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Embryonic Neurons of the Developing Optic Chiasm Express 11 and CD44, Cell Surface Molecules with Opposing Effects on Retinal Axon Growth

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Summary

The first retinal ganglion cell axons arriving at the embryonic mouse ventral diencephalon encounter an inverted V-shaped neuronal array defining the midline and posterior boundaries of the future optic chiasm. These neurons express L1, an immunoglobulin superfamily molecule known to promote retinal axon outgrowth, and CD44, a cell surface molecule that we find inhibits embryonic retinal axon growth in vitro. Incoming retinal axons do not penetrate this L1/CD44 neuron array, but turn to establish the characteristic X-shaped optic chiasm along the anterior border of this array. These results suggest that L1/CD44 neurons may serve as an anatomical template for retinal axon pathways at the embryonic mouse ventral diencephalon.

Introduction

The nervous system is characterized by long axon projections that connect populations of neurons with specific targets. An example of this in the mammalian CNS is retinal ganglion cell axon projections from the eyes conveying visual information to major targets in the diencephalon and the midbrain. These retinal projections are formed during embryonic development as axons from the two optic stalks enter the brain laterally from opposite sides at the anterior part of the ventral diencephalon. Ingrowing retinal axons do not project randomly into the ventral diencephalon, but instead grow toward each other to meet and form a striking X-shaped pattern of axon pathways known as the optic chiasm. Axons emerging from the chiasm then run within the optic tracts along the lateral walls of the diencephalon to reach visual target nuclei.

In mammals, retinal axons from the two eyes do not merely cross over one another at the X-shaped optic chiasm. The chiasm also represents a pathway decision point where retinal axons originating from the nasal retina of each eye cross the midline to innervate targets on the contralateral side of the brain. Axons from a group of ganglion cells in temporal retina do not cross, but instead turn away from the midline region and grow into the ipsilateral optic tract to innervate targets on the same side. This highly specific axon routing pattern ensures that visual information received by corresponding regions of the retina in the two eyes is conveyed to

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target nuclei on the same side of the brain for processing, a feature associated with binocular vision in mammals (Guillery, 1983).

The cellular and molecular mechanisms underlying the formation of retinal axon pathways at the mammalian ventral diencephalon are unclear. It is not known, for example, how retinal axons from the two eyes entering opposite sides of the embryonic brain find their way toward one another and meet at the midline region. Once retinal axons arrive at the midline region, the pathfinding cues that guide retinal axons to cross or turn away from the midline have yet to be identified. Finally, following this pathfinding decision in the region of the midline, it is not clear what cellular cues operate to then direct retinal axons into the two optic tracts.

Although the molecular mechanisms underlying these retinal axon pathfinding tasks are poorly understood, recent work has begun to describe the process by which axons decide whether to cross the midline or to remain on the same side. Examination of retinal axon trajectories in fixed tissues (Godement et al., 1990; Sretavan, 1990) and live retinal axon pathfinding using video microscopy (Sretavan and Reichardt, 1993) have shown that ipsilaterally projecting axons turn within a 100 μm wide region centered on the midline, indicating that pathfinding cues are likely to be located within this area. In vitro, cell membrane fragments isolated from the chiasm region have been shown not to support growth of ipsilaterally projecting retinal axons from ventral-temporal retina, but do support growth by contralaterally projecting axons from nasal retina (Wisemann et al., 1993). This suggests that membrane-associated molecules that inhibit growth of ipsilaterally projecting axons are involved in retinal axon decisions to cross or not to cross the midline.

Clues as to the cellular origin of these cues were provided in a study examining the effects of interactions between axons from opposite eyes in determining axon routing at the chiasm. Analysis of embryos with one eye removed early in embryonic life has shown that axons from the remaining eye still find their way into the correct optic tract, indicating that pathfinding decisions at the midline establishing the initial adult-like pattern of chiasmatic routing do not depend on interactions between axons from opposite eyes but instead depend on pathfinding cues expressed by local chiasm cells (Sretavan and Reichardt, 1993). Furthermore, time-lapse analyses of ipsilaterally projecting retinal axons in one eye embryos have shown that pathfinding cues at the central chiasm region do not trigger total ipsilateral growth cone collapse, but instead cause axons to turn 90° away from the midline area in 10–20 min into the ipsilateral optic tract (Sretavan and Reichardt, 1993).

