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Expression of Chondroitin Sulfate Proteoglycans in the Chiasm of Mouse Embryos

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ABSTRACT

Chondroitin sulfate (CS) proteoglycans have been implicated as molecules that are involved in axon guidance in the developing neural pathways. The spatiotemporal expression of CS was investigated in the developing retinofugal pathway in mouse embryos by using the CS-56 antibody. Immunoreactive CS was detected in inner regions of the retina as early as embryonic day 11 (E11). Its expression in subsequent stages of development followed a centrifugal, receding gradient that appeared to correlate with the sequence of axogenesis in the retina. In the chiasm, immunoreactive CS was expressed at E12, before the arrival of retinal axons. When the retinal axons navigated in the chiasm at E13–E14, immunoreactive CS remained at a low level in the optic fiber layer of the chiasm but was observed prominently in the caudal parts of the ventral diencephalon. This pattern followed closely the array of stage-specific-embryonic-antigen-1-positive neurons in the ventral diencephalon, with a V-shaped configuration that bordered the posterior boundary of the retinal axons, and a rostral raphe extension that ran across the decussating axons in the chiasm. Thus, the CS epitope is implicated in patterning the course of early retinal axons and in regulating axon divergence in the chiasm. At the lateral region of the chiasm, where the retinal axons cross the midline and approach the optic tract, a CS-immunopositive region coincided with the region in which active sorting of dorsal retinal axons from ventral retinal axons occurs. Moreover, at the threshold of the optic tract, the immunoreactive CS was restricted only to the deep part of the optic fiber layer, suggesting an inhibitory role of the CS epitope in repelling newly arrived axons to superficial regions of the optic tract during the development of chronotopic order at this part of the retinofugal pathway. J. Comp. Neurol. 417:153–163, 2000.

Indexing terms: retina; axon guidance; midline; optic tract; retinotopic order

Retinal axons undergo distinct rearrangements of fiber order at the chiasm. These changes in fiber order segregate retinal axons according to their positions along the nasal-temporal and dorsal-ventral axes of the retina and also reposition the fibers according to their ages in different parts of the chiasm (Godement et al., 1990; Guillery, 1991; Chan and Guillery, 1994; Colello and Coleman, 1997; Colello and Guillery, 1998; Chan and Chung, 1999). One control for such axon patterning is the interaction of retinal axons with guidance molecules at the chiasm. Recent studies of axon growth at the mouse chiasm have shown that the chiasm exerts a general dampening effect on axon growth and that this effect acts on both crossed and uncrossed axons (Godement et al., 1994; Mason and Wang, 1997; Chan et al., 1998). Retinal axons have an overall increase in pause frequency and a reduction in growth rate as well as an extensive remodeling of growth cone morphology at the chiasm. These characteristic growth behaviors of retinal axons may be caused by a contact-mediated interaction with cellular elements in the

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chiasm, such as the radial glial cells and chiasmatic neurons (Sretavan et al., 1994; Marcus et al., 1995; Mason and Sretavan, 1997), and with axons from the other eye (Godement et al., 1990; Chan and Guillery, 1993; Chan et al., 1999). Moreover, the cells of the chiasm may produce soluble factors that act to slow down the growth of retinal axons and prompt the axons for other guidance signals within the chiasm (Wang et al., 1996). However, the molecules that are involved in these guidance processes are largely unknown.

The chondroitin sulfate (CS) proteoglycans (PGs), as components of multidimensional mechanisms that regulate cell-cell and cell-matrix interactions, are likely candidates that guide axon growth and pathfinding in the developing central nervous system (for reviews, see Lander, 1993; Letourneau et al., 1994; Margolis and Margolis, 1997). In vitro studies have indicated the importance of the core proteins of the extracellular PGs as binding partners of the immunoglobulin (Ig) superfamily of neural cell adhesion molecules and the extracellular matrix tenascins and, thus, influence cell adhesion or neurite outgrowth (Margolis and Margolis, 1997). Immunocytochemical evidence of colocalization of these binding partners in the developing central nervous system underlines the biological relevance of the partnership. The CSs normally exist in tissues as substituents on core proteins of the PGs and, as such, also have been implicated in initiating retinal ganglion cell differentiation and defining the front of axonal outgrowth toward the optic disk in the rat (Snow et al., 1991; Brittis et al., 1992). Similar CS epitopes that are marked by the antibody CS-56 (Avnur and Geiger, 1984) also have been observed in the chiasm of fetal ferrets: the distribution of these moieties appears to correlate with the development of chronotopic order in the optic tract (Reese et al., 1997). In this study, we asked 1) whether similar CS moieties are expressed in the chiasm of mouse embryos at the time when retinal axons first grow into this decision region of the retinofugal pathway; and 2) in addition to their possible roles in controlling the age-related order in the tract, whether the expressions of these CS moieties are correlated with other fiber-reordering processes at the developing mouse retinofugal pathway.

MATERIALS AND METHODS

Animal and tissue preparations

The experimental procedures in this study were approved by the University Animal Ethics Committee. Timemated C57 mice were obtained from the Animal House of the Chinese University of Hong Kong. The day that the vaginal plug was found was designated as embryonic day 0 (E0). Pregnant mothers were killed by cervical dislocation. Embryos at E11–E16 were taken out by Cesarean section, washed briefly in 0.1 M phosphate buffered saline (PBS), pH7.4, and decapitated. The heads were immersed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and were stored overnight at 4°C. The fixed tissues were embedded in a gelatin-albumen mixture. After a cut was made in the block to mark the orientation of the head, the blocks were sectioned at 100 μm thickness with a Vibratome to yield horizontal sections in some embryos and coronal sections in others. Serial sections of the retinofugal pathway from the eyes to the proximal parts of the optic tract were collected in PBS, pH7.4.

Immunocytochemistry

The sections of the retinofugal pathway were washed in PBS and then incubated for 1 hour in blocking buffer containing 10% normal goat serum (NGS; Sigma, St. Louis, MO). After a brief wash with PBS, the sections were incubated overnight at 4°C in the primary antibody CS-56 (a monoclonal antibody raised against CSs; Sigma; diluted 1:200 in PBS with 1% NGS and 0.5% Triton X-100) to reveal the distribution of CS. Some sections were reacted with a primary antibody against stage-specific-embryonic-antigen-1 [SSEA-1; diluted 1:5; Developmental Studies Hybridoma Bank, IA (under contract N01-HD-6-2915 from NICHD); Solter and Knowles, 1978], which marks a population of early neurons in the ventral diencephalon (Marcus et al., 1995). After several rinses in PBS, the sections were incubated in a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (anti-mouse IgM; diluted 1:100 in PBS; Jackson Laboratories, Bar Harbor, ME) for 3 hours at room temperature. Control sections were processed similarly but without the addition of primary antibody. The sections were washed briefly in PBS and coverslipped with a mounting medium containing 0.1% p-phenylenediamine.

Confocal microscopy and image analyses

The images were captured with a confocal imaging system (MRC600; Bio-Rad, Cambridge, MA) connected to a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany) by using a blue excitation filter set (BHS; 488 nm excitation and 515 nm emission long pass). The digital images were stored in SparQ disks (SyQuest Technology Inc.) and were processed by using the Confocal Assistant software (Bio-Rad). Sections from the ventral diencephalon from 27 embryos ages E12–E16 were included in this study.

