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Proteoglycans as Cues for Axonal Guidance in Formation of Retinotectal or Retinocollicular Projections

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Abstract

Understanding the formation of neuronal circuits has long been one of the basic problems in developmental neurobiology. Projections from the retina to their higher center, the optic tectum in nonmammalian vertebrates and the superior colliculus in mammals, are most amenable to experimental approaches; thus, much information has been accumulated about the mechanisms of axonal guidance. The retinal axons navigate along the appropriate pathway with the help of a series of guidance cues. Although much of the work has focused on proteinaceous factors, proteoglycans have been identified as playing important roles in retinal axon guidance. Chondroitin sulfate proteoglycans and heparan sulfate proteoglycans are involved in essential decisions of axon steering along the pathway. However, it has not been determined whether diversity of the carbohydrate chains results in differential effects and how their diversity is recognized by growth cones, which represent an important area of future research.

Index Entries: Axon; growth cone; guidance; retina; retinal ganglion cell; retinotectal; retinocollicular; proteoglycan; glycosaminoglycan; chondroitin sulfate; heparan sulfate.

Introduction

In the nervous system, neurons extend their axons through precise and stereotyped pathways in an environment with a variety of cells and their extracellular matrices; finally, the axons navigate and make synapses with their targets. The formation of neuronal circuits has long been one of the basic problems because it constitutes the structural basis for higher brain functions *(1,2)*. Recent findings show that a series of guidance cues help axons navigate along the appropriate pathway. In general, guidance mechanisms are classified into four groups: chemoattraction, chemorepulsion, contact attraction, and contact repulsion. Most of the known guidance cues are proteinaceous factors identified using genetic and molecular biological approaches. The receptors for these proteinaceous factors are also being examined

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Fig. 1. Trajectories of the RGC axons labeled with the axonal tracer HRP in a lateral view of the embryonic day 7 (E7) chick brain **(A),** and with the DiI in a lateral view of the stage 40 *Xenopus* brain **(B).** After forming the optic chiasm at the ventral midline, the axons run on the pial surface of the diencephalon dorsocaudally toward the dorsal mesencephalon, optic tectum.

Schematic drawing of the axonal pathway in a vertebrate brain model from the retina to the optic chiasm in a frontal view **(C),** and from the optic chiasm to the optic tectum in a lateral view **(D).** Numbers of retinal axons uncrossed depend on the species. The following are decision points of the axons along the pathway: initial elongation to the central retina (i), exiting the eye at the optic nerve head (ii), running on the optic stalk to form the optic nerve (iii), deciding whether to cross or not at the optic chiasm (iv), delimiting the anterior border of the trajectory at the diencephalotelencephalic boundary (v), turning caudally on the diencephalon (vi), targeting to the diencephalic nuclei (vii), targeting to the dorsal mesencephalon (viii), the neurotrophic effects of the dorsal mesencephalon (ix), and the dorsal midline barrier between the dorsal mesencephalons (x). Chiasm, the optic chiasm; Di, the diencephalon; dorsal Mes, the dorsal mesencephalon; Tectum, the optic tectum; Tel, the telencephalon.

Schematic drawing of the pathway defects by perturbing function of the intrinsic GAGs, depicted on the brain model in the frontal **(E)** and lateral **(F)** views: (i) random orientation of the axons, (iv) decreasing the uncrossed axons, (v) aberrant invasion into the telencephalon, (vi) failure in the growth on the diencephalon, (vii) aberrant invasion into the nonretinorecipient diencephalic nuclei, and (viii) bifurcation of the trajectory at the diencephalomesencephalic boundary.