In the current study, we show that during embryonic development, a group of early generated neurons is present at the ventral diencephalon organized as an inverted V-shaped array defining the midline and the posterior boundary of the future optic chiasm. Incoming retinal axons turn in close proximity to the embryonic chiasm neurons, and both chiasm neurons and retinal axons express L1, a cell surface molecule shown previously to promote neurite outgrowth by retinal ganglion cells in vitro (Drazba and Lemmon, 1990). In addition, embryonic chiasm neurons also express CD44, a molecule involved in cell–cell interactions in the immune system. We demonstrate that CD44 has an opposing inhibitory effect on embryonic retinal axon growth in vitro. Retinal axons from the two eyes after encountering the L1/CD44 chiasm neurons form the X-shaped pathways along the anterior part of the inverted V-shaped array, suggesting that early generated neurons may play an important role in the development of retinal axon pathways at the mammalian ventral diencephalon.

Results

Figure 1 shows a schematic diagram depicting the development of the retinal axon pathways in mice from embryonic day 11(E11) to E16 and illustrates the spatial relationship between

the retinas, optic nerves, and optic chiasm, as viewed from the ventral surface of the embryonic diencephalon. Retinal ganglion cells are generated starting at E11 (Dräger, 1985) and begin to extend axons into the optic nerves at E12 (Godement et al., 1990; Silver, 1984; Sretavan, 1990). By E12.5, the first retinal axons have arrived at the ventral diencephalon (Colello and Guillery, 1990; Godement et al., 1990; Silver, 1984; Sretavan, 1990) and have begun to establish the X-shaped optic chiasm. By E15–E16, large numbers of retinal axons from the two eyes have grown through the ventral diencephalon into the two optic tracts, creating an X-shaped optic chiasm, within which an adult-like pattern of ipsilateral and contralateral axon projection is present. From E16 to birth, axons from later generated retinal ganglion cells continue to join the optic chiasm, enlarging the specific axon routing pattern established earlier to give rise to the mature pattern of retinal projection (Colello and Guillery, 1990; Godement et al., 1990; Sretavan, 1990).

The Future Region of the Optic Chiasm Contains an Early Neuronal Population

The anterior region of the ventral diencephalon in E11.0–E12 mouse embryos, prior to the arrival of any retinal axons, contains a population of cells that are immunoreactive for neuronal markers microtubule-associated protein 2 (MAP2) and β III tubulin. In E12 embryos, the spatial relationship of this cell population with respect to the optic stalks and the diencephalon midline is best seen in the horizontal plane, where cells positive for MAP2 and β III tubulin are distributed symmetrically about the midline as an inverted V-shaped array (Figures 2A and 2B). The tip of the V (Figures 2A and 2B, arrows) is located at the midline and points anteriorly, and the two legs of the V extend posteriorly and laterally. The location of this early neuronal population in the anterior region of the ventral diencephalon, close to the junction between the optic stalks and the ventral diencephalon, places it in the path of ingrowing retinal axons.

Cells Positive for MAP2 and β III Tubulin Have a long Axon and Multiple Dendrite-like Processes

The identification of this cell population as neuronal in origin on the basis of MAP2 and β III tubulin immunoreactivity was supported by the finding that these cells morphologically resembled neurons in having long axons and dendrite-like processes. The axon projections from these embryonic optic chiasm cells can be visualized in E12 mouse embryos by placing Dil crystals into the ventral diencephalon at the site of the inverted V-shaped array. These anterograde tracing experiments resulted in the robust labeling of long axon projections extending from the inverted V-shaped array around the midline, extending to both sides of the brain. These axon projections were best seen in the coronal plane, where they can be followed from their labeling site at the midline and projecting up to 1.2 mm dorsally along the lateral wall of the diencephalon (Figure 2C).

