In the hippocampus, specific patterns of synaptic activity can either facilitate (*16*) or, as shown here, inhibit LTP induction. Further clarifying the rules that govern the generation of LTP will be necessary for a comprehensive understanding of the role of LTP in nervous system function and in addition should provide important information for biologically based neural network models that incorporate Hebbian synaptic modifications.

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11. Standard intracellular or whole-cell patch-clamp recording techniques were used (*5, 6*), and EPSPs were recorded under current clamp with an Axoclamp 2A (Axon Instruments). Cells were held at −75 to −90 mV throughout the course of the experiment. We observed no significant differences between cells recorded with either technique, so we have combined results from all cells.
12. To activate the NMDA receptor during depolarization in the test but not control input, we gave a 400- to 800-ms depolarizing current step (0.4 to 1.0 nA) 5 to 10 ms before stimulation of the test input. Before LTP induction, this was repeated either (i) once every 90 s, six to ten times or (ii) three to six times at 0.1 Hz, which was then repeated four to five times at 3- to 7-min intervals. We induced LTP by depolarizing the cell (from −20 to 0 mV) with continuous current injection and applying 20 to 30 stimuli to both pathways at 0.25 Hz. A strong induction protocol was used to ensure that the occurrence of LTP in either pathway could be measured above the baseline, which is inherently noisier when recording single-cell EPSPs rather than field EPSPs.
13. The initial pairing protocol sometimes caused increases in both the test and control EPSPs that lasted for over 10 min. Therefore, in Fig. 4B, cells (n = 13) were included only if the net change in the test pathway EPSP following the inactivation protocol was not significantly different (<10%) from any net change in the control pathway. This may have resulted in the selection of cells in which the probability of generating LTP was intrinsically less in the test input than the control input, independent of any experimental manipulation. Therefore, for Fig. 4C, we included all cells (n = 25), even those in which the initial pairing protocol caused a stable increase in the test EPSP. If the differences in Fig. 4B were due to the selection process, then the total potentiation in the two inputs (Fig. 4C) would not be significantly different.
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Chondroitin Sulfate as a Regulator of Neuronal Patterning in the Retina

PERRY A. BRITTIS*, DAVID R. CANNING, JERRY SILVER

Highly sulfated proteoglycans are correlated with axon boundaries in the developing central nervous system which suggests that these molecules affect neural pattern formation. In the developing mammalian retina, gradual regression of chondroitin sulfate may help control the onset of ganglion cell differentiation and initial direction of their axons. Changes induced by the removal of chondroitin sulfate from intact retinas in culture confirm the function of chondroitin sulfate in retinal histogenesis.

DURING THE EARLY STAGES OF VERtebrate retinal histogenesis, undifferentiated neuroepithelial cells in the eye undergo a change in cytodifferentiation predominantly toward a committed ganglion cell fate (*1, 2*). It is believed that retinal ganglion cells cease dividing when their somata are located at the ventricular surface, that those located near the optic fissure achieve maturity first, and that differentiation proceeds in a center-to-periphery sequence (*2*). At some time near the last cell division, ganglion cells project axons directly toward the optic fissure (*3, 4*). Although the relationship between morphogenesis of the optic fissure and egress of axons from the eye is known (*5, 6*), the mechanisms that control retinal ganglion cell differentiation and direct the growth of axons back toward the fissure are unknown.

Molecules that potentially promote axon growth in the retina are not distributed in a way that could impart precise directional information. Thus, molecules that are repulsive to axon growth may be instrumental in neural patterning (*7*). Structurally diverse proteoglycans are abundant in the developing central nervous system (*8–10*) and are found in the embryonic retina (*11, 12*). When sulfated proteoglycans are enriched relative to growth-promoting molecules in the same territory, a boundary is formed that inhibits advancing growth cones (*13, 14*). We have localized a chondroitin sulfate–containing proteoglycan within the embryonic rat retinal extracellular spaces that may help determine aspects of ganglion cell differentiation such as the polarity of retinal ganglion cell bodies and the initial direction of their axons.