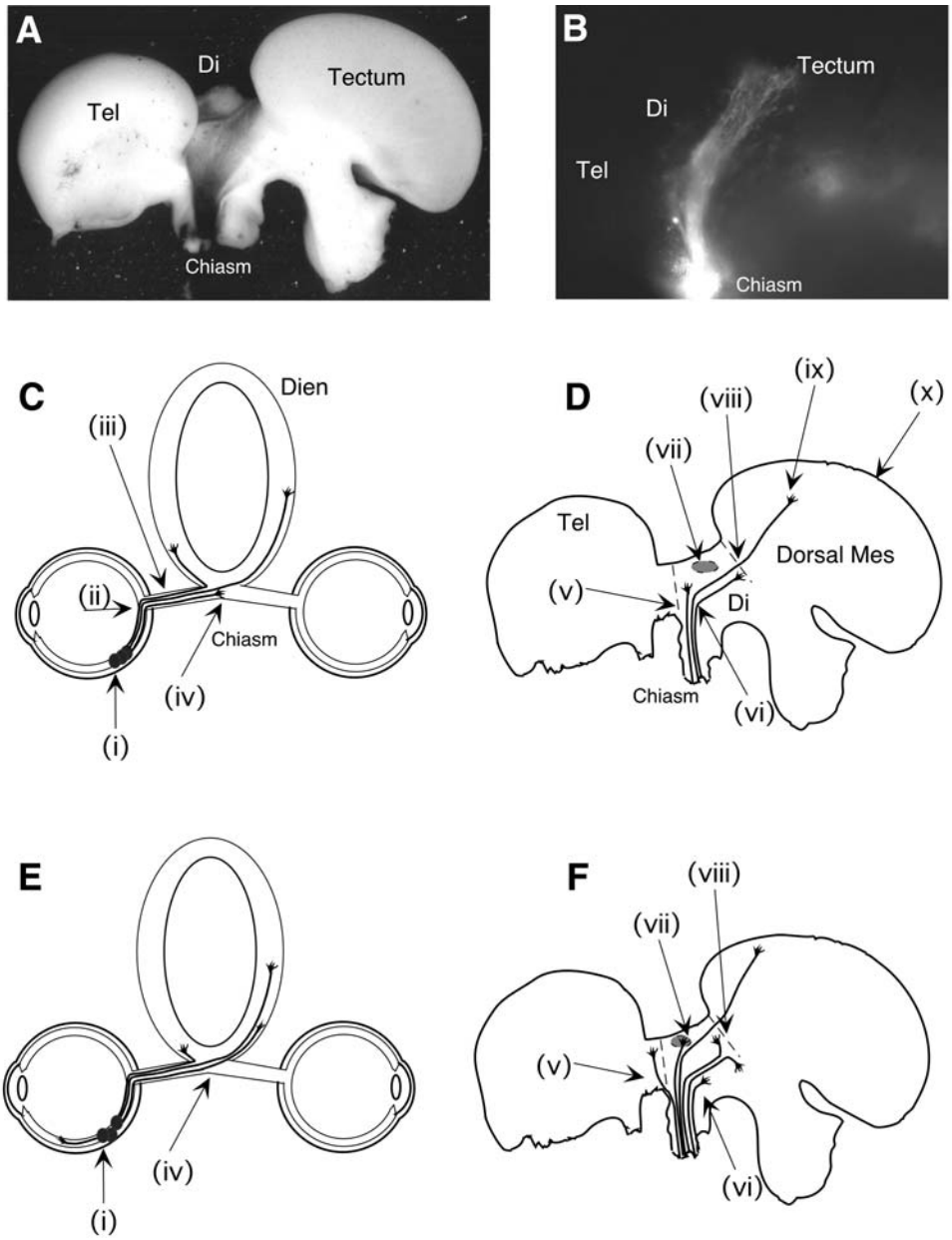
in detail. The effects of these guidance cues are not rigid but are regulated by relevant factors and by modulation of intracellular signals *(3).* Moreover, it has also been found that the effects of the guidance cues are regulated by lateral interactions between ligands and their receptors on an axon *(4,5).*

