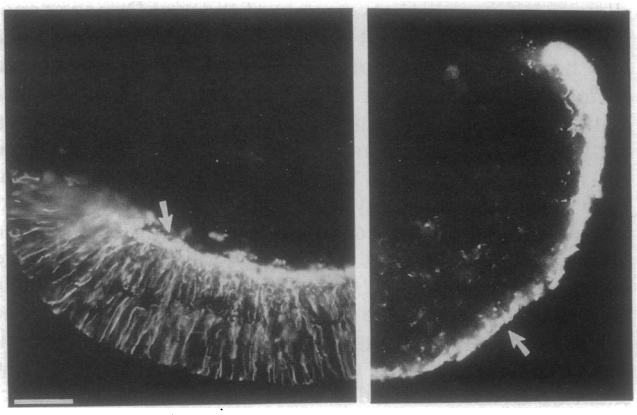

FIG. 2. Complete inversion of the retinal ganglion cell and nerve fiber layer by exogenous CS. (*Left*) In untreated retinas cultured for 48 hr, the TUJ1-positive cell and nerve fiber layer is located within the vitreal surface (closest to the lens) of the retinal neuroepithelium (arrow). (*Right*) In direct contrast, the retinas incubated in CS (10 mg/ml) for 48 hr displayed a completely repolarized TUJ1 ganglion cell and nerve fiber layer (arrow). (Bar = 50 μm.)