We took advantage of the presence of long axons on these early generated ventral diencephalon cells to label them retrogradely with Dil and examine their morphology. Cells backfilled in this manner were located in a layer-like fashion just below the pial surface at the ventral diencephalon (Figures 2D–2F; see also Figure 5D). Unlike cells with radial glial morphology at the ventral diencephalon, which span the whole thickness of the neuroepithelium with endfeet attached to both the ventricular and pial surfaces (Colello and Guillery, 1992; Mason et al., 1991, *Sot. Neurosci.*, abstract), retrogradely labeled embryonic optic chiasm cells are multipolar in shape and have a number of dendrite-like processes emanating from their cell bodies (Figures 2D–2F).

It is of note that embryonic optic chiasm neurons, by their location just beneath the ventral pial surface, are situated in a region of the ventral diencephalon through which ingrowing retinal ganglion cell axons navigate. Previous studies at the light microscope level have shown that ingrowing retinal axons do not course throughout the entire thickness of the neuroepithelium.

Instead, retinal axons entering the mammalian ventral diencephalon grow between the endfeet of the neuroepithelial cells, just below the pial surface (Colello and Guillery, 1992; Guillery and Walsh, 1987; Silver, 1984). Our present findings show that this region is occupied by the embryonic optic chiasm neurons and their processes prior to the arrival of the retinal ganglion cell axons.

Incoming Retinal Axons Grow toward Chiasm Neurons and Their Processes

The relationship between incoming retinal ganglion cell axons and embryonic chiasm neurons was examined following immunostaining of ventral diencephalon whole mounts. The results in Figure 3 show that axons of embryonic chiasm neurons express L1, a cell surface molecule of the immunoglobulin family.

At E11.0, as the first born retinal ganglion cells are undergoing their last mitotic division (Dräger, 1985) and before the arrival of any retinal ganglion cell axons at the ventral diencephalon, the L1-immunoreactive processes of the embryonic chiasm neurons are already present in an inverted V-shaped pattern on the ventral diencephalon surface (Figure 3A). The location of this inverted V-shaped pattern of L1 staining corresponds in size, shape, and location to the inverted V-shaped array of embryonic chiasm neuronal cell bodies visualized using anti-MAP2 and anti- β III tubulin antibodies (compare Figures 2A and 2B with Figure 3A). The similarities in staining pattern show that the processes of the chiasm neurons, like their cell bodies, are located at a site to meet incoming retinal ganglion cell axons.

By E12, retinal ganglion cell axons have begun to enter the ventral diencephalon (Figure 3B, arrowheads). Retinal ganglion cell axons at these early stages already express L1 and are labeled using anti-L1 antibody. At E12, a few L1-positive retinal axons (Figure 3B, arrowheads) can be seen leaving the optic nerves to grow toward the future region of the chiasm. However, at this stage, none have yet grown into the array of L1-positive embryonic chiasm neurons and their processes.

A day later, at E13, significant numbers of retinal axons have entered the future region of the chiasm (Figure 3C). It is of note that retinal ganglion cell axons from the two eyes, upon leaving the optic nerves, do not continue on their trajectory to grow straight toward each other to meet at the ventral midline. Instead, the population of retinal axons as a whole, upon entering the diencephalon, make gradual turns of roughly 45° in a posterior direction to orient toward and grow into the array of embryonic optic chiasm neurons and their processes (see Figure 3C, arrows; note that a few retinal axons make turns of up to 90°). This 45° turn in axon trajectory at the junction of the optic nerves with the ventral diencephalon is quite apparent during early axon ingrowth. It is, however, difficult to see later in development, by E15–E16, after large numbers of retinal axons have joined the pathway.

Retinal Axons Do Not Penetrate the Embryonic Optic Chiasm Neuronal Array

Whereas anti-L1 immunostaining in whole mounts illustrated the global relationship between ingrowing retinal ganglion cell axons and embryonic chiasm neurons during early stages of development, their relationship at E13 and later cannot be clearly determined using this method, since retinal axons and the processes of the embryonic chiasm neurons are both L1 immunopositive. To examine the spatial relationship between retinal ganglion cell axons and embryonic chiasm neurons after they have encountered each other, retinal axons were anterogradely labeled using Dil crystals implanted into the retina, and embryonic chiasm neurons and their processes were retrogradely labeled by DiO placed into the region of their axons along the lateral wall of the diencephalon.