Among the various experimental systems, the projections from the retina to their higher center, the optic tectum in nonmammalian vertebrates and the superior colliculus in the mammals, are the most amenable to experimental approaches *(6–14).* Axons of retinal ganglion cells (RGCs) grow in the nerve-fiber layer of the retina centrally toward the optic nerve head or optic fissure. They exit the eye through the optic fissure to form the optic nerve *(15).* They run toward the ventral midline of the diencephalon to form the optic chiasm, in which the axons decide whether to cross the midline. Subsequently, the retinal axons run on the pial surface of the diencephalon dorsocaudally. They do not invade the telencephalon anteriorly, the epithalamus dorsally, or the hypothalamus ventrally at this stage of development, but run on the middle part of the diencephalon, forming the optic tract. They arrive at the boundary between the diencephalon and the dorsal mesencephalon (the optic tectum in nonmammalian vertebrates or the superior colliculus in mammals), and target the dorsal mesencephalon. One of the most fruitful achievements of retinotectal research has been the discovery of ephrins and their Eph receptors, which are involved in the formation of the retinotectal topographic map *(16,17).*

Not only proteinaceous factors but also more complex factors—composites of a protein core and carbohydrate chains, proteoglycans—play roles in the formation of neuronal circuits *(18).* However, much remains unknown about the functions of the proteoglycans, especially their carbohydrate chains, in the formation of the neuronal circuits because their carbohydrate chains are structurally heterogeneous, their analysis is difficult, and their artificial synthesis is impossible so far. Here I review the effects of the proteoglycans and their carbohydrate chains on the guidance of the retinal axons along the pathway.

Initial Outgrowth of RGC Axons to Retinal Center

Being generated in the ventricular zone of neuroepithelium, the RGCs extend axons



toward intermediate targets. RGC axons always grow toward the retinal center to the optic nerve head, never toward the retinal periphery to the ciliary margin or the lens (Fig. 1C), suggesting the existence of mechanisms guiding the axons to the center.

Snow et al. showed that in the rat and chick the monoclonal antibody CS56 recognizing carbohydrate chains of chondroitin sulfate proteoglycans (chondroitin sulfates, CSs) labels a decreasing gradient from the peripheral to the central region of the retina *(19)*. The RGC axons did not invade a territory coated with chondroitin sulfate proteoglycans (CSPGs) in vitro, and the inhibitory effect was abolished by treatment with chondroitinase ABC. Furthermore,

when endogenous CSs were removed from the retinal whole-mount culture by treatment with chondroitinase ABC, the axons were not directed to the central retina, but grew in random orientations *(20).* These results suggest that CSs play roles in regulating the direction of axonal growth to the central retina; the axons do not elongate to the peripheral retina where more CSs are distributed, but elongate to the central retina where fewer CSs are distributed.

Although the CSs play roles in regulating the directed growth of the retinal axons to the central retina, the regulation might be indirect because not only the directionality of the axonal growth but also the cellular polarity of the RGCs were affected by the treatment with chondroitinase ABC; the RGCs did not migrate to the vitreous surface of the retina but stayed on the ventricular side. In addition, the application of soluble CSs affected the histogenesis in retinal organ cultures and induced random orientation of the axonal growth of RGCs *(21).*

Ohta et al. found that lens epithelial cells secrete a repulsive factor that is distinct from CSs. This lens repulsive factor is likely to initially direct the retinal axons to the central retina by its repulsive effect through the ciliary margin of the retina from the lens. In some experimental preparations of the retinal whole-mount cultures *(19,20),* the lens was removed; thus, one is not able to determine whether the random axonal growth was attributed to the removal of CSs or to the removal of the lens factor *(22).*

RGC Axons Growing on Mueller Glial Endfeet in Retinal Basal Lamina

Tyrosine phosphorylation is one of the key events in the growth cone guidance, and is regulated by receptor tyrosine kinases and protein tyrosine phosphatases. Chick protein tyrosine phosphatase σ (cPTP σ, originally called CPYP α), is distributed on the RGC axons. To examine the roles of cPTP σ, Mueller et al. produced their receptor-AP fusion proteins *(23).* Addition of cPTP σ-AP or antibody against cPTP σ disturbed interactions between cPTP σ on the axons and its intrinsic ligands on the retinal basal lamina in the culture; the RGCs growth cones lost lamellipodia, and their advancement was inhibited.