To study the distribution of the CS epitope in relation to the axonal course in the diencephalon, the retinas of some embryos (three at E13 and three at E14) were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [DiI; $DiIC_{18}(3)$; Molecular Probes, Eugene, OR). The injections were made into brain slice preparations of the retinofugal pathway as described in an earlier study (Chan et al., 1998). The DiI solution (dissolved in dimethyl formamide) was injected into the vitreous of the eye by using a Picospritzer (General Valve Co., NJ). The injected brain slices were cultured in Dulbecco's modified Eagle's medium (DMEM/F12; Life Technologies, Bethesda, MD) supplemented with 10% fetal bovine serum at 37°C for 5 hours to allow sufficient time for label to reach retinal axons in the optic tract. The brain slices were then fixed in 4% paraformaldehyde in 0.1 M PBS, pH7.4. The dye-filled retinal axons were examined in the exposed chiasm under a confocal microscope (MRC600; Bio-Rad) by using a green excitation filter set (GHS; 514 nm excitation and 550 nm long pass). After images of the retinal axons at the chiasm had been captured, the brain slices were embedded in a gelatin-albumen mixture and sectioned horizontally into 100-μm sections. The sections that contained the chiasm and the optic tract were reacted with the primary antibody CS-56 and then with the FITC-conjugated secondary antibody, as described above. These sections, which were double-labeled with DiI for retinal axons and with FITC for CS, were then imaged with a confocal imaging system (MRC1024; Bio-Rad) by using a

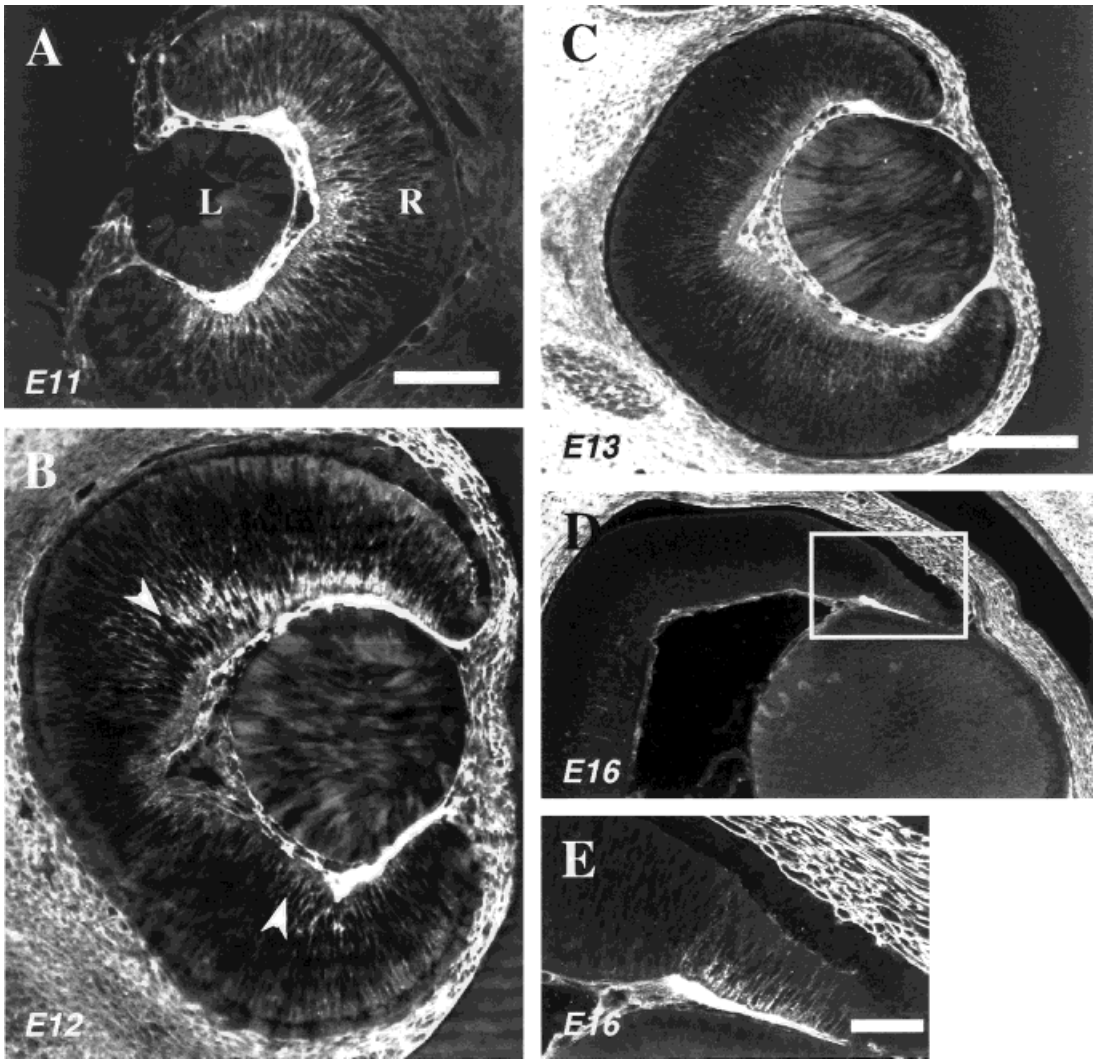

Fig. 1. Confocal photomicrographs showing immunoreactivity to CS-56 in cross sections of the retinas in C57 mouse embryos. Anterior is up. **A:** At embryonic day 11 (E11), the immunoreactive chondroitin sulfate (CS) is expressed preferentially in inner retinal layers of the central retina (R) that include the future ganglion cell layer and the optic fiber layer. Positive staining also is observed in the lens (L) and the vitreous. **B:** At E12, immunoreactivity for CS in the central retina is lower than that in the peripheral regions (indicated by arrowheads). **C:** At E13, the intensity of immunopositive CS is distributed evenly throughout the inner retinal layer. **D:** By E16, the immunoreactivity is concentrated in the inner layers of the peripheral retina (as indicated in **E**) and apparently is absent from the central retina. Scale bars = 100 μm in A (also applies to B); 200 μm in C (also applies to D); 50 μm in E.

combination of filter sets (T1 and T2 blocks) for simultaneous imaging of DiI-labeled structures (with 488 nm excitation and 522 nm emission) and FITC-labeled structures (with 568 nm excitation and 585 nm emission long pass). The dual-excited images were stored in Jaz disks (Iomega) and processed with the Confocal Assistant software.

RESULTS

Expression pattern of immunoreactive CS in the retina

Expression of the CS epitope in the retina of mouse embryos was present already at E11, the earliest stage that we examined, when most ganglion cells generated in the retina have not yet sent out axons into the optic pathway (Silver and Sidman, 1980; Dräger, 1985). The staining appeared to mark the migrating cells in the retina and their pericellular space and was most prominent at inner retinal regions that, later, are occupied by the ganglion cell layer and the optic fiber layer (Fig. 1A). At E12, when the first retinal axons emerge from the central retina around the optic disk, the expression of the CS epitope in the central retina was markedly lower than that in the peripheral retina (Fig. 1B). By E13, when most ganglion cells are sending axons into the optic pathway, this difference in staining intensity in the inner retinal regions was no longer apparent. The inner retinal regions were stained evenly with the antibody (Fig. 1C). The immunoreactive CS in the retina

