Roles of the Telencephalic Cells and their Chondroitin Sulfate Proteoglycans in Delimiting an Anterior Border of the Retinal Pathway

Hiroyuki Ichijo and Izumi Kawabata

Department of Anatomy, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

The Journal of Neuroscience, December 1, 2001, 21(23):9304–9314

The axons of the retinal ganglion cells run on the diencephalotelencephalic boundary on their way to the tectum; however, they do not invade the telencephalon anteriorly. To investigate the mechanisms that prevent the retinal axons from entering the telencephalic territory, the effects of the telencephalic cells were examined on the outgrowth of the retinal axons in vitro; the retinal outgrowth was selectively inhibited by the cellular substrate derived from the telencephalon. The responsible factor for the selective inhibition was, furthermore, found in the telencephalic membranes and the fraction of peripheral membrane molecules from the telencephalon. Because the inhibitory effect was destroyed by chondroitinase ABC but not by heat, this inhibition was attributable to the carbohydrate chains of chondroitin sulfate proteoglycans (CSPGs) adhering to the membranes of the telencephalic cells. To understand the function of the telencephalic CSPGs on the retinal pathfinding in vivo, their carbohydrate chains [chondroitin sulfate glycosaminoglycan (CS-GAG)] were removed from the embryonic brains by intraventricular injection of chondroitinase ABC; the removal of CS-GAG resulted in an anterior enlargement of the optic tract. The results indicate that the telencephalic cells delimit the anterior border of the optic tract with their CSPGs and prevent the retinal axons from aberrantly entering the anterior territory.

Key words: axon guidance; pathfinding; retinotectal projection; optic tract; retinal ganglion cell; chondroitin sulfate proteoglycan; telencephalon

Neurons extend their axons through a precise and stereotyped pathway and project to their target during development. The formation of neuronal circuits is one of the important problems in developmental neurobiology, which is the structural basis of brain functions (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Retinotectal projections have long served as a good model for the formation of neuronal circuits (Holt and Harris, 1993; Dingwell et al., 2000; Mey and Thanos, 2000).

It is shown that sequential presentation of guidance cues directs the retinal axons to their final target (Carlstrom et al., 1996; Trowe et al., 1996). Retinal ganglion cells (RGCs) extend their axons to the center of retina; lens epithelial cells secrete a repulsive factor and are likely to initially direct the retinal axons to the central retina (Ohta et al., 1999). Furthermore, because chondroitin sulfate proteoglycans (CSPGs) are expressed in a decreasing gradient from the retinal periphery to its center and inhibit the growth of the retinal axons, they are also likely to direct the axonal growth to the central retina (Snow et al., 1991; Britts et al., 1992). The retinal axons turn their direction at the optic nerve head and exit the eyeball; their turning is thought to be induced by Netrin-1 (Deiner et al., 1997). The retinal axons form the optic chiasm at the ventral midline of the diencephalon (Guillery et al., 1995; Mason and Sretavan, 1997); it has been reported that Ephrin-As, Ephrin-Bs, Netrin-1, Slit-2, and CSPGs are involved in the formation of the optic chiasm (Deiner and Sretavan, 1999; Dutting et al., 1999; Chung et al., 2000ab; Erskine et al., 2000; Marcus et al., 2000; Nakagawa et al., 2000; Niclou et al., 2000). The retinal axons run dorsocaudally in the middle part of the diencephalon; they are guided by an early-generated axonal scaffold, the tract of the postoptic commissure (Wilson et al., 1990; Taylor, 1991; Easter et al., 1993; Chedotal et al., 1995; Mastick and Easter, 1996; Anderson and Key, 1999). In addition, the carbohydrate chains of heparan sulfate proteoglycans regulate the guidance of the retinal axon in the diencephalon through their binding to fibroblast growth factors (Walz et al., 1997). In contrast, the retinal axons do not invade the dorsal and ventral diencephalon during early development, in which Slit-2 is expressed. Because Slit-2 repels the retinal axons in vitro, this suggests that Slit-2 prevents the retinal axons from invading the dorsal and ventral diencephalon (Tuttle et al., 1998; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000).