Aricescu et al. identified candidates for cPTP σ ligands in vitro: heparan sulfate proteoglycans (HSPGs), agrin, and collagen type XVII, in the retinal basal lamina *(24).* The binding of cPTP σ to agrin and collagen type XVIII was abolished by treatment with heparitinase, which suggests that carbohydrate chains of HSPGs (heparan sulfates, HSs) are responsible for the binding. They further demonstrated by site-directed mutagenesis that the HSs bind the Ig-1 domain in the cPTP σ. cPTP σ recognizes HSPGs on the Mueller glial endfeet in the retinal basal lamina, and is likely to be involved in the intraretinal pathfinding of the RGC axons.

The binding of cPTP σ-AP indicates that their ligands were not only on the Mueller glial endfeet in the retinal basal membrane, but also on the RGC axons themselves *(23).* The coexistence of cPTP σ and its ligands on the axons is thought to function in regulating the sensitivity of the growth cones to the ligands, as has been shown for Eph receptors and ephrins *(4,5).*

Formation of Intraretinal Circuits

RGCs receive input from the bipolar cells and amacrine cells on their dendrites in the inner plexiform layer, and extend their axons in the optic fiber layer for their output. Inatani et al. showed that neurite outgrowth of the RGCs is inhibited by neurocan and phosphacan, major proteoglycans in the nervous system; their inhibitory effects were mainly attributed to their protein cores *(25).* They were highly expressed in the neuropil of the rat retina from postnatal day 7 to during the late stage of intraretinal circuit formation. This suggests that they terminate the circuit formation by inhibiting the neurite outgrowth *(26).*

RGC Axons at Optic Fissure and in the Optic Nerve

The direction of the RGC axons changes at the optic nerve head; these axons exit the eyeball *(15)* and subsequently run on the optic stalk to form the optic nerve (Fig. 1C). Henke-Fahre et al. identified a soluble proteoglycan, Te38 antigen, (370 kDa core protein with CS and keratan sulfate [KS] side chains) from the chick brain. Te38 was distributed in the optic fissure, in the dorsal part of the optic nerve where there were neuroepithelial cells but no axons, and in the nonneuronal cells surrounding the axons at the optic chiasm *(27).* It inhibited the axonal outgrowth of RGCs in vitro; this inhibitory effect was attributable mainly to the protein core or KS. The results indicate that Te38 antigen defines territories for axonal growth in the optic nerve and the chiasm.

RGC Axons at the Optic Chiasm

Passing through the optic nerve, the RGC axons approach the ventral midline of the diencephalon to form the optic chiasm (Fig. 1C,D). It has been reported that various factors are involved in the formation of the optic chiasm, for example, Ephrin-As, Ephrin-Bs, Netrin-1, and CSPGs *(28–32).* Chung et al. showed the distribution of CSs around the optic chiasm during the pathfinding stage in mice *(33);* they established slice cultures for the eye and the ventral diencephalon, and examined the axonal trajectory around the chiasm after the removal of intrinsic CSs with chondroitinase ABC *(34).* In the slices without the intrinsic CSs, the axons approached the diencephalic midline through an aberrant anterior path, forming a broad bundle of axons that crossed the midline in the early stage of chiasm formation. They did not transiently project to the ipsilateral side in the middle stage; subsequently, the number of ipsilaterally projecting axons was significantly decreased in the late stage. In contrast, in the normal embryos and in the slices with the intrinsic CSs, a number of axons turned ipsilaterally to form the transient uncrossed projection in the middle stage; subsequently, the axons arising from the ventrotemporal retina permanently projected ipsilaterally in the late stage.