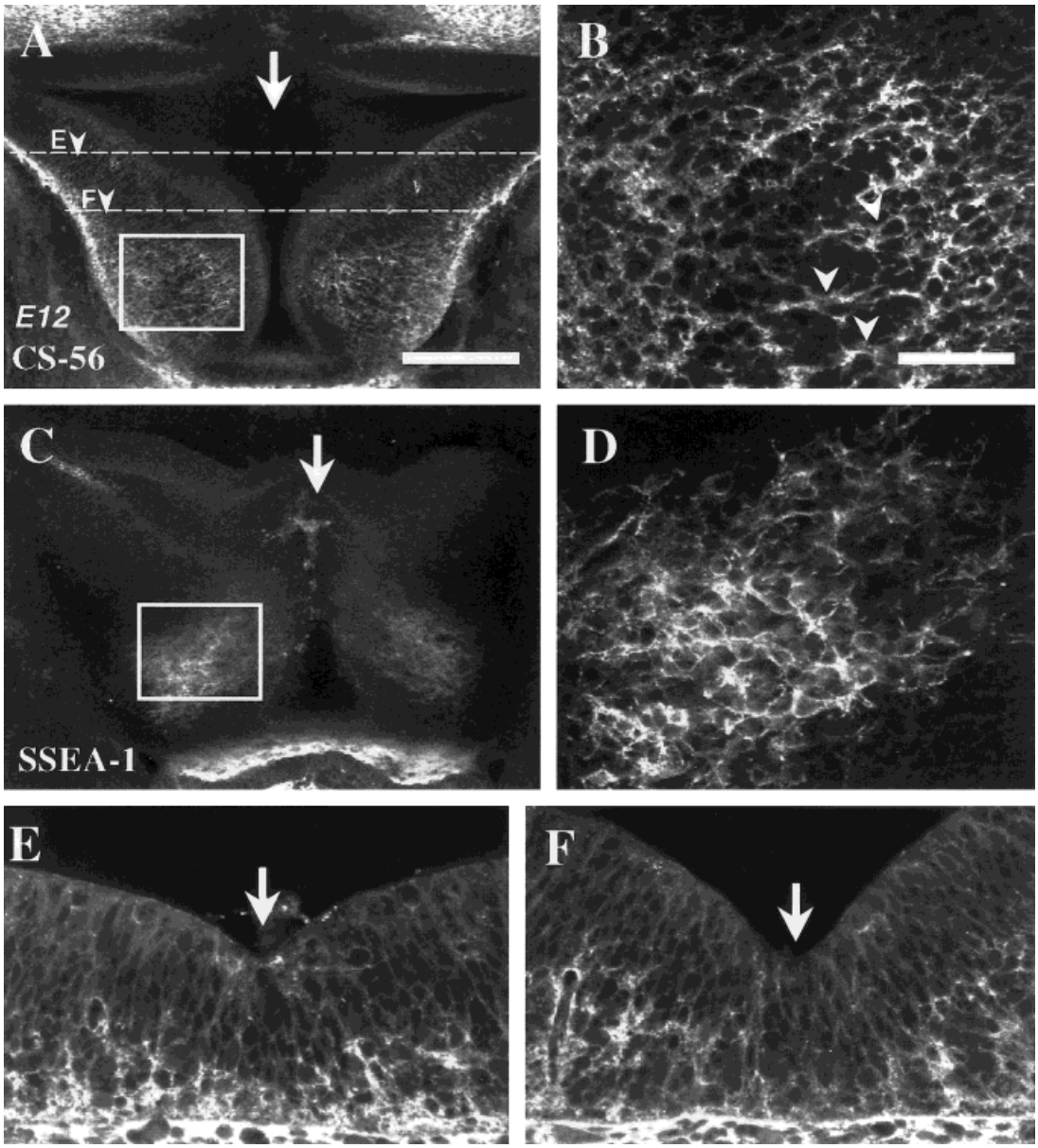

Fig. 2. Confocal photomicrographs showing immunoreactive CS and stage-specific antigen-1 (SSEA-1) in the chiasm of E12 mouse embryos. In the horizontal sections (**A–D**), anterior is up; in the coronal sections (**E,F**), dorsal is up. The arrow points to the midline of the brain. A: At E12, immunoreactive CS is distributed as two groups of cells in the caudal regions of the ventral diencephalon. It is localized on the surface of the cells (indicated by arrowheads in B) and in the pericellular space around the cells. This staining pattern appears to coincide with the cells that are immunopositive for SSEA-1 (C,D). E,F: Coronal sections correspond to the regions indicated by the dashed lines in A. Cells immunopositive for CS are located in the regions flanking the midline of the future chiasm and occupy the ventral tier of the neuroepithelium (E). These cells occupy the lateral regions but not at the midline, as shown in the caudal section of the chiasm (F). The optic fiber layer could not be observed during this stage. Scale bars = 200 μm in A (also applies to C), 50 μm in B (also applies to D, E, and F, but in E,F the scale bar is 25 μm).

gradually declined in subsequent stages, until, at E16, positive staining was detectable only in the inner regions of the retina at the peripheral margins, where some axons may still be added (Fig. 1D,E). The central retina was virtually devoid of staining.

Expression pattern of immunoreactive CS in the ventral diencephalon

Immunoreactivity to CS was detected first in the ventral diencephalon at E12. At this stage, most of the first retinal axons are growing in the stalk and have not yet arrived at the chiasm (Silver and Sidman, 1980). Staining of the CS epitope in horizontal sections of the ventral diencephalon revealed a symmetric distribution of staining in caudal regions of the ventral diencephalon (Fig. 2A). The staining appeared to mark the cells and their pericellular environment. In some regions in which staining of single cells was identifiable, the CS-positive cells were irregular in shape and had several short processes (Fig. 2B). In more ventral sections, immunopositive cells were