Results from these double labeling experiments showed that retinal axons entering the ventral diencephalon in E13 embryos clearly grow into the array of embryonic chiasm neurons and their processes (Figures 4A and 4B). Retinal axons enter approximately the anterior one-third to one-half of the inverted V-shaped region (Figure 4A, see arrows), but do not completely penetrate through the entire neuron array. After entering the anterior part of the array, retinal axons turn either toward the midline and grow through the anterior tip of the inverted V-shaped array into the opposite side of the brain (Figure 4A), or turn in the other direction, away from the midline region and back toward the ipsilateral side (Figure 4B). As a result, retinal axons begin to establish an X-shaped optic chiasm along the anterior border of the inverted V-shaped neuron array at the ventral diencephalon.

Embryonic Optic Chiasm Neurons Express CD44

The presence of early generated neurons at the future region of the chiasm before the arrival of retinal ganglion cell axons and the subsequent close relationship between these cells and ingrowing retinal axons raise the possibility that embryonic chiasm neurons play a role in retinal axon guidance at the ventral diencephalon. If so, it is likely that embryonic chiasm neurons express cell surface molecules capable of influencing retinal axon growth. As shown in Figures 3A–3C above, embryonic optic chiasm neurons do in fact express L1, a cell surface molecule known to be a potent promoter of axon outgrowth (Fischer et al., 1986; Lemmon et al., 1989), including outgrowth from retinal ganglion cells (Drazba and Lemmon, 1990). Furthermore, L1 is known to be capable of promoting neurite elongation in a homophilic manner (Lemmon et al., 1989), and expression of L1 on both chiasm neuron processes and retinal axons provides a potential basis for such a homophilic interaction.

In addition to L1, we have found that embryonic chiasm neurons express CD44, a cell surface molecule known to be involved in a number of cell–cell interactions in the immune system, including lymphocyte binding to specialized endothelial cells (Jalkanen et al., 1986, 1987), up-regulation of lymphocyte–macrophage interactions (Denning et al., 1990; Shimizu et al., 1989), and changes in lymphocyte adhesion following exposure to chemokines (Tanaka et al., 1993). As illustrated in Figure 5, CD44 expression on embryonic optic chiasm neurons and their processes in E12 embryos can be detected by immunostaining using three different monoclonal antibodies (MAbs) against mouse CD44. The staining patterns observed with all three antibodies were similar, and all showed the inverted V-shaped pattern of staining characteristic of embryonic optic chiasm neurons and their processes (Figures 5A–5C). Furthermore, CD44 expressing cells, like embryonic chiasm neurons, were also located in a layer-like fashion under the pial surface at the ventral diencephalon (Figure 5D). Double immunostaining experiments demonstrated colocalization of β III tubulin and CD44 immunoreactivity on the same cells, indicating that CD44 is indeed present on early generated optic chiasm neurons (Figures 5E–5H). CD44 on embryonic chiasm neurons and their processes can be detected by MAb immunostaining starting at about E10.5–E11 (data not shown), well before the arrival of retinal axons at E12.5.

CD44 on Embryonic Chiasm Neurons Exists as an 85K Sialated Glycoprotein

Different isoforms of CD44 are known to be generated by posttranslational modifications involving N-, O-glycosylations, addition of glycosaminoglycan side chains (Brown et al., 1991; Camp et al., 1991), and alternative splicing in the extracellular domain (Gunthert et al., 1991; Screaton et al., 1992, 1993; Stamenkovic et al., 1991). The molecular weight of the CD44 isoform expressed by embryonic chiasm neurons was determined in immunoblots using membrane preparations from ventral diencephalon tissue containing embryonic chiasm neurons and their processes. The results showed a broad immunoreactive band at about 85K (Figure 6A), consistent with expression of the low molecular weight isoform of this molecule at the ventral diencephalon. Although similar, CD44 at the ventral embryonic diencephalon

exists at a lower molecular weight compared with CD44 on circulating lymphocytes in adult mice of the same strain, which appears as a major band of about 95K and a minor band of about 115K (Figure 6A).