On their way to the tectum, the retinal axons run on the diencephalotelencephalic boundary and cross over the suprapptic tract (SOT), which is another axonal scaffold between the telencephalon and diencephalon, but they do not invade the telencephalon anteriorly (see Fig. 1). Among the zebrafish mutants, five mutants (bal, gup, sly, cyc, and ast) show retinal axons that turn anteriorly and aberrantly invade the telencephalon (Karlstrom et al., 1996). 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 retinal axons from invading the telencephalon (Silver et al., 1987). These observations indicate the mechanisms that prevent the retinal axons from aberrantly invading the telencephalon; however, their cellular and molecular bases have not been elucidated.
To understand the mechanisms that prevent the retinal axons from invading the telencephalon, the effects of telencephalic cells were examined on outgrowth of the retinal axons in vitro with a coculture method devised previously, the *ryomen* assay (Ichijo and Bonhoeffer, 1998). Here we show in the chick embryo that (1) the retinal outgrowth was selectively inhibited by the telencephalic cells, (2) CSPGs adhering to the telencephalic membranes selectively inhibited the outgrowth of the retinal axons in vitro via their carbohydrate chains [chondroitin sulfate glycosaminoglycans (CS-GAGs)], and (3) removal of the CS-GAGs by enzymatic treatment induced an anterior enlargement of the optic tract toward the telencephalon in vivo. The results indicate that the telencephalic cells delimit the anterior border of the optic tract by their CSPGs.

**MATERIALS AND METHODS**

The *ryomen* chamber assay. The coculture assay, the *ryomen* chamber assay, was developed by Ichijo and Bonhoeffer (1998) to inspect interactions between the RGC axons and the processes of their target cells, mediated through either contact or diffusion; this assay was applied to investigating the pathfinding of retinal axons (Fig. 1). “*Ryomen*” is the Japanese term for “double-sided.” The chamber consisted of a pair of stainless steel rings holding the Nuclepore filter. B. An axon of a retinal ganglion cell grows on the processes of telencephalic cells that have penetrated the filter. C. A scanning electron micrograph shows the telencephalic substrate on the retinal side of the filter. The telencephalic substrates consist of cellular processes that are positive for MAP2 (D), vimentin (E), and G4/NgCAM (F). Scale bars, 25 μm.
a trypsin-EDTA solution (Sigma, St. Louis, MO) for 10 min at 37°C. Trypsinization was stopped by adding fetal bovine serum. Cell suspensions were prepared by trituration and washed twice in an F-12 culture medium consisting of an F-12 nutrient mixture, 10% fetal bovine serum, 2% chick serum, 2 mM glutamine, and penicillin-streptomycin (all reagents in the F-12 culture medium were from Life Technologies, Rockville, MD). Five hundred microliters of the cell suspensions (1 × 10^6 cells/ml) were cultured on the cellular side of the filter overnight at 37.5°C in 4% CO_2. The next day, the cultured media were removed; then, 25 μl of Matrigel was added to the tectal side. After incubation for 30 min at 37.5°C in 4% CO_2, the chambers were turned upside down to make the other side, the retinal side, of the filter available. The recovered media were centrifuged to remove cellular debris and mixed with the same volume of fresh F-12 culture medium.

Neutral retinas from E6 or E7 chickens were prepared (Halfter et al., 1983). The retina was chopped perpendicular to the optic fissure with a tissue chopper (The Mickle Laboratory Engineering, Gomshall, UK) to make 0.3-mm-wide retinal strips. The explants (0.3 × 0.6 mm) were obtained from the central part of the retina for the experiments. Dorsal root ganglia (DRGs) were also prepared from E7 chickens. The retinal and DRG explants were cultured for 24 hr in 300 μl of the F-12 minimum medium with the peripheral membrane molecules (0.5 mg/ml protein) for separate peripheral membrane molecules from the integral ones. The membranes were phase-partitioned with Triton X-114 (Bursa and Rodolf, 1994; Nomura et al., 1998). The membranes were dissolved in 2% Triton X-114 (Sigma) in the homogenization buffer (1 ml/mg protein) and extracted overnight at 4°C. The solutions were incubated at 37°C for 10 min and centrifuged at 16,000 × g for 10 min at 25°C. After centrifugation, peripheral and integral membrane molecules were concentrated in an upper aqueous and a lower detergent phase, respectively. The upper aqueous phases were recovered and treated with 1% β-mercaptoethanol. The retinal and DRG explants were cultured for 24 hr in 300 μl of the F-12 minimum medium with the peripheral membrane molecules (0.5 mg/ml protein).