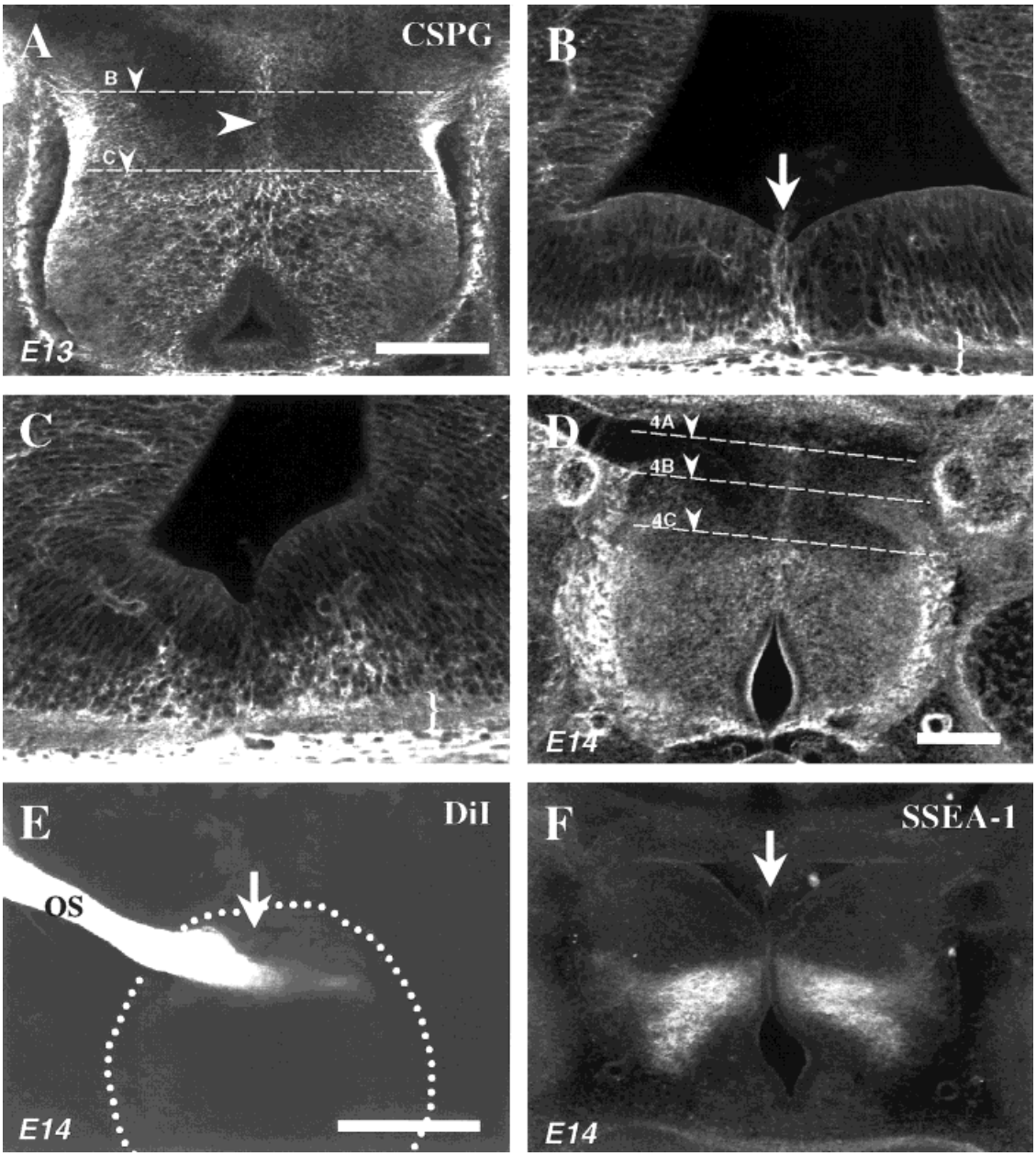

Fig. 3. **A–F:** Confocal photomicrographs showing immunoreactivity for CS and SSEA-1 as well as the position of retinal axons in the chiasm of E13 and E14 embryos. A,D–F: Horizontal sections. The arrows point to the midline of the brain, and anterior is up. A: At E13, immunostaining for CS is seen as a broad, inverted, V-shaped array opening caudally in the caudal half of the ventral diencephalon. Moreover, a midline extension (indicated by arrowhead) stems out at the rostral end of the CS-immunopositive region. A similar staining pattern is found at E14 (D). These CS-immunopositive regions appear to mark the caudal border of the retinal axons shown in E as the 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled optic stalk (OS) leading from the left eye to the chiasm of an E14 embryo. The rostral part of the CS-immunopositive array likely coincides with the SSEA-1-positive cells (F). B,C: Coronal sections of the chiasm (E13) at regions indicated by the dashed lines in A. The optic fiber layer can be observed at the ventral floor (marked by brackets) of the diencephalon. The expression of the CS epitope is localized about the cells at the chiasm and is particularly prominent at the midline (indicated by arrow in B) but is spread widely in a more caudal level at the chiasm (C). Staining also was detected in the optic fiber layer. Scale bars = 200 μm in A (also applies to B,C); 100 μm in D; 500 μm in E (also applies to F).

found at the regions flanking the midline, anterior to the immunopositive cells in the caudal diencephalon. Coronal sections through the position of the future chiasm confirmed that immunopositive cells were found in the midline regions (Fig. 2E). They occupied the ventral tier of the neuroepithelium, where axons grow at later developmental stages. In a more caudal section of the chiasm, these cells occupied more lateral regions of the chiasm (Fig. 2F). This staining pattern of CS in the ventral diencephalon coincides with the distribution of chiasmatic neurons that

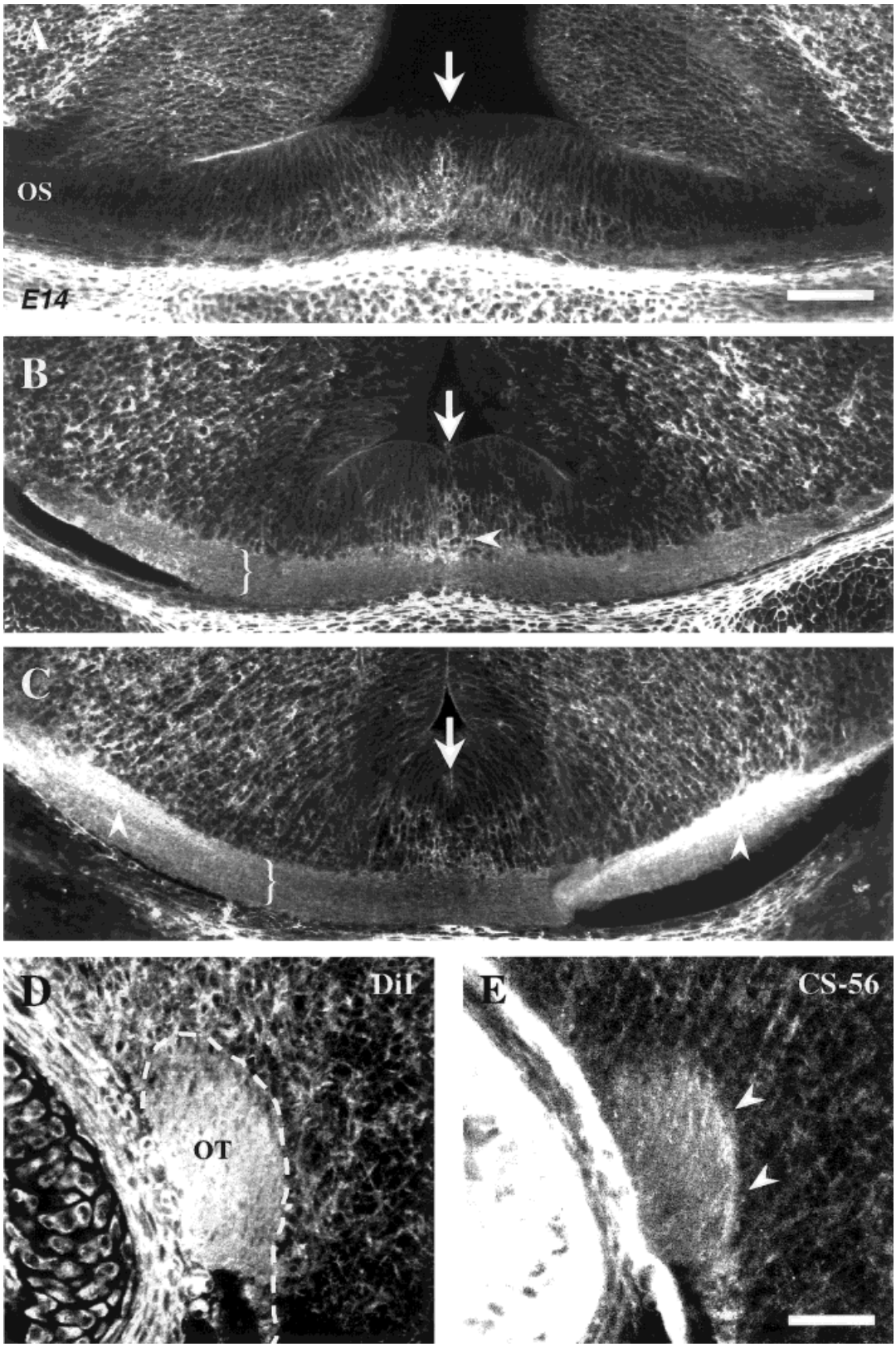

Figure 4

are immunopositive for SSEA-1 (compare Fig. 2C with Fig. 2A; see also Fig. 2D). These SSEA-1-positive neurons have been implicated in the patterning of the earliest axons that arrive at the ventral diencephalon at subsequent stages of development (Marcus and Mason, 1995; Marcus et al., 1995).