Results in Figure 6B show that CD44 is present as the low molecular weight 85K form at the ventral diencephalon between E12 and postnatal day 0 (P0). Thymidine birth dating studies have shown that retinal ganglion cells are born over an extended period of time from E11 to E19 (Dräger, 1985), and retinal axons grow into the ventral diencephalon starting at E12.5 and continuing until around birth. During this entire period, CD44 in the ventral diencephalon exists as an 85K protein. Protein immunoblots did not show detectable levels of higher molecular weight forms of this molecule in the ventral diencephalon at any stage of embryonic development.

The CD44-immunoreactive band obtained from ventral diencephalon tissue was diffuse in character, consistent with CD44 being a highly glycosylated molecule (Goldstein et al., 1989; Nottenburg et al., 1989; Stamenkovic et al., 1989). Enzymatic digestions of CD44 isolated from the embryonic mouse ventral diencephalon using neuraminidase revealed that glycosyl additions to CD44 in this region included sialic acid (~15K; Figure 6C). In addition to glycosylation sites, the primary sequence of CD44 is also known to contain potential sites for glycosaminoglycan addition (Goldstein et al., 1989; Nottenburg et al., 1989), and the addition of chondroitin sulfate side chains transforms this molecule into a cell surface proteoglycan of 180K–200K (Brown et al., 1991; Carter and Wayner, 1988; Gallatin et al., 1989; Jalkanen et al., 1988; Stamenkovic et al., 1989). The presence of the 85K form of CD44 in the ventral diencephalon suggests that CD44 in this region is not modified extensively with glycosaminoglycan chains. This is supported by our finding that enzymatic digests using chondroitinase ABC failed to produce a detectable shift in the molecular weight of CD44 isolated from the embryonic ventral diencephalon (Figure 6C).

Embryonic Chiasm Neurons Express the Hematopoietic Form of CD44

Reverse transcription polymerase chain reaction (PCR) analysis was carried out to identify the CD44 splice variant present in the ventral diencephalon. To do so, poly(A)⁺ mRNA from ventral diencephalon tissue of E4 mouse embryos was used to generate cDNAs, which were then used as templates for PCR analysis employing primers located in both constitutive and alternatively spliced exons (Figure 7B). Previous studies in rats have shown that CD44 isoforms with alternatively spliced exons in the extracellular domain confer metastatic potential to carcinoma cells (Gunthert et al., 1991), whereas the short (low molecular weight) form of CD44 with constitutively expressed exons is found on hematopoietic cells (Brown et al., 1991; Stamenkovic et al., 1989). The sizes of the PCR products obtained from this analysis were consistent with the presence of mRNA at the ventral diencephalon encoding the short form of CD44 (Figure 7C, lanes 1–4). No amplified products were detected in PCRs using primers internal to the alternatively spliced large extracellular insert (Figure 7C, lanes 5 and 6). Thus both reverse transcription PCR analysis of RNA and SDS gel analysis of protein show that the short form of CD44 is expressed at the embryonic chiasm. These neurons do not express detectable amounts of the large isoform commonly found on cells of the epithelial lineage (Brown et al., 1991; Stamenkovic et al., 1989, 1991).

CD44 Defines the Midline of the Optic Chiasm during Ipsilateral and Contralateral Axon Pathfinding

The inverted V-shaped pattern of CD44 expression at the future region of the chiasm in E12 embryos undergoes modification as retinal axons make specific pathfinding choices into the ipsilateral and contralateral optic tracts. In E14 embryos, CD44 immunoreactivity at the ventral diencephalon was present as an inverted V shape, with the legs of the V extending laterally

away from the midline in a posterior direction, similar to the pattern seen in younger embryos. In addition, however, there is a pronounced anterior extension of CD44 immunoreactivity at the midline (Figure 8, arrow). This midline strip of CD44 expression is only approximately 25 μm wide, in contrast with CD44 expression at E12, when the anterior tip of the inverted V array at the midline measures approximately 100 μm wide (compare Figures 5A–5C [E12] with Figure 8 [E14]).