In the rat, the retina is devoid of ganglion cells until day 12.5 of embryonic development (E12.5). At this time, the first retinal ganglion cells with axons appear just dorsal to the optic fissure. Thereafter, axons emerge from ganglion cell bodies located progressively more peripherally. This process continues until E16.5 when the vitreal (inner, facing the lens) surface of the retina is completely populated with fasciculated axons. On tissue sec-

Department of Neurosciences, Case Western Reserve University, Cleveland, OH 44106.

*To whom correspondence should be addressed.

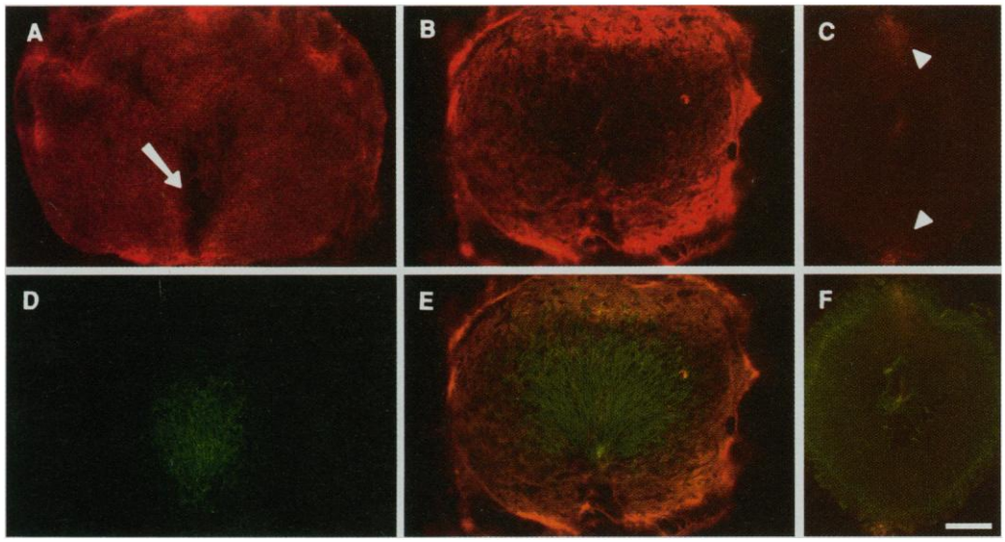

Fig. 1. Immunocytochemical localization of chondroitin sulfate by MAb CS-56 in (**A**) E13.0, (**B**) E14.5, and (**C**) E16.5 retinal whole mounts. TUJ1 localization at (**D**) E13.0, (**E**) E14.5, and (**F**) E16.5. At E13.0 (A), chondroitin sulfate staining has cleared from the center of the retina dorsal to the optic fissure (arrow). By E14.5 (B), CS-56 immunoreactivity is only present at the retinal periphery. TUJ1-positive retinal ganglion cells are present in areas of reduced CS-56 staining. By E16.5 (F), the retina is covered with mature fasciculated axons and chondroitin sulfate is restricted to the dorsal and ventral poles (arrowheads in C). F, fissure. Scale bar represents 50 μm (A and D); 100 μm (B and E); 300 μm (C and F).

tions and whole mounts we used two monoclonal antibodies (MAbs): TUJ1 (*15*) to stain neuron-specific β-tubulin of both primitive ganglion cells and older, more mature neurons with well-developed axons and MAb CS-56 to localize glycosaminoglycan chains of the chondroitin sulfate proteoglycan (*16*).

On E12.5, the chondroitin sulfate epitope was detected throughout the retina. When the retinal neuroepithelium was viewed in cross section, more chondroitin sulfate was detected in the vitreal side than in the ventricular side. In whole mounts (*17*), radial neuroepithelial cells that had begun to differentiate toward a neuronal fate expressed β-tubulin and were found in the most central portion of the retina, dorsal to the fissure (Fig. 1). At the same time of development, chondroitin sulfate in that region was markedly diminished. Axons grew in the vitreal margin, but only within the path cleared of chondroitin sulfate leading to the fissure (Fig. 1A). The pattern of neurons encircled by chondroitin sulfate became more distinct by E14.5 as the axonal population of the retina expanded (Fig. 1E). At the border of the chondroitin sulfate–positive regions, within a band ~75 μm in width encompassing the region of newly recruited neurons, TUJ1-positive retinal ganglion cell bodies lacking distinct axons could be seen positioned where there was variable chondroitin sulfate expression (Fig. 2, A and B). Based upon staining intensity, we concluded that this zone contained less chondroitin sulfate than more peripheral regions of the retina devoid of TUJ1-positive cells, and more than regions where more mature neurons containing β-tubulin and with definitive axons were found (Fig. 2A). Thus, the centrifugal distribution of chondroitin sulfate staining across this zone may be indicative of a local gradient of proteoglycan.