At E13, the first retinal axons start to grow into the chiasm (Godement et al., 1987). In horizontal sections of the ventral diencephalon, again, the most prominent region that expressed CS immunoreactivity was the caudal half of the ventral diencephalon, where they formed a broad, inverted-V-shaped configuration opening caudally in the confocal photomicrographs (Fig. 3A). Moreover, a prominent raphe extension was found at the rostral end of this immunoreactive CS region (Fig. 3A, arrowhead). This rostral extension marks the immunoreactive CS-rich zone at the midline of the chiasm where retinal axons decussate. Strong immunostaining also was detected at the pial surface of the lateral diencephalon, which includes the position of the future optic tract. There are very few axons in the tract at this stage. Coronal sections of the regions shown in Figure 3A demonstrate that immunoreactive CS was localized in neuron-like cells at the ventral diencephalon and that this was particularly prominent at the midline (Fig. 3B). A broad array of cells at a more caudal level in the chiasm also was immunopositive for CS (Fig. 3C). Some immunostaining was detected in the thin optic fiber layer in the chiasm. The radial glial cells in the chiasm did not show obvious staining at these early stages of retinal axon growth in the chiasm.

The immunostaining pattern of the CS epitope in the ventral diencephalon at E14 was similar to that seen at E13 (Fig. 3D). At this stage, in coronal sections of the ventral diencephalon, a weakly stained, X-shaped configuration of the chiasm was identified rostral to the immunoreactive CS-rich regions (Fig. 3D,E). The rostral parts of the immunoreactive CS regions in the caudal diencephalon are likely immunopositive for the SSEA-1 antibody (compare Fig. 3D with Fig. 3F). These CS immunopositive regions appear to mark the caudal border of the retinal axons in the chiasm and may participate in the development of the partial decussation pattern.

In E14 embryos, coronal sections from the rostral regions of the chiasm showed a substantial expression of the CS epitope among the cellular elements at the midline (Fig. 4A). Punctate staining of CS was found among these cell-rich regions anterior to the retinal fibers in the chiasm. At a midlevel within the chiasm, immunoreactive CS expression at the midline was confined largely to the cellular elements immediately above the optic fiber layer (Fig. 4B). A slight elevation in staining intensity was noted in the fiber layer at the midline, whereas fibers in the regions flanking both sides of the midline showed a weak basal level of staining. At a caudal level in the chiasm, this weak staining at the midline was not discernible (Fig. 4C). In addition to this change in expression of the CS epitope along the midline, expression of the CS epitope also was changed at different levels in the course of the axons through the chiasm. At the entry zones of retinal axons into the chiasm, at the junction of the optic stalk and the diencephalon, the optic fiber layer was stained only weakly with the CS-56 antibody (Fig. 4A,B). The staining was confined to the connective tissue enclosing the stalk and the cellular structures in the ventral diencephalon dorsal to the optic fiber layer. It is noteworthy that, at the regions of the chiasm where most retinal fibers cross the midline (and that also contain axons that have turned away from the midline) and grow toward the optic tract, intense immunostaining of the CS epitope was detected in the fiber layer at the junction of the chiasm and the optic tract (Fig. 4C). This staining appeared in the chiasm ≈300 μm from the midline, extended laterally to the optic tract, and it was strongest at the deep parts of the optic fiber layer. The staining diminished gradually toward the subpial surface of the optic fiber layer. This differential distribution of immunoreactive CS also was characterized in cross sections of the optic tract. In four embryos at E14, the retinal fibers in the tract were examined for dual label with DiI and the CS-56 antibody. In coronal sections of the tract, the dye-filled optic tract appeared as a discrete bundle of fibers at the lateral diencephalon (Fig. 4D). Immunostaining of the CS epitope was detected only in the deep regions, and not in the superficial regions, of the tract (Fig. 4E).

At E15 and E16, most retinal axons have arrived at the chiasm and entered the optic tract, including those from the ventral temporal retina that turn before reaching the midline of the chiasm. At both ages, there was very little staining of the CS epitope in the optic stalk and the rostral chiasm (Fig. 5A). Obvious staining could be seen at the pia surrounding the stalk and in the cells of the lateral diencephalon. At a more caudal level, where fibers from the two eyes mingle extensively at the midline, a strong label of the CS epitope was detected at the lateral margin of the chiasm, ≈300 μm from the midline (Fig. 5B). Substantial immunostaining of the CS epitope also was seen in other regions of the optic fiber layer, particularly at the pial surface at the midline. Moreover, a slight but obvious elevation in staining intensity was observed at the fiber layer at the midline. At the level of the caudal chiasm and the tracts, the lateral regions of the fiber layer in the chiasm, again, were stained the most intensely (Fig. 5C). This expression extended medially to the subpial region around the midline, but it tapered off laterally in the subpial regions of the fiber layer at the threshold of the optic tract. A high level of CS immunoreactivity was detected in the deep regions at the threshold of the tract, and

Fig. 4. Confocal photomicrographs showing the CS-56 immunoreactivity in the chiasm of E14 embryos. Coronal sections (A–C) correspond to the regions indicated by the dashed lines in Figure 3D. The arrow points to the midline of the brain, and dorsal is up. **A:** Substantial expression of the CS epitope is localized about the cellular elements at the midline anterior to the retinal fibers in the chiasm. The optic stalks, however, are devoid of CS-56 immunostaining. **B:** At a midlevel within the chiasm, the staining is confined to the cells immediately above the optic fiber layer (indicated by arrowhead). A slight elevation in staining intensity is noted in the optic fiber layer (marked by brackets) at the midline, whereas fibers in regions flanking both sides of the midline show a basal level of staining. **C:** At the caudal part of the chiasm, intense immunostaining of CS is detected in the fiber layer at the junctures of the chiasm and the optic tract (indicated by arrowheads), which starts at ≈300 μm from the midline and is most obvious at the deep parts of the chiasm and tract. **D,E:** Horizontal sections of the ventral diencephalon doubled stained with DiI at retinal axons and CS-56 antibody. The optic tract (OT) shows up as a discrete bundle of fibers at the lateral diencephalon (D), and CS immunoreactivity is localized predominantly in the deep regions of the tract (E; indicated by arrowheads). Anterior is up, and medial is to the right. Scale bars = 50 μm in A (also applies to B,C); 25 μm in E (also applies to D).

this extended laterally into the tract. In addition to the staining in the chiasm, many cellular elements in the ventral diencephalon were immunopositive for the CS-56 antibody. These cells were similar morphologically to the radial glial cells, with radially oriented cell bodies and processes extending from the ventricle toward the pial surfaces of the diencephalon.