In the experiments with the conditioned media, the cultures were fixed and stained with antibodies against G4/NgCAM antibody. In the experiments with the membrane fractions, they were observed under a phase-contrast microscope (Nikon) because the G4/NgCAM immunoreactivity was so intense in the membranes that it disturbed the observation of the retinal axons; in the experiments with the peripheral membrane molecules, they were also observed under the phase-contrast microscope. They were photographed, and their images were digitized with a laser scanning confocal microscope (Leica, Nussloch, Germany). The sections were stained with 4',6-diamidino-2-phenylindole (DAPI) and photographed under a fluorescence microscope (Zeiss). Neural retinas from E6 or E7 chickens were prepared (Halfter et al., 1983). The retina was chopped perpendicular to the optic fissure with a tissue chopper (The Mickle Laboratory Engineering, Gomshall, UK) to make 0.3-mm-wide retinal strips. The explants (0.3 × 0.6 mm) were obtained from the central part of the retina for the experiments. Dorsal root ganglia (DRGs) were also prepared from E7 chickens. The retinal and DRG explants were cultured for 24 hr in 300 μl of the F-12 minimum medium with the peripheral membrane molecules (0.5 mg/ml protein). In the experiments with the conditioned media, the cultures were fixed and stained with antibodies against G4/NgCAM antibody. In the experiments with the membrane fractions, they were observed under a phase-contrast microscope (Nikon) because the G4/NgCAM immunoreactivity was so intense in the membranes that it disturbed the observation of the retinal axons; in the experiments with the peripheral membrane molecules, they were also observed under the phase-contrast microscope. They were photographed, and their images were digitized with a laser scanning confocal microscope (Leica, Nussloch, Germany). The sections were stained with 4',6-diamidino-2-phenylindole (DAPI) and photographed under a fluorescence microscope (Zeiss).
and the groove above the thin layer of cells on the SOT, the diencephalotelencephalic boundary. The data were expressed as the mean ± SE and compared with an unpaired t test.

RESULTS

The telencephalic substrate consists of cellular processes of various origins

Cells from the E8 telencephalon were cultured overnight on one side of the Nuclepore filter (the cellular side) in the ryomen chamber (Fig. 2B); the next day, the chamber was turned upside down. Processes from the telencephalic cells penetrated the filter to generate a cellular substrate on the side of the filter opposite to their cell bodies (the retinal side). This telencephalic substrate consisted of three types of processes: thin, flat, and round ones (Fig. 2C). The round processes were 5–10 μm in diameter. The expression of the following cell-type markers was examined with immunocytochemistry: MAP2 for neuronal dendrites (Fig. 2D), G4/NgCAM for axons (Fig. 2F), GFAP for mature astrocytic processes (data not shown), and vimentin for immature glial processes (Fig. 2E). The elongated and flat processes were mainly neuronal, based on MAP2 and G4/NgCAM immunoreactivity. The round processes showed vimentin immunoreactivity. No processes were immunopositive for GFAP. The results showed the presence of both neuronal and immature glial processes in the substrate after 3 d in vitro.

Differential outgrowth of the retinal axons on the cellular substrates

The retinal axons grew well on the anterior tectal substrates (Fig. 3D,E) and immediately on the diencephalic substrates (picture not shown; see Fig. 6A); however, the axons from the central, nasal, or temporal retina did not grow on the telencephalic substrates (Fig. 3A,B). To quantify the axonal outgrowth, the diameters of the axonal halos radiating from the retinal explants were measured; it was not feasible to calculate total areas covered with axonal bundles from an explant because the neurites were stained by G4/NgCAM in the cellular substrates (Fig. 2F). The diameters of axonal halos were significantly smaller on the telencephalic substrates than on the anterior tectal substrates [1.4 ± 0.1 mm on the E6 telencephalic substrates (n = 11); 3.6 ± 0.3 mm on the E6 anterior tectal substrates (n = 11); p < 0.0001] [1.4 ± 0.1 mm on the E8 telencephalic substrates (n = 52); 3.8 ± 0.2 mm on the E8 anterior tectal substrates (n = 67); p < 0.0001] (see Fig. 6A). The DRG axons grew well on either the telencephalic or anterior tectal substrates (Fig. 3C,F).