Chondroitin sulfate was progressively lost from the central retina (Fig. 1), so that by E16.5 chondroitin sulfate was only located at the most dorsal and ventral poles of the retinal neuroepithelium. Axons filled the retina in the characteristic spoke pattern and at this stage became highly fasciculated.

Neurons in vitro avoid areas where chondroitin sulfate has been artificially laid in their path (*13, 18*), which suggests that the in vivo distribution of chondroitin sulfate could influence differentiating retinal ganglion cells. To test this, E12.5 and E13.5 retinas were

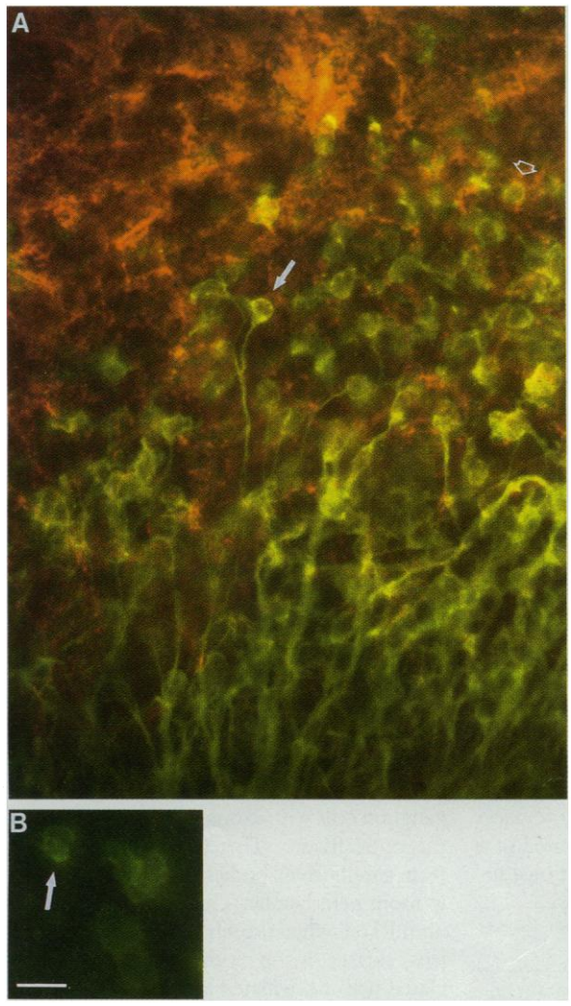

Fig. 2. The border between high and low chondroitin sulfate expression in an E14.5 retinal whole mount. (**A**) Chondroitin sulfate (orange) is abundant in the periphery of the neuroepithelium (top of photograph). Few TUJ1-positive cells populate this region. In the chondroitin sulfate gradient area, TUJ1-positive cells (green) have various configurations. TUJ1-positive retinal ganglion cells that have not sent out axons are found where chondroitin sulfate is present at intermediate amounts (open arrowhead). In areas of lower chondroitin sulfate immunoreactivity, retinal ganglion cells send axons that curve toward the optic fissure (arrow). Axons eventually merge into fascicles (lower portion of photograph). (**B**) TUJ1-immunoreactive cells in the retinal periphery found in a region similar to that marked by the open arrowhead in (A). Within the peripheral parts of the retinal neuroepithelium, TUJ1-positive neurons lacking detectible axonal processes were situated in the chondroitin sulfate matrix (arrow). Scale bar represents 30 μm (A); 10 μm (B).

treated in culture with chondroitin sulfate ABC lyase (*19*) (E.C. 4.2.2.4).

Control retinas, which had no treatment or were treated with keratanase or toxin-free endoneuraminidase, had the normal CS-56 staining pattern and did not display morphological abnormalities (Fig. 3, A and B) (*20*). Axons in control preparations consistently grew toward the fissure as in retinas taken directly from embryos of corresponding embryonic stages (*21*).