Although CD44 is clearly expressed on embryonic chiasm neurons at the midline at younger ages, such as E12, we do not know for certain that the midline expression at E14 is present on the same population of neurons or on other cell types. Nevertheless, the midline location of CD44 immunoreactivity at E14 is of note, since it is during this period that ipsilaterally projecting retinal axons avoid the midline and turn back into the ipsilateral pathway, whereas contralaterally projecting axons cross the midline to reach the contralateral optic tract.

Generation of CD44⁺ Cell lines

As a first step toward addressing the possibility that CD44 plays a role in axon guidance, we examined whether this molecule is capable of influencing embryonic retinal axon growth. To do so, we generated cell lines expressing CD44 and used membrane fragments from transfected cells in assays of embryonic retinal explant axon outgrowth in vitro.

CD44⁺ cell lines were generated from parental K562 erythroleukemia cells, which do not express CD44 (see Figure 9E). K562 cells were transfected using a plasmid vector containing the cytomegalovirus promoter (pCDNA/neo; Invitrogen) and encoding the full-length cDNA for the short form of CD44. Immunostaining showed that this molecule is displayed on the surface of transfected cells (Figures 9A–9D), and protein immunoblots showed that transfected cells expressed an 85K form of CD44 (Figure 9E), a form comparable in molecular weight to CD44 found on embryonic optic chiasm neurons in vivo (compare lanes 1 with 3 and 4). This similarity in size suggested that CD44 from these two sources were glycosylated to a similar degree.

CD44 Exerts an inhibitory Influence on Retinal Axon Growth In Vitro

To study whether retinal axons can interact with CD44, we used retinal explants from E12.5 to E13.0 embryos, ages corresponding to the times during normal development when ingrowing retinal axons are in a position to first encounter CD44-expressing neurons at the ventral diencephalon. To recreate a situation in vitro in which retinal axon fascicles growing in a three-dimensional matrix encounter CD44 displayed on the surface of cells, we examined the growth of axon fascicles from retinal explants placed into collagen gels seeded with membrane fragments from transfected cells expressing CD44.

Retinal explants grown in the presence of membranes from CD44[−] parental K562 cells extended numerous axon fascicles in a radial fashion (Figure 10, row A). Retinal explants cultured in the presence of membranes from CD44⁺ cells also extended axon fascicles radially. The axon fascicles formed under this latter condition were of approximately the same thickness but were on the average shorter, resulting in a smaller total amount of axon fascicle growth (Figure 10, row B). This negative effect of CD44 on embryonic retinal axon growth is quantified in Figure 11A, which shows that the presence of CD44 results in about a 60% decrease in average explant fascicle length.

The N-terminal extracellular region of CD44 has homologies with cartilage link proteins (Goldstein et al., 1989; Nottenburg et al., 1989; Stamenkovic et al., 1989), and CD44 has been shown to be capable of binding hyaluronic acid (Aruffo et al., 1990; Culty et al., 1990; Lesley et al., 1990; Miyake et al., 1990b; Peach et al., 1993). One possible explanation for CD44's

growth inhibitory effect is that it binds hyaluronic acid, which may be expressed on retinal axons, and thereby inhibits their growth. Experiments were carried out in which hyaluronidase was added to the collagen gels and culture medium to eliminate any possible hyaluronate on retinal axons or other hyaluronate that may have bound to CD44. The results showed that even in the presence of hyaluronidase, CD44⁺ membranes still exhibited an inhibitory effect on embryonic retinal axon growth in vitro, suggesting that CD44 exerts its effects independently of hyaluronate binding (Figure 11A).