A factor inhibiting the outgrowth of retinal axons in the medium conditioned by the telencephalic cells

To characterize the effect of the telencephalic substrates on the outgrowth of the retinal axons, the retinal and DRG explants were cultured in media conditioned by the anterior tectal or telencephalic cells. The retinal axons grew profusely in the F-12 culture medium and the medium conditioned by anterior tectal cells (Fig. 4A,B); however, they did not grow in the medium conditioned by telencephalic cells (Fig. 4C). With respect to the total area covered with axonal bundles from an explant, which is comparable with the product between the number and length of the axons, the outgrowth in the medium conditioned by telencephalic cells [0.22 ± 0.03 mm² (n = 33)] was 53% of that in the medium conditioned by anterior tectal cells [0.42 ± 0.03 mm² (n = 14)] (p < 0.0001) (see Fig. 6B); the outgrowth of retinal axons in the medium conditioned by telencephalic cells was significantly less than that in the medium conditioned by anterior tectal cells. In contrast, the DRG axons grew equally well in the F-12 culture medium and the medium conditioned by anterior tectal cells or by telencephalic cells (Fig. 4E–G).

In the case in which the medium conditioned by telencephalic cells was heat treated, the retinal axons did not grow well either; only small numbers of thin axonal bundles were highly fascicu-
Anterior tectal cells (55%; conditioned media. The retinal (A–D) and DRG (E–H) explants are cultured in the F-12 culture medium (A, E), in the medium conditioned by the E8 anterior tectal cells (B, F), in the medium conditioned by the E8 telencephalic cells (C, G), and in the heat-treated medium conditioned by the E8 telencephalic cells (D, H). Scale bars, 300 μm.

Figure 4. Axonal outgrowth in the conditioned media. The retinal (A–D) and DRG (E–H) explants are cultured in the F-12 culture medium (A, E), in the medium conditioned by the E8 anterior tectal cells (B, F), in the medium conditioned by the E8 telencephalic cells (C, G), and in the heat-treated medium conditioned by the E8 telencephalic cells (D, H). Scale bars, 300 μm.

A factor inhibiting the outgrowth of retinal axons in a fraction of peripheral membrane molecules from the telencephalon

To further inspect the selective inhibition of axonal outgrowth, membrane fractions were prepared from the anterior tecta and telencephalons, and their effects on the retinal outgrowth were examined. The retinal axons grew well in media with the E8 anterior tectal membranes (Fig. 5B); however, they did not grow in media with the E8 telencephalic membranes (Fig. 5C). The retinal outgrowth was significantly less in the medium with telencephalic membranes than in the medium with anterior tectal membranes with respect to the total area covered with axonal bundles [0.05 ± 0.008 mm² with the E8 telencephalic membranes (n = 12); 0.27 ± 0.04 mm² with the E8 anterior tectal membranes (n = 12); p < 0.0001] (Fig. 6C). The retinal axons also did not grow in media with the E13 telencephalic membranes [0.08 ± 0.015 mm² (n = 12)] (Fig. 6C). In contrast, the DRG axons grew well in the media with either anterior tectal or telencephalic membranes (Fig. 5E, F).

To examine whether the inhibitory factor adheres to surfaces of the telencephalic membranes, a fraction of peripheral membrane molecules was prepared with the phase-partitioning method using Triton X-114, and its effect was investigated on the retinal outgrowth. The retinal axons did not grow in media with the peripheral membrane molecules from the E8 telencephalon [0.13 ± 0.012 mm² (n = 12); p < 0.002; the total area covered with axonal bundles with the E8 telencephalic peripheral membrane molecules] (Figs. 5H, 6C), although they grew in media with the peripheral membrane molecules from the E8 anterior tecta (Fig. 5G). The DRG axons grew well in the media with peripheral membrane molecules from either the anterior tecta or telencephalon (Fig. 5J, K).