In retinas taken from E12.5 rat embryos and cultured in the presence of chondroitin sulfate ABC lyase for 48 hours, ganglion cells differentiated ectopically and their location in the retinal periphery suggested they had differentiated prematurely (Fig. 3, C and D, and Fig. 4) (*22*). Numerous TUJ1-positive ganglion cell bodies were found at the ventricular side of the retina with their axons oriented in all directions, including directly away from the fissure and toward the retinal periphery. Thus, the peripheral marginal zone can support axon growth, but normally fibers are inhibited from entering these regions at early stages. When chondroitin sulfate was removed from older retinas (E13.5) (Fig. 3E), the altered regions were shifted peripherally relative to those seen when treatment was begun at E12.5. Looping axons were found near the pupil and some axons were found on the ventricular side of the retina. The previously formed centrally located axons were unaffected by the enzyme treatment.

As the wave of chondroitin sulfate recedes from the fissure during development, it leaves at its dissipating edge TUJ1-positive cell bodies that are shaped like neuroepithelial cells that have vitreal and ventricular attachments (end feet) but lack obvious axons. These radially shaped cells express the TUJ1 epitope and therefore may have been primed toward a full commitment of the ganglion cell fate. When the chondroitin sulfate wave passes further over these primed cells, they may become fully determined to project an axonal process, while a new cohort of axonless neurons is recruited more peripherally. We suggest that retinal ganglion cell development has two generalized phases: cytodifferentiation of the neuroepithelium into radially shaped TUJ1-positive retinal ganglion cells, followed by orientation of the axons they produce. Thus, the environment could interact with a postmitotic cell, allowing it to express its neuronal phenotype in synchrony with its TUJ1-positive neighbors. Removal of the chondroitin sulfate matrix would disrupt this synchronized development.

Our experimental removal of native chondroitin sulfate from the retina would tend to enhance the role of growth-promoting molecules (*23–25*). In vitro, chondroitin sulfate proteoglycan inhibits axon initiation and elongation even in the presence of NCAM and laminin (*13*). The inhibition can be overcome by increasing the concentration of laminin or by enzymatically removing the glycosaminoglycan side chains from the core protein (*13*). The disorganized orientation of axon outgrowth in vivo, after ABC lyase treatment, probably reflected a shift in the ratio of growth-promoting to growth-inhibiting molecules.

In enzyme-treated preparations, TUJ1-positive cell bodies were found not only in the far periphery but also along the ventricular side of the retina, where they would not normally differentiate. This may be the result of cell body migration along the associated ectopic axon. Conversely, ectopic differentiation could have also affected axon directionality (*6*). We suggest that the enzyme treatment may have altered the signal from the extracellular matrix in the marginal zone to