Inhibitory Effect on Retinal Axon Growth Is Partially Reversed by an Anti-CD44 Antibody

Results in Figure 12 show that the inhibitory effects of CD44 on embryonic retinal axon growth are blocked by an anti-CD44 MAb, demonstrating that the observed axon growth inhibition by transfected cells is indeed mediated through CD44. Three different MAbs against CD44 were studied. Two antibodies, KM201 and IM7, did not block the inhibitory influence of CD44 on retinal axon growth (Figure 10, rows C and D, respectively; Figure 12). KM201 recognizes an epitope in the hyaluronic acid-binding region of CD44, and its binding to its epitope blocks the hyaluronic acid binding ability of CD44 (Culty et al., 1990). This lack of effect of KM201 suggested that the inhibitory influence of CD44 on retinal axon growth was independent of CD44 binding to hyaluronic acid and is consistent with the inability of hyaluronidase treatment to reverse the inhibitory effects of CD44 (see above), providing additional evidence that CD44 does not exert its effects on retinal axons by binding hyaluronic acid. The epitope for IM7 has not been identified, but this antibody is known to block hyaluronate binding by CD44 (Miyake et al., 1990b).

Membranes from CD44⁺ cells preincubated with the anti-CD44 MAb IRAW (Lesley et al., 1992) extended longer axon fascicles compared with explants grown in CD44⁺ membranes alone (Figure 10, row E; Figure 12). The inhibitory effects of CD44 were, however, only partially reversed by this antibody, as axons in the presence of IRAW were on average still somewhat shorter than those seen with untransfected cell membranes (Figure 12).

IRAW has been shown to be an activating antibody that enhances CD44 binding to hyaluronic acid (Lesley et al., 1992). With this in mind, one explanation for the ability of the IRAW antibody to reverse the inhibitory effects of CD44 is that IRAW increases hyaluronic acid binding by CD44 and this complex in turn promotes retinal axon outgrowth. Two lines of evidence suggest that this is not the case. First, CD44⁺ membranes preincubated with hyaluronate still demonstrated inhibitory effects on retinal axon growth to a degree similar to that seen with CD44⁺ membranes alone, suggesting that the CD44-hyaluronate complex does not promote retinal axon growth (Figure 11B). Second, the use of hyaluronidase to digest away any hyaluronate that may be present in the culture did not affect the ability of the IRAW antibody to reverse the inhibitory effects of CD44 (Figure 11B). This result indicates that ability of the IRAW antibody to block CD44 inhibition on retinal axon growth is not mediated through increased hyaluronate binding. Given these results, it seems likely that IRAW antibody attachment to CD44 directly interferes with retinal axon interaction with CD44. The mechanisms that could mediate this effect include the possibility that IRAW sterically hinders retinal axon interaction with CD44 and shields the inhibitory effects of this molecule. This suggests that a retinal axon inhibitory epitope lies near the epitope recognized by IRAW. Alternatively, since IRAW is able to activate CD44 binding to hyaluronic acid, its binding to CD44 may induce conformational changes in this molecule, which in turn affect retinal axon interaction with CD44.

Discussion

Results from the present study show that in mouse embryos the site of the future optic chiasm is populated by early generated neurons expressing L1, a molecule shown previously to

promote neurite outgrowth by retinal ganglion cells, and CD44, a glycosylated cell surface molecule known to regulate interactions among leukocytes and, in the present study, shown to exert an opposing inhibitory effect on embryonic retinal axon outgrowth in vitro. These CD44-expressing neurons exist as an inverted V-shaped array that appears to define the midline and the posterior boundary of the optic chiasm region and may serve as a template for the growth of retinal axons growth as they establish the X-shaped optic chiasm along the anterior border of this array. In addition, the presence of CD44 at the midline region during the time when retinal axons make decisions to cross the midline to the contralateral side or to turn away into the ipsilateral optic tract raises the possibility that this cell surface molecule may be involved in setting up the characteristic ipsilateral and contralateral axon routing pattern at the mammalian optic chiasm (see summary Figure 13).

Embryonic L1/CD44 Chiasm Neurons

During the course of our studies, Easter and colleagues independently identified a “scaffold” of early axon tracts in the embryonic mouse brain (Easter et al., 1993). An examination of that work revealed that the embryonic optic chiasm neurons we describe in this paper likely belong to this set of first generated or “pioneer” neurons of the embryonic mammalian brain. In addition, axons of the embryonic chiasm neurons course along the lateral wall of the diencephalon in an area corresponding to the location of an early axon tract these authors have named the “tract of the postoptic commissure” (TPOC). In this study we have not equated the embryonic chiasm neurons with “TPOC neurons,” since we have noted that at the ages we have studied, axons that make up the TPOC originate from neurons located in different sites of the embryonic CNS (D. W. S., L. F., and L. F. R., unpublished data). Thus, although the axons of the chiasm neurons contribute to this early pathway, they do not form the entire TPOC.