Destruction of the factor inhibiting the outgrowth of retinal axons by chondroitinase ABC

To further characterize the factor that selectively inhibits the retinal outgrowth, the E8 telencephalic membranes were treated with heat or enzymes, and their effects on the retinal outgrowth were examined. The retinal axons did not grow in media with the heat-treated telencephalic membranes (100°C, 5 min) [0.05 ± 0.013 mm² (n = 12); p < 0.0001; with respect to the area covered with axonal bundles] (Fig. 6C). The retinal axons also did not grow in media with the telencephalic membranes that had been treated with keratanase (0.5 U/mg protein at 37°C for 1 hr) (data not shown). In contrast, in media with the telencephalic membranes that had been treated with chondroitinase ABC (0.5 U/mg protein at 37°C for 1 hr), the retinal axons grew profusely [0.31 ± 0.040 mm² (n = 12); p = 0.43; with respect to the area covered with axonal bundles] (Figs. 5I, 6C). The DRG axons grew well in the media with the telencephalic membranes that had been treated with heat, keratanase (data not shown), or chondroitinase ABC (Fig. 5L).

Aberrant routing of the retinal axons at the diencephalotelencephalic boundary after enzymatic removal of chondroitin sulfate from embryonic brain in ovo

To understand the biological relevance of CS-GAG as the factor that selectively inhibits retinal outgrowth, the distribution of CS-GAG was examined immunohistochemically in the brains of E7 chickens with an anti-CS-GAG antibody (Fig. 7A, C). In the telencephalon, CS-GAG immunoreactivity was intense in general, especially on the pial side, except that it was moderate in the ventricular zone. In the diencephalon, the optic tract situated below the pia was labeled, whereas the ventricular zone was moderately labeled. In contrast, the SOT wiring between telencephalon and diencephalon was not labeled by the anti-CS-GAG antibody. Between the pial surface and SOT, there was a layer of cells that were intensely labeled. Over the thinnest part of the positive cells, there existed a sharp groove between the telencephalon and diencephalon on the pial side, which is referred to as a boundary between the diencephalon and telencephalon. In the neural retina, a gradient of CS-GAG distribution was con-
firmed; it was intense in the peripheral retina and was less intense in the central retina (data not shown) (Snow et al., 1991; Ring et al., 1995). The distribution of CS-GAG was the same between the normal brains and the brains in which a solution of the chondroitinase ABC that had been inactivated by heat (100°C, 5 min) had been injected.

The solution of chondroitinase ABC (5 µl, 1 µU/µl) was injected into the right lateral ventricle of the E6 chickens. To verify the removal of CS-GAG by the injection, their brains were fixed on E7, sectioned, and stained with anti-CS-GAG antibody. The CS-GAG immunoreactivity was greatly reduced bilaterally in the telencephalon, diencephalon, and tectum. Around the diencephalomotorial boundary, the CS-GAG immunoreactivity was lost at the layer of cells between the optic tract and SOT (Fig. 7B). The sections were double-stained with DAPI; their cytoarchitecture was preserved (Fig. 7D). On the other hand, the gradient of CS-GAG distribution was retained in the neural retina even after the enzymatic treatment; CS-GAG immunoreactivity was intense in the peripheral retina and less intense in the central retina (Fig. 7E,F).

Figure 5. Axonal outgrowth in the media with the membrane fractions or peripheral membrane molecules. The retinal (A-C, G-I) and DRG (D-F, J-L) explants are cultured in the F-12 minimum medium (A, D), in the medium with the anterior tectal membranes (B, E), in the medium with the E8 telencephalic membranes (C, F), in the medium with peripheral membrane molecules extracted from E8 anterior telencephalic membranes (G, J), in the medium with peripheral membrane molecules extracted from E8 telencephalic membranes (H, K), and in the medium with E8 telencephalic membranes treated with chondroitinase ABC (I, L). Scale bar, 300 µm.
To examine the effects of the CS-GAG removal on pathway formation of the retinal axons, they were anterogradely labeled with HRP after injection of chondroitinase ABC or the heat-inactivated enzyme. By the whole-mount observation, it was shown in the embryos injected with the chondroitinase ABC that an anterior margin of the optic tract seemed to be neither sharp nor straight, but to curve convexly toward the telencephalon (14 cases of the 16 embryos injected with the chondroitinase ABC, 87.5%) (pictures not shown). This curving was seldom seen in the control embryos (two cases of the 15 embryos injected with the heat-inactivated enzyme, 13.3%). Those embryos were subjected to the histological examinations; in the embryos injected with the chondroitinase ABC, the retinal axons were situated over the anterior groove above the thin layer of cells on the SOT, which is likely to cause this groove to become shallow (Fig. 7H,J). To quantify the aberrant anterior shift of the optic tract, it was measured that the distances between the most anteriorly situated retinal axon and the groove above the thin layer of cells on the SOT, the diencephalotelencephalic boundary. In the control embryos injected with the heat-inactivated enzyme, the retinal axon was situated at 28.5 ± 13.4 μm (n = 8) anterior to the boundary (Figs. 7G–I, 8). In contrast, the retinal axon was situated at 78.9 ± 7.7 μm (n = 8) anterior to the boundary in the embryos injected with the chondroitinase ABC (Fig. 8), which was significantly different (p < 0.01).