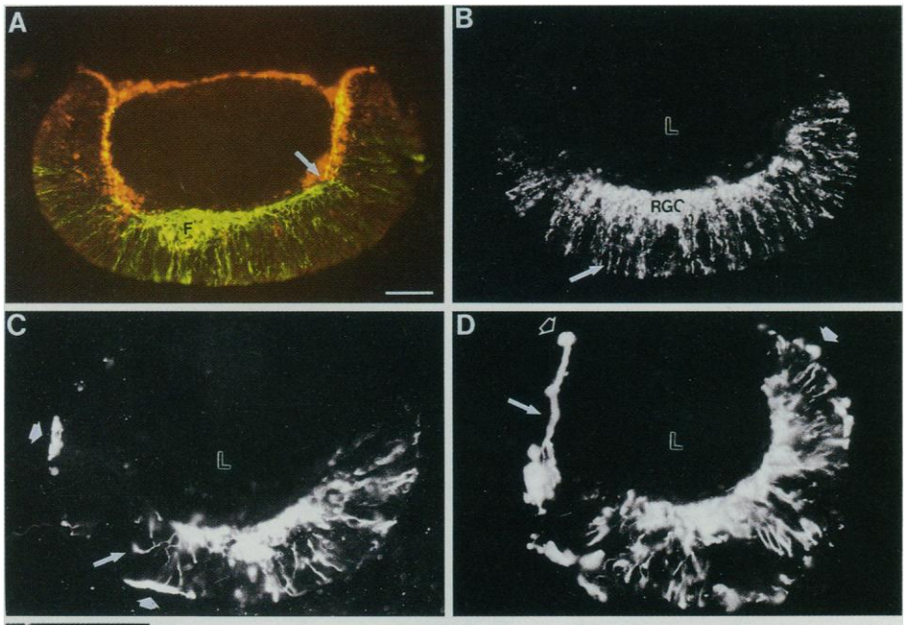

Fig. 3. Immunocytochemical localization of chondroitin sulfate and β-tubulin in cross sections of cultured retinas. (**A**) Chondroitin sulfate immunoreactivity (yellow) and TUJ1-positive cell bodies, processes, and axons (green) in a retina cultured for 48 hours beginning at E12.5. Chondroitin sulfate immunoreactivity was highest at the vitreal surface and formed a border to the expanding nerve fiber layer (arrow). (**B**) TUJ1-positive radial ganglion cells and their axons in a normal retina cultured for 48 hours beginning at E12.5. Axons were confined to the nerve fiber layer. The majority of ganglion cell bodies were situated close to the nerve fiber layer and were therefore hidden. Primitive radial ventricular processes (*26*) (arrow) still persist at this stage of development. (**C** and **D**) E12.5 retinas cultured for 48 hours in the presence of chondroitin sulfate ABC lyase. When treatment began, the retina lacked TUJ1-positive cells. After culture, many retinal ganglion cell bodies were located ectopically at the retinal periphery in the middle of the retinal neuroepithelium (D, open arrowhead) and on the ventricular surface in both the center and periphery of the retina (C and D, closed arrowheads). Axonal guidance was also severely perturbed. Some axons were directed away from the fissure and were located at incorrect depths of the retinal neuroepithelium (C and D, arrows). (**E**) Periphery of an E13.5 retina cultured for 24 hours in the presence of chondroitin ABC lyase and immunostained with TUJ1. Ectopic fibers (arrows) were identified as axons by tracing their length through successive planes of focus. F, fissure; RGC, retinal ganglion cell body layer; L, lens; VIT, vitreal surface; VENT, ventricular surface. Scale bar represents 50 μm (A to D); 20 μm (E).

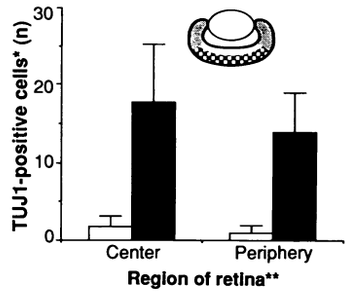

Fig. 4. The effect of chondroitin ABC lyase on the number of differentiating retinal ganglion cells in different regions of the retina not including the optic fiber layer. TUJ1-positive cell counts were taken from serial sections of five eyes cultured in chondroitin ABC lyase (treated, filled bars) and five eyes in media alone (control, open bars). *Number of cells located midway within the retinal neuroepithelium and on the ventricular surface. **As viewed in serial sagittal sections (see inset top). "Center" refers to the central arc (120°, checkered) of the retina, which corresponds to the region populated by retinal ganglion cells in controls after 48 hours in culture. "Periphery" refers to the regions (shaded, dorsal plus ventral retina) not included in the central arc.

the neuroepithelial end feet, which in turn would signal neuronal precursor cells to polarize and differentiate in the wrong place or time. The attachment of both vitreal and ventricular end feet and the correct timing of their detachment are believed to be critical to retinal ganglion cell differentiation, resulting in the localization of the cell body at the vitreal surface (*2, 26*). Thus, chondroitin sulfate, perhaps along with other glycosaminoglycans, may be a key regulatory factor in these phenomena.

Our results suggest that the graded front of chondroitin sulfate that recedes centrifugally across the retina, perhaps in combination with bound tropic and trophic factors (*27*), allows retinal ganglion cells to differentiate sequentially and polarize their cell bodies and axons in their proper orientation.