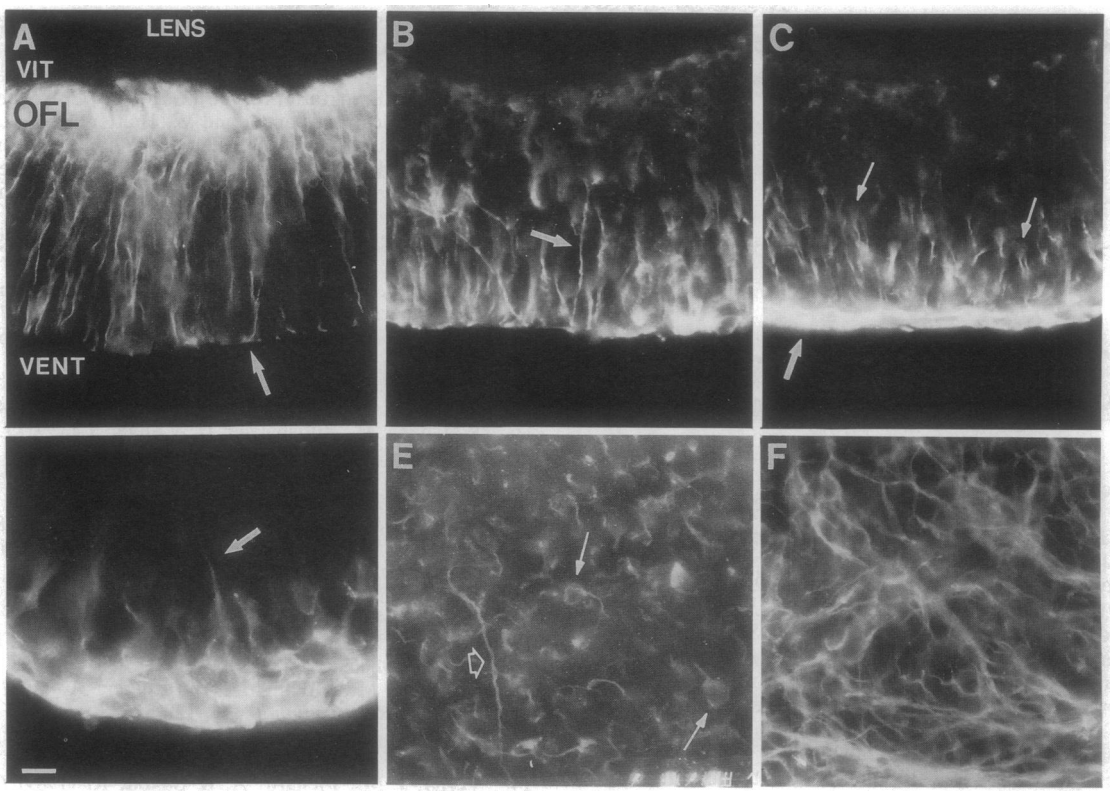


FIG. 3. The temporal sequence of optic fiber and cell body layer repolarization to the ventricular surface as marked by mAb TUJ1 in cultured retinas beginning at embryonic day 13.0. (*A*) Control optic fiber and cell body layers polarize to the vitreal surface in retinas cultured for 48 hr. Primitive retinal ganglion cells retain a radial configuration while maintaining ventricular (arrow) and vitreal endfeet attachments. (*B*) After 24 hr in culture retinal ganglion cells in GAG (10 mg/ml)-treated retinas retain their radial configuration while losing their vitreal endfoot attachments (arrow). (*C*) After 36 hours in culture, the ganglion cell bodies (small arrows) translocate to the ventricular surface as the optic fiber layer forms along the ventricular surface (large arrow). (*D*) Higher magnification of the repolarized cell body and optic fiber layers in a retina cultured for 48 hr. Both the novel cell body and the thickened optic fiber layer completely repolarized to the ventricular surface. Some ganglion cells still retain transient detached radial processes (arrow). (*E* and *F*) Inverted whole-mounted retinas (ventricular surface facing up) treated with CS (10 mg/ml) for 24 and 48 hr, respectively, and labeled with MAb TUJ1. After 24 hours in culture (*E*), a subset of retinal ganglion cell bodies (small arrows) have repolarized to the ventricular surface and send axons in random directions (arrowhead). By 48 hr (*F*), long, highly fasciculated axons with no directional orientation cover the ventricular surface. OFL, optic fiber layer; VIT, vitreal surface; VENT, ventricular surface. (Bar = 20 $\mu$m in *A–C*, *E*, and *F* and 10 $\mu$m in *D*.)

A variety of Extracellular matrix (ECM) molecules have been shown to play important roles in establishing polarity in many different cell types *in vitro* (9, 10). Our data suggest that CS, a major component of retinal ECM (3), when localized preferentially around the endfeet of young radially shaped neurons, is a potent signal *in vivo* that conveys critical information to the cell about its axon initiation site and location of its soma. Whether other GAG components of the ECM *in vivo* can also contribute this type of polarity information is unknown. However, both hyaluronic acid, a repeating disaccharide composed of an unsulfated amino sugar with uronic acids, and keratan sulfate, a sulfated amino sugar with hexose moieties, did not induce an axon from or cause the cell body to translocate toward the ventricular endfeet.

A consistent effect of CS addition was the abnormal differentiation of ganglion cell bodies and axons on the wrong side of the retina, suggesting a possible role for native GAG moieties in retinal ganglion cell polarization (Fig. 4). Linker *et al.* have recently shown that cultured keratinocytes, fibroblasts, and endothelial cells synthesize both proteoglycans and free GAGs and have hypothesized that the processing that creates biologically active free GAG chains occurs on cell surfaces (11–13). Although it is conceivable that enzymatic cleavage of CSPG or *de novo* synthesis could present CS at the retinal margin, the question of whether biologically active free GAGs are normally manufactured and/or liberated at the vitreal surface of the retinal neuroepithelium during development remains to be determined. In accord with our results, only free GAGs, and not those bound to substrates, have recently been reported *in vitro* to induce rapid morphological changes in neurons (14–16) and enhance neurite outgrowth (17). This phenomenon is unlike the regulatory action of the entire proteoglycan (core protein and GAG chain) bound to a substrate (18–21). The ways in which free GAG chains manifest their actions are unclear. In primary liver cultures, GAG chains can induce gap junction expression and restore transcription of tissue-specific mRNAs (22, 23). In addition, the ability of GAGs to bind and thereby potentiate and/or inhibit the action of different growth factors has been well documented (24–27). Thus, ectopic axon initiation from the ventricular endfoot could be due to this type of synergistic mechanism. An alternative explanation is that GAGs could act independently through a distinct receptor. Indeed, CS acting independently in serum-free conditions can cause a rapid induction of tyrosine phosphorylation in whole retinas (unpublished data). Thus, it is possible that CS could signal the endfeet to polarize the cytoskeletal machinery necessary to make an axon and translocate the cell body (28).