DISCUSSION

In this study, the distribution of chondroitin sulfate proteoglycans (CSPGs), as depicted by the CS-56 antibody, has been characterized in the retinofugal pathway of mouse embryos. The current results on the mouse confirm earlier findings in the rat that the CS epitope is expressed as a gradient receding from the more central retinal layers out to the periphery (Brittis et al., 1992). Furthermore, it was demonstrated in this study that the CS epitope exists at the chiasm at E12, before the arrival of any retinal axons. At this early stage and in the course of subsequent development, the pattern of CS immunoreactivity in the ventral diencephalon follows closely the distribution of neurons that are immunopositive for SSEA-1. These SSEA-1-positive neurons have been attributed with a role in the shaping of trajectory of the earliest retinal axons in the chiasm (Marcus and Mason, 1995). Expression patterns of the CS epitope at later stages, i.e., E13–E15, when most axons are growing through the chiasm, suggest that these moieties are involved in the control of distinct fiber orders at the chiasm. The abundant expression of the CS epitope at the midline indicates that these moieties may play a part in the development of partial decussation. This expression at the midline appears to diminish gradually, whereas expression in fibers at the threshold of the optic tract is elevated in later stages of development, when most axons are growing through this region of the pathway. The distinct distribution of the CS epitope in the caudal chiasm and in deep parts of the optic tract suggests a role for these molecules in controlling the development of age-related order and retinotopic order in the retinofugal pathway.

CSPGs in the retina

The retina of mouse embryos is strongly immunopositive for the CS epitope at the earliest stage studied, i.e., E11; thereafter, the immunoreactivity gradually decreases. With subsequent development, the expression of the CS epitope moves from the center toward the peripheral margin of the retina. This developmentally regulated expression of the CS epitope is in agreement with the findings in rat embryos, which showed similar distribution of CSPGs in the developing retina. A centrifugal, receding gradient of CSPG expression, which also was depicted by immunoreactivity to the CS-56 antibody, exists in the retina of rat embryos, which is coincident with and peripheral to the retinal axon outgrowth toward the optic disk (Brittis et al., 1992). Furthermore, the pattern of retinal axon outgrowth was perturbed when explant cultures of the retina were maintained in a medium containing chondroitinase ABC, which digests the CS chains of the PGs (Brittis et al., 1992). Time-lapse videomicroscopy of axonal outgrowth from retinal explants has shown that the growth cones turned and continued to grow along the border of the CSPG-coated substratum, indicating an inhibitory function of CSPG on retinal neurite outgrowth (Snow et al., 1991). Based on these findings, it is concluded that CSPGs provide guidance to developing axons in the retina by directing and restricting axonal growth toward the center of the retina.

In contrast to the findings of Brittis et al. (1992), immunohistochemical studies on the chick visual pathway have shown that CSPGs not only were found in the basal lamina and neuroepithelium but also were colocalized with developing axons in the optic fiber layer (McAdams and McLoon, 1995; Ring et al., 1995). In the latter, the expression of CSPGs coincides temporally and spatially with the onset and cessation of retinal axon growth, suggesting a supportive role rather than an inhibitory role of these molecules in patterning the axonal course in the chick retina. These various roles of CSPGs in guiding axonal outgrowth in the retinofugal pathways remain unresolved, but they may represent differences in response of retinal growth cones to the balance of growth-promoting and inhibitory factors in species-specific environments.

Roles of CSPGs in the chiasm

Many studies have suggested that CSPGs are the active components in structures that act as barriers for growing axons (e.g., posterior sclerotome: Oakley and Tosney, 1991; Perris et al., 1991; Landolt et al., 1995; roof plate of the spinal cord: Snow et al., 1990; retina: Snow et al., 1991; Brittis et al., 1992; optic tectum: Hoffman-Kim et al., 1998). Retinal axons in culture are shown to avoid substratum-bound CSPGs when presented as a border against growth-promoting laminin or fibronectin, indicating that these PGs, as the less permissive partner to growth cone exploration, guide and deviate the growth of axons (Snow et al., 1991; Snow and Letourneau, 1992). This inhibitory role of CSPGs is supported by our demonstration of characteristic spatial and temporal expression of the CS epitope in relation to the pattern of axonal growth in the chiasm. First, axons appear to navigate along, but not across, the anterior border of the CSPG-rich regions in the caudal diencephalon, indicating that, whereas the CS-lined border is supportive to axon growth, the CS-enriched territory beyond the border discourages axonal growth, a phenomenon similar to the lining up of neurites along the border of the CSPG-enriched regions in vitro (Snow et al., 1991; Snow and Letourneau, 1992). Second, the presence of a thin CS-positive raphe at the chiasmatic midline at E13 and E14 suggests its role as a functional barrier that stops the growth of uncrossed axons and allows the crossed axons to pass through. The high frequency of pauses in the crossing axons at the midline of the chiasm in mouse embryos also may be related to the abundant expression of these CSPG molecules (Godement et al., 1994). The uncrossed axons that arrive at the chiasm at these ages (E13–E14) are largely from the central retina (Godement et al., 1987; Colello and Guillery, 1990; Chan et al., 1999). Although previous studies have suggested that turning of these early uncrossed axons does not rely on specific guidance cues at the chiasm (Godement et al., 1990; Taylor and Guillery, 1995; Chan et al., 1999), our finding of the CS-positive region at the chiasmatic midline in E13 and E14 embryos raises the possibility that some, if not all, uncrossed axons from the central retina rely on this functional barrier to turn at the chiasm. In contrast, axons that arise from the ventral temporal retina at later stages require positional information in the retina and interaction with fibers from the