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16. MAb CS-56 is specific for the GAG portion of native chondroitin sulfate proteoglycan and binds to both the 4- and 6-sulfated moieties.
17. Embryonic eyes were dissected and extraocular tissues removed. Retinas were mounted vitreal side up on sartorius filters, fixed with 4% NBF, permeabilized with 0.3% Triton X-100 and incubated with CS-56 or TUJ1 overnight at 4°C. For double labeling, primary and secondary antibodies were added sequentially. Whole mounts were incubated in goat antibody to immunoglobulins M and G (gamma chain–specific).
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19. Chondroitin ABC lyase purified from *Proteus vulgaris* catalyzes the eliminative cleavage of *N*-acetylhexosaminide linkages in chondroitin 4- and 6-sulfate, yields mainly disaccharides with Δ4-hexuronate at the non-reducing ends, does not act on keratan sulfate, heparin, and heparan sulfate, and leaves the core protein intact.
20. Control retinas were grown in media with no chondroitinase (n = 30). In addition, retinas were cultured in the presence of keratanase (Sigma, 0.5 to 1.0 U/ml), which did not remove CS-56 immunostaining (n = 15). Retinas were also cultured in the presence of toxin-free phage endoneuraminidase (n = 10) that specifically cleaves alpha-2, 8-linked polysialic acid from NCAM. These experiments with endoneuraminidase indicated that the removal of sugars unrelated to the glycosaminoglycan family does not give comparable results as seen following glycosaminoglycan removal. A separate group of retinas (n = 10) after ABC lyase treatment stained positive with MAb HNK-1, suggesting that the enzyme did not remove all carbohydrate side chains.
21. Rat eyes were taken at E12.5 and E13.5. Retinas along with intact lens and vitreous were cultured in DMEF-12 supplemented with 10% FCS in 24-well dishes at 37°C in a humidified environment with 5% CO_2 for 24 or 48 hours. The media used for enzyme perturbations included chondroitin ABC lyase (ICN, 1 U/ml). In some perturbation experiments a broad spectrum protease inhibitor was added (2-Makroglobulin, ICN, 1 mg/ml). Under these conditions, retinas remained healthy: no differences in numbers of pycnotic nuclei visualized in serial 1-μm plastic sections were observed between control and treated retinas. In preparations that were cultured for 48 hours with chondroitin ABC lyase, chondroitin sulfate could not be detected with CS-56 immunostaining.
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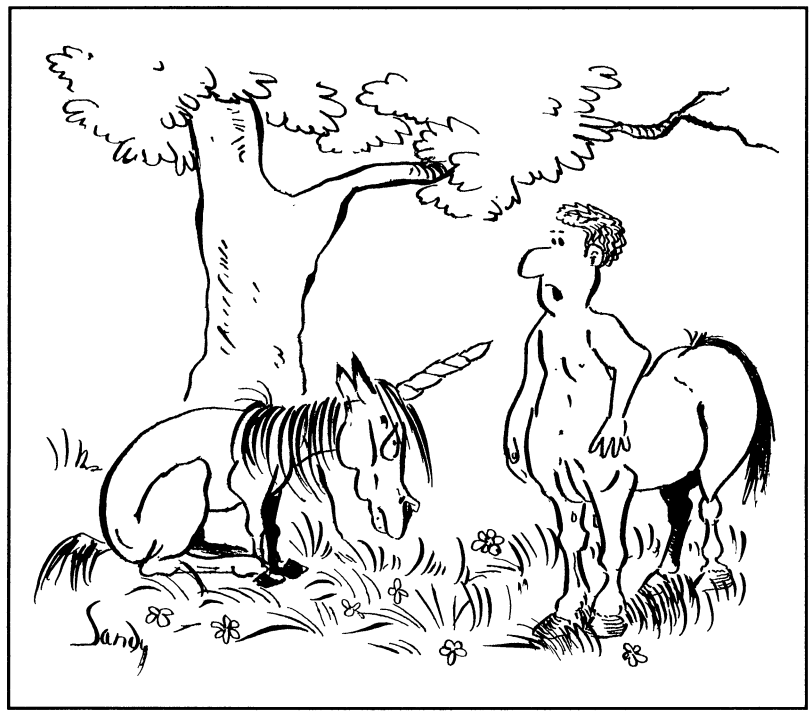

"Frankly, I'd rather be mythical than extinct."