Similar aberrant pathfinding has also been observed in *slit-1* and *-2* double-mutant mice *(35).* Slits are expressed in complementary domains surrounding the retinal pathway, and they have a repulsive influence on outgrowth of the retinal axons in vitro; thus, they are likely to form a corridor for the retinal trajectory. The similarity between the phenotype caused by removal of the CSs and that of the double mutants of *slits* suggests that Slits are involved in the common context of regulation for the retinal pathfinding in the chiasm, which raises the question of whether they interact in order to increase the efficiency of the pathfinding, for example, whether Slits bind to CSs. In addition, the expression of HSPGs occurred around the optic chiasm, although the roles of HSPGs are unknown *(36).*

Effects of CSPGs on RGC Axons in the Optic Tract

RGC axons run dorsocaudally in the middle part of the diencephalon to form the optic tract *(37–42).* On their way to the dorsal mesencephalon, the axons run on the diencephalotelencephalic boundary, but they never invade the telencephalon anteriorly (Fig. 1A,B,D). In several zebrafish mutants (for example, *bal, gup, sly, cyc,* and *ast*), RGC axons turn anteriorly and aberrantly invade the telencephalon *(43).* In addition, at the border between the telencephalon and diencephalon, there is a region with a dense cluster of non-neuronal cells, which may function as a barrier that prevents the axons from invading the telencephalon *(44).* These facts imply that there are mechanisms that prevent the retinal axons from aberrantly invading the telencephalon.

The effects of the telencephalic cells on the formation of the retinal pathway were examined in the chick with a co-culture model

(45,46); the axonal outgrowth of RGCs was selectively inhibited by the telencephalic cells. The responsible factor was found in the fraction of peripheral membrane molecules of the telencephalon. Because the inhibitory effect was destroyed by chondroitinase ABC but not by heat treatment, the inhibition was attributed to the carbohydrate chains of CSPG secreted by the telencephalic cells. The function of the telencephalic CSPGs was further examined by treatment with chondroitinase ABC *in ovo;* the enzyme solution was injected into a lateral ventricle of the chick embryos, and then it diffused into the brain wall to degrade the intrinsic CSs in the telencephalon, diencephalon, and tectum. Transient removal of the CSs resulted in anterior enlargement of the optic tract; thus, the retinal axons are released from the outgrowth inhibition by the telencephalic CSPGs, and are allowed to enter a foreign territory. This suggests that the telencephalic cells prevent the retinal axons from aberrantly invading the anterior territory via their CSPGs.

Similar effects of CSs were reported in *Xenopus* by Walz et al. *(47).* They established a method for exposed brain preparations in which a tadpole without the epidermis over its diencephalon is kept in a chamber, and they examined the effects of exogenous CSs on the retinal trajectory. The application of CSs increased the complexity of the growth cones, and induced intermittent stalling thereof, causing aberrant extension of the retinal axons into the forebrain; this indicated that CSs are involved in the guidance of the retinal axons on the diencephalon, delimiting the anterior border of the optic tract.

In addition, RGC axons exhibit various abnormal wiring, including anterior invasion to the telencephalon in the zebrafish mutant, *ast.* Its responsible gene is shown to be *robo2,* Roundabout family of axon guidance receptors, suggesting that Robo2 on the RGC axons recognizes Slit family of axon guidance cues along the visual pathway *(48).* However, it is presently unknown whether Robo2, Slits, and CSs interact with one another.