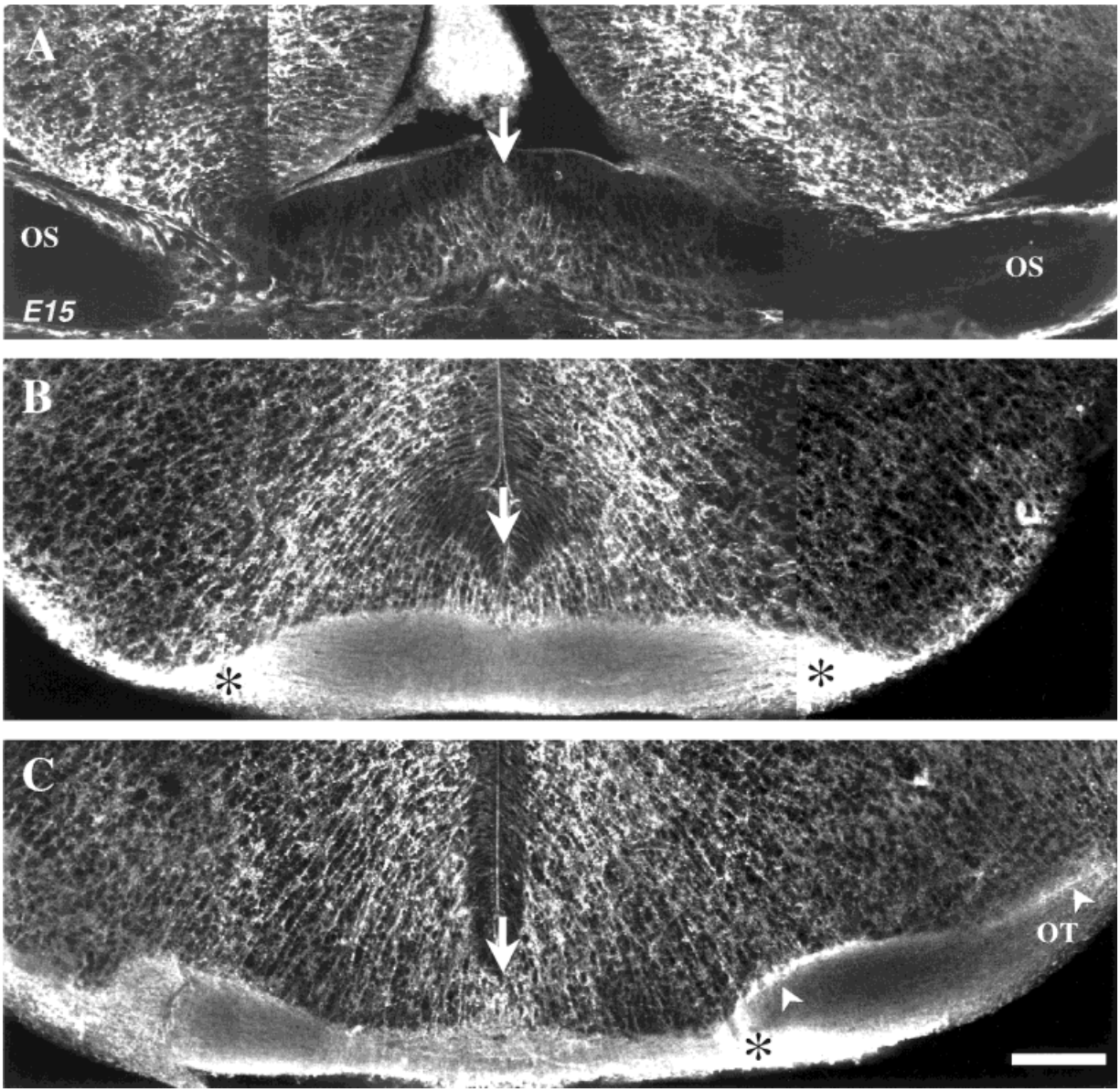

Fig. 5. Confocal photomicrographs showing CS-56 immunoreactivity in the chiasm of an E15 embryo. In these coronal sections of the ventral diencephalon, the arrows point to the midline of the brain, and dorsal is up. **A:** The rostral chiasm is weakly positive for CS-56 at E15, whereas the optic stalk (OS) is devoid of labeling. In contrast, obvious staining is seen at the pia surrounding the stalk and about the cells at the lateral diencephalon. **B:** In a more caudal section, dense labeling of CS-56 is detected at the lateral margin of the chiasm ≈300 μm from the midline (marked by asterisks). A slight but obvious elevation in staining is observed at the deep regions of the midline. **C:** At the level of the caudal chiasm and the tracts, the CS epitope again is expressed preferentially at the lateral regions of the fiber layer in the chiasm (the slight asymmetry in the optic fiber layer is a sectioning artifact). This expression extends medially along the subpial region around the midline but tapers off laterally toward the threshold of the optic tract (OT). Immunostaining for CS is found in the deepest regions at the threshold and also in the proximal region of the optic tract (indicated by arrowheads). In addition, many cellular elements in the ventral diencephalon express the CS epitope, and these cells show the morphology of radial glia. Scale bar = 50 μm.

opposite eye to make their turns in the chiasm (Godement et al., 1990; Taylor and Guillery, 1995; Chan et al., 1999).

At the threshold of the optic tract, expression of the CS epitope is most prominent in the deeper parts of the chiasm and tract. This restricted distribution of CSPGs is observed in embryos at E14–E16, when most axons are navigating toward the optic tract. It has been shown in many studies that there is a chronotopic order of retinal fibers in the tract, with the youngest axons closest to and oldest axons farthest from the pial surface of the tract (Guillery and Walsh, 1987; Colello and Guillery, 1992; Colello and Coleman, 1997; Colello and Guillery, 1998). This order reflects the time of arrival of retinal axons in the tract. This age-related order also has been reported in the juxtachiasmatic part of the optic stalk (Guillery and Walsh, 1987; Colello and Guillery, 1992, 1998), which is

lost at the midline regions of the chiasm (Reese et al., 1994; Colello and Coleman, 1997) and reestablished in the tract. The loss of the juxtachiasmatic order in the midline regions may be related to a concentration of the CS epitope at the subpial regions of the optic fiber layer that is particularly prominent in E15–E16 chiasms. This concentration of PGs may deflect the growth cones away from the pial surface. At the junction of the chiasm and the tract, the restricted, intense expression of the CS epitope in the deep parts of the pathway appears to signal the growth cones to deviate toward the subpial surface of the tract, so that the age-related order, similar to that at the juxtachiasmatic region, is reestablished again. Similar findings of an elevated expression of CSPGs has been reported in the chiasm and tract of developing ferrets (Reese et al., 1997), suggesting a general role of the CS epitope in the development of age-related fiber order in the tract. However, the lack of expression of the CS epitope at the junction of the optic nerve and the chiasm suggests that the age-related order at this position is under the control of a mechanism that is different from that at the threshold of the tract, which appears to involve the CS epitope as one of the guidance molecules.

Although the exact structure of the CS epitope recognized by the CS-56 antibody has not been determined, it is known that the antibody is specific for chondroitin-4 sulfates and chondroitin-6 sulfates of native CSPGs (Avnur and Geiger, 1984). In this study on the mouse, as well as in other studies on rats, hamsters, and ferrets, the epitope invariably has been associated with barrier function in the course of developing retinal afferents (Snow et al., 1991; Reese et al., 1997; Hoffman-Kim et al., 1998). For example, the tectal midline in hamsters that acts as a barrier to prevent the growth of retinal axons across the midline is highly immunoreactive for the CS epitope (Hoffman-Kim et al., 1998). Attempts to identify the core protein that bears the CS epitope found one or more candidates, but none was specific to the region of interest (Reese et al., 1997; Hoffman-Kim et al., 1998). It appears that the distinctive patterns of CS immunoreactivity is due not so much to increased expression of core proteins but to increased synthesis of sulfated glycosaminoglycans bearing the CS epitope as component substituents of perhaps one of the core proteins identified. It remains possible that the cells that normally produce core proteins substituted with low-sulfated chondroitins respond locally to invading growth cones with the production of core proteins bearing an altered sulfation pattern of the chondroitins, as depicted by the CS-56 antibody. Indeed, in our study of postcrush sciatic nerves, we found water-soluble, glycosaminoglycan-containing forms enriched in 6-sulfated chondroitins peaking at a phase coincident with axonal regrowth in the regenerating nerve (Shum and Chau, 1996; Chau and Shum, 1997). It would be interesting to determine how developing and regrowing axons stimulate the change in sulfation pattern of the chondroitins and whether growth cones react directly to the changed CS or to changed, CS-bound factors as guidance cues.

Another interesting finding in this study is that there is a marked CS-positive zone in the lateral regions of the chiasm where the crossed retinal axons have to travel through before entering the tracts. These regions are located $\approx$300 μm from the midline. It has been reported that a complicated change in fiber arrangement occurs at these positions in the chiasm. This fiber reordering establishes a segregation of dorsal retinal fibers from ventral retinal fibers in the optic tract (Chan and Chung, 1999). It is possible that this active fiber repositioning after axons cross the midline is related to the abundant CS epitope in these chiasmatic regions, and the interaction of axons with the CS epitope may be mediated by a signaling mechanism that involves the growth-associated protein GAP-43 (Kruger et al., 1998). However, the extent to which the development of the dorsal ventral order in the tract relies on the expression of the CS epitope in the chiasm and whether the control for the development of dorsal ventral order is related to that for the age-related order in the tract remain unknown.