**DISCUSSION**

We found that the retinal outgrowth was selectively inhibited by the telencephalic cells *in vitro*. The responsible factor for the selective inhibition was found in the fraction of peripheral membrane molecules of the telencephalon. Because the inhibitory effect was destroyed by chondroitinase ABC but not by the heat treatment, this inhibition was attributable to carbohydrate chains of CSPGs (CS-GAG) adhering to the telencephalic membranes. To understand the function of the telencephalic CSPGs on path-finding of the retinal axons *in vivo*, CS-GAG was removed from the embryonic brains by intraventricular injection of chondroitinase ABC. The removal of CS-GAG resulted in an anterior enlargement of the optic tract.

**Technical considerations**

The *ryomen* chamber assay has been developed previously for investigating the formation of the topographic map in the retinotectal projection (Ichijo and Bonhoeffer, 1998). Cellular substrates mimicked the *in vitro* situation more closely in the *ryomen* chamber than in the conventional cultures; this might be because the cells are packed between the filter and a gel matrix at a high density with a sufficient supply of gases, which allows them to locally interact with each other. As can be seen in Figure 2, the telencephalic substrates were composed of a variety of cellular processes derived from neurons and immature glia but not from mature glia; thus, the composition of the substrates reflected the cellular components of the telencephalon during the early stage of development. Although the *ryomen* chamber assay is a sensitive method for investigating the neuron–target interactions, this model is not necessarily feasible for biochemical approaches.

*heat-Tel*, the medium with the E8 telencephalic membranes treated with heat; *chondroitinase ABC-Tel*, the medium with the E8 telencephalic membranes treated with chondroitinase ABC; *PM-Tel*, the medium with the peripheral membrane molecules from the E8 telencephalon. *Asterisks* indicate the significant differences from the ATs in each graph (p < 0.01).
because of the small scale of the culture. This limitation was complemented by the retinal explant culture with membrane fractions; it enabled us to characterize the factor with the phase-partitioning method and to destroy the factor with chondroitinase ABC.

**Carbohydrate chains of CSPGs on the telencephalic membranes are the factor inhibiting the outgrowth of the retinal axons**

The telencephalic effect on the retinal outgrowth was observed during the stages in which the retinal axons find their path on the diencephalon, in the telencephalic substrates on E6 and E8, or in the telencephalic membranes on E8 and E13 (Figs. 3, 5, 6A,C). Moreover, the retinal axons ran on the boundary between the telencephalon and diencephalon (Fig. 1); it is probable that their growth cones or side branches sense the surface of telencephalic cells or their extracellular matrix. These results in vitro suggest that the telencephalic cells operate during the pathway formation of the retinal axons.

In the **ryomen** chambers, the outgrowth of the retinal axons was selectively inhibited by the telencephalic substrates (Fig. 3A,B);
Involvement of CS-GAG in pathfinding of retinal axons around the diencephalotelencephalic boundary

Injection of chondroitinase ABC into the right lateral ventricle removed CS-GAG from telencephalon, diencephalon, and telencephalon, although their cytoarchitecture was retained (Fig. 7B,D). On the other hand, the CS-GAG was not removed in the eyeball; the graded distribution of CS-GAG was kept in the retinas (Fig. 7E,F) (Chung et al., 2000b); thus, the removal of CS-GAG was not likely to influence differentiation of the retinal ganglion cells (Brittis and Silver, 1994) but was likely to affect the local environment around the retinal axons. The enzymatic treatment did not only lower the concentrations of the CS-GAG around the retinal pathway but also cancelled the spatial pattern of its distribution. Immunoreactivity against CS-GAG was lost in the layer of cells between the optic tract and SOT and on the pial side of the telencephalon (Fig. 7B). Although the enzymatic treatment was effective, residual expression of CS-GAG was observed sporadically, which might be attributable to either incomplete removal or recovered deposits because of the gradual decay of the enzymatic activity and its transient action. By transiently lowering the pathfinding of the retinal axons because it has been reported that the CSPGs bind to some of the cell adhesion molecules and growth factors (Grumet et al., 1993; Friedlander et al., 1994; Emerling and Lander, 1996; Milev et al., 1996; Sakurai et al., 1996; Anderson et al., 1998; Soussi-Yanicostas et al., 1998). In the present study, however, the inhibitory effect on axonal outgrowth does not seem to be caused by the core proteins of CSPGs or the proteaceous factors associated with CSPGs because the effect was stable even after the treatment with heat (100°C, 5 min).