Effects of HSPGs on RGC Axons in the Optic Tract

After passing the diencephalotelencephalic boundary in the optic tract, the RGC axons turn caudally toward the dorsal mesencephalon (Fig. 1A,B,D). Fibroblast growth factor 2 (FGF2) is expressed on the pial surface of the diencephalon in the presumptive region of the optic tract in *Xenopus,* but at low levels on the tectum; FGF receptors (FGFR) are localized on the retinal growth cones. These facts suggest the involvement of FGF2 in the formation of the pathway of the optic tract. The involvement of FGF and FGFR signaling was examined by transfecting the dominant-negative form of FGFR into the RGCs. It inhibited the elongation of the retinal axons on the optic tract, indicating that the axons recognize the presumptive region of the optic tract through the FGF-FGFR signaling pathway *(49).*

Holt and colleagues examined the effects of exogenous application of FGF2 on the exposed brain preparation *(50,51).* In the treated tadpoles, the retinal trajectory bifurcated dorsocaudally at the diencephalomesencephalic boundary and the axons did not enter the tectum. These results suggest that the axons perceive the tectum as their target because of the low level of FGF2, and that in the treated tadpoles, the higher concentration of the applied FGF2 prevented the axons from recognizing the tectum as their target. Therefore, signaling through FGFR regulates not only the pathfinding in the optic tract but also the target recognition on the tectum.

Because HSPGs and their carbohydrate side chains (HSs) are known to be co-receptors for FGFs-FGFR interaction and are colocalized in the presumptive region of the optic tract, HSs were applied to the exposed brain; heparin and FGF2-binding HS induced the bifurcation of the retinal trajectory at the diencephalomesencephalic boundary, but an FGF2-nonbinding HS fragment did not. In addition, early and late removal of endogenous HSs by heparitinase induced failure of the axonal elongation in the optic tract and the bifurcation of the trajectory,

respectively *(52).* The bifurcation of the retinal trajectory was, furthermore, induced by chlorate treatment, which inhibits proteoglycan sulfation, and by chemically modified heparins, which indicates the importance of 2-o- and 6-o-sulfate groups in HSs longer than decasaccharides *(53).* Although the *N*-sulfate group in HSs is essential for the binding between HSs and FGF2, it was not essential for retinal pathfinding. The application of HSs or FGF2 was shown to induce a similar but significantly different bifurcation of the retinal trajectory; therefore, it also seemed unlikely that HSs mediate the FGF2 signal. This series of experiments indicated that FGF2, FGFR, and HSs are involved in the common context of the guidance mechanism for the formation of the optic tract and the target recognition of the optic tectum.

As discussed above, HSPGs are likely to be involved in the intraretinal pathfinding of the RGC axons via interactions with cPTP σ; thus, one intriguing question is whether cPTP σ functions in the formation of the *Xenopus* optic tract.

Projection of RGC Axons to Diencephalic Nuclei

Because neurons and glial cells are continuously generated in the ciliary margin of the retina in the zebrafish, pathfinding of the RGC axons continues throughout its life; therefore the guidance cues are constitutively expressed in the adult, which may also allow regeneration of RGC axons after injury in the adult. Along the optic tract, RGC axons project to particular nuclei on the diencephalons, but do not invade nonretinorecipient nuclei. During their regeneration, the axons do not invade the nonretinorecipient nuclei, in which the CSs are densely distributed. The axons do not invade a territory coated with CSs in vitro, and treatment with chondroitinase ABC during axonal regeneration induces aberrant invasion of the axons into the nonretinorecipient nuclei in vivo, indicating that CSs contribute to target recognition in the diencephalic nuclei by preventing the axons from invading nonretinorecipient regions. Furthermore, it has been suggested that the CSs function not only during regeneration but also during development because the CSs in the developing diencephalon are distributed in comparable regions to the nonretinorecipient nuclei *(54).*

Trophic Effects of CSPGs in the Superior Colliculus on RGCs

The RGC axons target the dorsal mesencephalon, in which the axons are retrogradely influenced by neurotrophic factors. The axons compete to acquire a limited amount of neurotrophic factors for their survival; consequently, a massive loss of the RGCs takes place. Schulz et al. purified CSPG with a molecular weight of 480 kDa from the rat superior colliculus, and showed that it promotes survival of the RGCs in culture *(55,56).* Its intraocular injection inhibits loss of the RGCs in early postnatal development *(57),* and rescues axonotomized RGCs in the adult *(58),* suggesting that the 480-kDa CSPG is one of the target-derived neurotrophic factors in the retinocollicular projection.