An interesting paradox concerning axon guidance in the eye exists upon comparing our present results using exogenous GAGs with our previous GAG chondroitinase perturbation experiments (3). We have previously shown that a matrix-bound CSPG could regulate intraretinal axon initiation towards the optic fissure by its repulsive characteristics (21). A CSPG inhibits retinal ganglion cell axon elongation *in*

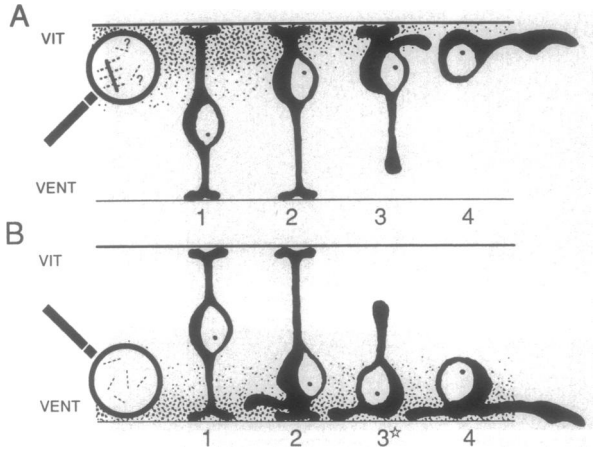


FIG. 4. Schematic depiction of the temporal stages of retinal ganglion cell differentiation in normal and CS-treated retinas revealed by TUJ1 staining. (*A*) Stages: 1, during normal development, primitive TUJ1-positive radially shaped retinal ganglion cells lack axons and have ventricular and vitreal endfeet; 2, the cell bodies translocate to the vitreal surface toward the CSPG matrix; 3, the cells eventually bud processes in an area of decreased CSPG matrix while retracting their ventricular endfoot; and 4, the cells lose their endfeet and, within a low concentration of CSPG matrix, project axons toward the optic fissure. Whether free GAGs are sequestered within the matrix as the CSPG is receding is not known (see magnifying glass). (*B*) Retinal ganglion cells differentiate on the wrong side of the retina when exogenous CS is distributed uniformly along the ventricular surface (see magnifying glass). Stages: 1, TUJ1-positive radial retinal ganglion cells lack axons and retain vitreal and ventricular endfeet attachments while (stage 2) repolarizing their soma towards the exogenous CS matrix; 3, these cells retract their vitreal endfeet and randomly project axons [it is possible that CS addition affected some differentiating neurons whose somata had not yet left the ventricular surface (star)]; 4, the retinal ganglion cells lose both endfeet and become part of the newly polarized cell and nerve fiber layers. VIT, vitreal surface; VENT, ventricular surface. In the magnifying glasses, the broken line represents free CS GAG and the solid line represents bound proteoglycans.

*vitro* by an inhibitory response that can be overcome by enzymatically cleaving the GAG chains from the core protein (14, 29, 30). We now show that CS, not bound to a core protein, has the potential to modulate cell body polarity and also can be a potent stimulant for axon elongation. To our surprise the ventricular surface was an excellent substrate for axon outgrowth. However, the repolarized axons did not have any particular orientation, thus confirming the concept that the pial surface supplies all of the proper directional information (31, 32). In the developing retina, it is possible that the core protein component of the proteoglycan at the pial surface functions indirectly to impart repulsive information to the growth cone by concentrating and correctly orienting the carbohydrate chains at the vitreal surface. Of course, the core protein may also have functions that are independent of its sugar moieties (33).

We have discovered that one class of primitive neuroepithelia, the retinal rudiment, is quite plastic in that it retains the potential to initiate axons from alternative sites when provoked experimentally by presenting a CS matrix in a novel location. Therefore, it is likely that during embryogenesis, as in the retina, other classes of developing neurons (34) could use preformed strata of GAG-containing matrix as positional cues for building the gross patterns of neuropil and fiber layers in the central nervous system.

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