The effect of the telencephalic membranes was dose dependent; high concentrations of the telencephalic membranes inhibited the outgrowth of the DRG neurites as well as the retinal axons (data not shown). The retinal axons are, therefore, more sensitive to CSPGs than the DRG neurites as shown by Snow et al. (1991). In addition, higher concentrations of even the anterior telencephalic membranes inhibited the outgrowth of both the retinal and DRG axons (data not shown). The immunohistochemical examinations did not show that distribution of CS-GAG was specific in the telencephalon, but the CS-GAG was broadly distributed with variations in the signal intensity; the immunoreactivity was intense in the telencephalon and moderate in the diencephalon and tectum (Fig. 7A). Although it is difficult to elucidate local concentrations of CS-GAG, the density of CS-GAG in the telencephalic membranes is likely to be higher than that of the anterior telencephalic membranes; the high content of the CS-GAG might cause the selective inhibition of retinal outgrowth by the telencephalic substrates, telencephalic membranes, and the fraction of peripheral membrane molecules from telencephalon in the cultures. In the normal embryos, the CS-GAG was intensely distributed on the pial side of the telencephalon. At the diencephalotelencephalic boundary, the CS-GAG immunoreactivity was intense at the thin layer of cells between the optic tract and SOT (Fig. 7A), suggesting that this layer separates the optic tract from the SOT.

It has been suggested that the CSPGs are thought to function not only in pathfinding but also in differentiation (Brittis and Silver, 1994; Nichol et al., 1994; Canoll et al., 1996; Maeda and Noda, 1996). The abundant distribution of CS-GAG indicates that the CSPGs are involved with various steps in pathfinding and differentiation; the spatial pattern of CS-GAG distribution would not necessarily have to be specific in the telencephalon or at the demarcation of the diencephalotelencephalic boundary.
the local concentration and erasing the spatial pattern of CS-GAG distribution, the enzymatic treatment seemed to affect pathfinding of a fraction of the retinal axons that ran around the diencephalotelencephalic boundary on their way to the tectum.

The enzymatic removal of CS-GAG induced the anterior enlargement of the optic tract beyond the groove between the telencephalon and diencephalon, causing this groove to become shallow (Figs. 7H, J, 8). The retinal axons are likely to be released from outgrowth inhibition and allowed to enter foreign territories. The results indicate that the mechanism inhibiting the invasion of the retinal axons into the telencephalon is, at least in part, likely to be attributable to the function of CS-GAG, which delimits the anterior border of the optic tract. It is suggested that the telencephalic cells prevent the retinal axons from aberrantly invading the anterior territory and restrict the retinal pathway to the tectum.

The enzymatic treatment seemingly induced the restricted effects on the retinal trajectory at the diencephalotelencephalic boundary, although the CS-GAGs were widely distributed in the normal embryos and they were homogenously removed in the experimental embryos. The antibody (CS-56) and the chondroitinase ABC used in this study do not discriminate types of the CS-GAGs but recognize the various types of the CS-GAGs; a specific type of CS-GAGs might cause the anterior delimitation of the retinal trajectory.

Several mutants in the zebrafish show retinal axons that invade the telencephalon (Karlstrom et al., 1996). There is a similarity between the effects of intraventricular injection of chondroitinase ABC and the phenotypes of these mutants, although the retinal axons enter the anterior territory less dramatically in the embryos treated with chondroitinase ABC than in the mutants, presumably because of the transient action of the enzyme. The CS-GAG might be involved in the phenotypes of the mutants, in which its receptor or intracellular signaling might be affected.

REFERENCES