Effects of CSPGs as a Midline Barrier in Dorsal Mesencephalon

Because the radial glial cells in the dorsal midline show intense immunoreactivity with the CS56 antibody, it has been suggested that they form a physical barrier that prevents the RGC axons from invading the contralateral side (Fig. 1D) *(59).* Hoffman-Kim et al. *(60)* measured the amounts of CSPG core proteins in the dorsal midline or lateral region of the tectum and found no evidence for differential expression of the core proteins. On the other hand, metabolic labeling showed an increase of the synthesis of sulfated macromolecules. These results indicate that the intense immunoreactivity in the dorsal midline is caused by a higher rate of synthesis or sulfation of carbohydrate chains; for example, an increased number of carbohydrate

chains per core, longer carbohydrate chains, a higher amount of sulfation per carbohydrate chain, or higher levels of substrates for glycosaminoglycan biosynthesis, rather than the production of particular proteoglycans.

RGC Axons Sensing CSPGs as Guidance Cues

CSPGs were at one time regarded as repellents or inhibitors of neurite outgrowth. However, in the preceding sections I have reviewed that the various effects of proteoglycans on RGC axons. CSPGs promote the survival of RGCs; thus, they regulate axon wiring both negatively and positively. Similar duality of the CSPGs on cortical neurons was also observed. 6B4 proteoglycan/phosphacan inhibited adhesion to the culture substrate; on the other hand, it promoted the differentiation of dendrites *(61).*

The functional diversity of CSPGs is caused by their structural heterogeneity; it depends not only on their protein cores but also on their carbohydrate side chains, CSs. The CSs consist of repeating units of disaccharides: *N*-acetylgalactosamine and glucuronic acid. Different types of CSs are distinguished by the patterns of sulfation of the unit *(62,63).* For example, during development of the chick brain or the rat retina, the synthesis of CSs is developmentally regulated *(64,65).*

Although the CSs are widely distributed in the embryonic brain, their removal causes restricted effects on the retinal trajectory at the diencephalotelencephalic boundary in vivo, which suggests that specific types of CSs function in the pathfinding. It should be further examined how various types of CSs are spatially distributed in the embryonic brain, and whether they operate differentially in the retinal pathfinding *(46).* The functional specificity of the diverse structures of the CSs is a further problem to be solved, although there may be methodological obstacles in the way of approaching the problem. Walz et al. have attempted to examine the effects of the CSs' sulfation on the retinal pathfinding *(47).* In addition to their studies of the retinotectal pathway, Tanaka et al. have examined the effects of type-enriched CS in organotypic cultures of developing cerebellum *(66).* In contrast to the CSs, the structures of the HSs are not well characterized; however, Irie et al. have demonstrated effects of the HSs sulfation patterns on retinal pathfinding *(53).*

CSs, moreover, exhibit differential effects on different types of cells, for example, on cortical versus thalamic neurons and on retinal versus DRG axons *(46,61);* it is not known whether neurons recognize the carbohydrate chains directly or indirectly via the proteinaceous factor. Concerning intracellular signaling, it has been shown that Rho and its effector, ROCK, mediate an inhibitory effect of a mixture of CSPGs *(67).*

Conclusions

RGC axons navigate along the appropriate pathway using a series of guidance cues. Proteoglycans and their carbohydrate chains function in axon guidance at various decision points; however, we still do not know whether each of them participates directly or indirectly with proteinaceous factors. In addition, it has not been determined whether diversity of the carbohydrate chains results in differential effects on the steering of the axons and how their diversity is recognized *(68)*. These questions should be addressed experimentally in the near future.

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