The CS epitope, as discussed above, may be produced by the chiasmatic neurons during the initial phases of axon growth at the chiasm. However, at later developmental stages (at E15–E16), this CS immunoreactivity in the region of the SSEA-1 neurons is down-regulated dramatically, whereas the immunoreactivity in the region of the radial glia at the ventral diencephalon obviously is up-regulated. The prominent expression of the CS epitope in the chiasmatic neurons likely is involved in patterning the course of earliest retinal axons, whereas the expression of the CS epitope in the radial glia is involved in the development of chronotopic order and dorsal ventral fiber order in the optic tract, which take place at a later stage of pathway development. These results, together with findings in fetal ferrets that CS immunoreactivity follows closely the glial profiles in deep parts of the tract (Reese et al., 1997), support the idea that the CS epitope in radial glia is involved in patterning the fiber order in the optic tract.

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LITERATURE CITED

Avnur Z, Geiger B. 1984. Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell 38:811–822.

Brittis PA, Canning DR, Silver J. 1992. Chondroitin sulfate as a regulator of neuronal patterning in the retina. Science 255:733–736.

Chan SO, Chung KY. 1999. Changes in axon arrangement in the retinofugal pathway of mouse embryos: confocal microscopy study using single- and double-dye label. J Comp Neurol 406:251–262.

Chan SO, Guillery RW. 1993. Developmental changes produced in the retinofugal pathways of rats and ferrets by early monocular enucleations: the effects of age and the differences between normal and albino animals. J Neurosci 13:5277–5293.

Chan SO, Guillery RW. 1994. Changes in fiber order in the optic nerve and tract of rat embryos. J Comp Neurol 344:20–32.

Chan SO, Wong KF, Chung KY, Yung WH. 1998. Changes in morphology and behaviour of retinal growth cones before and after crossing the midline of the mouse chiasm—a confocal microscopy study. Eur J Neurosci 10:2511–2522.

Chan SO, Chung KY, Taylor JSH. 1999. Effects of prenatal monocular enucleation on the cellular specialization in the development of retinofugal pathway. Eur J Neurosci 11:3225–3235.

Chau CH, Shum DKY. 1997. Injury-associated increase in 6-sulfated chondroitin sulfates in post-crush sciatic nerves of guinea pigs. Glycoconjugate J 14:S9.

Colello SJ, Coleman L-A. 1997. Changing course of growing axons in the optic chiasm of the mouse. J Comp Neurol 379:495–514.

Colello RJ, Guillery RW. 1990. The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course. Development 108:515–523.

Colello RJ, Guillery RW. 1992. Observations on the early development of the optic nerve and tract of the mouse. J Comp Neurol 317:357–378.

Colello SJ, Guillery RW. 1998. The changing pattern of fiber bundles that pass through the optic chiasm of mice. Eur J Neurosci 10:3653–3663.

Dräger UC. 1985. Birth dates of retinal ganglion cells giving rise to the crossed and uncrossed optic projections in the mouse. Proc R Soc London [Biol] 224:57–77.

Godement P, Vanselow J, Thanos S, Bonhoeffer F. 1987. A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development 101:697–713.

Godement P, Salaün J, Mason CA. 1990. Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers. Neuron 5:173–186.

Godement P, Wang L-C, Mason CA. 1994. Retinal axon divergence in the optic chiasm: dynamics of growth cone behavior at the midline. J Neurosci 14:7024–7039.

Guillery RW. 1991. Rules that govern the development of the pathways from the eye to the optic tract in mammals. In: Lam DMK, Shatz CJ, editors. Development of the visual system, volume 3. Proc Retina Research Foundation Symposia. Cambridge, MA: Massachusetts Institute of Technology, p 153–171.

Guillery RW, Walsh C. 1987. Changing glial organization relates to changing fiber order in the developing optic nerve of ferrets. J Comp Neurol 265:203–217.

Hoffman-Kim D, Lander AD, Jhaveri S. 1998. Patterns of chondroitin sulfate immunoreactivity in the developing tectum reflect regional differences in glycosaminoglycan biosynthesis. J Neurosci 18:5881–5890.

Kruger K, Tan AS, Lu C, Sretavan DW. 1998. Retinal ganglion cell axon progression from the optic chiasm to initiate optic tract development requires cell autonomous function of GAP-43. J Neurosci 18:5692–5705.

Lander AD. 1993. Proteoglycans in the nervous system. Curr Opin Neurobiol 3:716–723.

Landolt RM, Vaughan L, Winterhalter KH, Zimmermann DR. 1995. Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth. Development 121: 2303–2312.

Letourneau PC, Condic ML, Snow DM. 1994. Interactions of developing neurons with the extracellular matrix. J Neurosci 14:915–928.

Marcus RC, Mason CA. 1995. The first retinal axon growth in the mouse optic chiasm: axon patterning and the cellular environment. J Neurosci 15:6389–6402.

Marcus RC, Blazeski R, Godement P, Mason CA. 1995. Retinal axon divergence in the optic chiasm: uncrossed axons diverge from crossed axons within a midline glial specialization. J Neurosci 15:3716–3729.

Margolis RU, Margolis RK. 1997. Chondroitin sulfate proteoglycans as mediators of axon growth and pathfinding. Cell Tissue Res 290:343–348.

Mason CA, Sretavan DW. 1997. Glia, neurons and axon pathfinding during optic chiasm development. Curr Opin Neurobiol 7:647–653.

Mason CA, Wang L-C. 1997. Growth cone form is behavior-specific and, consequently, position-specific along the retinal axon pathway. J Neurosci 17:1086–1100.

McAdams BD, McLoon SC. 1995. Expression of chondroitin sulfate and keratan sulfate proteoglycans in the path of growing retinal axons in the developing chick. J Comp Neurol 352:594–606.

Oakley RA, Tosney KW. 1991. Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. Dev Biol 147:187–206.

Perris R, Krotoski D, Lallier T, Domingo C, Sorrell JM, Bronner-Fraser M. 1991. Spatial and temporal changes in the distribution of proteoglycans during avian neural crest development. Development 111:583–599.

Reese BE, Maynard TM, Hocking DR. 1994. Glial domains and axonal reordering in the chiasmatic region of the developing ferret. J Comp Neurol 349:303–324.

Reese BE, Johnson PT, Hocking DR, Bolles AB. 1997. Chronotopic fiber reordering and the distribution of cell adhesion and extracellular matrix molecules in the optic pathway of fetal ferrets. J Comp Neurol 380:355–372.

Ring C, Lemmon V, Halfter W. 1995. Two chondroitin sulfate proteoglycans differentially expressed in the developing chick visual system. Dev Biol 168:11–27.

Shum DKY, Chau CH. 1996. Changes in glycosaminoglycans during regeneration of post-crush sciatic nerves of adult guinea pigs. J Neurosci Res 46:465–476.

Silver J, Sidman RL. 1980. A mechanism for the guidance and topographic patterning of retinal ganglion cell axons. J Comp Neurol 189:101–111.

Snow DM, Letourneau PC. 1992. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). J Neurobiol 23:322–336.

Snow DM, Steindler DA, Silver J. 1990. Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. Dev Biol 138:359–376.

Snow DM, Watanabe M, Letourneau PC, Silver J. 1991. A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Development 113:1473–1485.

Solter D, Knowles BB. 1978. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75:5565–5569.

Sretavan DW, Feng L, Puré E, Reichardt LF. 1994. Embryonic neurons of the developing optic chiasm express L1 and CD44, cell surface molecules with opposing effects on retinal axon growth. Neuron 12:957–975.

Taylor JSH, Guillery RW. 1995. Effect of a very early monocular enucleation upon the development of the uncrossed retinofugal pathway in ferrets. J Comp Neurol 357:331–340.

Wang L-C, Rachel RA, Marcus RC, Mason CA. 1996. Chemosuppression of retinal axon growth by the mouse optic chiasm. Neuron 17:849–862.