It should also be noted that the embryonic chiasm neurons and their processes are distinct from the supraoptic commissure (commissure of Gudden), a well-defined commissure present in the adult ventral diencephalon containing decussating axons originating from structures in dorsal regions of the diencephalon and the midbrain and projecting to targets on the opposite side of the brain (Gitler and Barraclough, 1988; Graybiel, 1978; Watanabe and Kawana, 1979). Embryonic chiasm neurons send axons up the lateral wall of the diencephalon, whereas the supraoptic commissure contains axons that project from dorsal structures down into the ventral diencephalon to cross to the other side. Given the data presented here demonstrating these early generated neurons in the future region of the optic chiasm and their expression of cell surface molecules L1 and CD44, we have referred to these neurons as L1/CD44 embryonic chiasm neurons to describe their location, molecular identity, and relationship to developing retinal pathways at the ventral diencephalons.

Cell Types at the Ventral Diencephalon

Previous studies examining retinal axon ingrowth into the embryonic chick diencephalon have shown that retinal axon growth cones grow between the endfeet of the local neuroepithelial cells, suggesting that neuroepithelial cells may serve a retinal axon guidance function (Silver and Rutishauser, 1984). Studies have also described the presence of glial elements in the ventral diencephalon whose processes span the thickness of the neuroepithelium (Colello and Guillery, 1992; Guillery and Walsh, 1987). Immunohistochemical methods have shown that some of these glial cells express antigens characteristic of cortical radial glia, and midline glial elements have been proposed to play a role in axon pathfinding in this region (Mason et al., 1991, *Sot. Neurosci.*, abstract). The possibility that glial cells may be involved in retinal axon guidance at the ventral diencephalon needs to be investigated, since glial cells are clearly involved in axon guidance in insects (Bastiani et al., 1987; Klämbt et al., 1991). In addition, glia have also been shown to support axon growth in the mammalian brain in vivo (Silver and Ogawa, 1983) and are often aligned with axon trajectories (Norris and Kalil, 1991; Vanselow et al.,

1989). These studies thus raise the possibility that glial elements are generally involved in axon guidance during neuronal development.

The results from the present study indicate that in addition to neuroepithelial cells and glial cells, the future region of the chiasm at the ventral diencephalon contains a population of early generated neurons. Given that the first retinal axons appear to make turns in the region of the embryonic chiasm neurons and that chiasm neurons express cell surface molecules capable of influencing embryonic retinal axon growth, L1/CD44 chiasm neurons may play an important role in the proper development of initial retinal axon pathways at the mammalian ventral diencephalon similar to guidepost cells in the grasshopper limb bud (Bentley and Caudy, 1983) and subplate neurons in the developing neocortex (Ghosh et al., 1990).

How May CD44 Function at the Ventral Diencephalon?

CD44 has been reported to mediate a number of cell–cell interactions in the immune system. These include lymphocyte binding to lymph node sections in vitro (Stamenkovic et al., 1991), a process thought to mimic lymphocyte homing, which involves specific cell recognition and adherence to specialized endothelial cells and leads to lymphocyte extravasation from the vascular circulation into the lymphatic system (Butcher, 1991). CD44 is also known to regulate the functional avidities of other cell surface ligand–receptor pairs, such as LFA3 and CD3, which mediate T cell adhesion to macrophages and lead to T cell activation (Denning et al., 1990; Shimizu et al., 1989). Finally, CD44 appears to regulate cell adhesion by binding and presenting diffusible chemokines to cells, which in turn can lead to enhancement of cell surface interactions involving integrins (Tanaka et al., 1993). Thus, CD44 has been implicated in cell recognition events and modulation of ligand–receptor interactions on leukocytes.

Previous studies of CD44 have noted the presence of this molecule in the human brain during adult life (Dalchau et al., 1980) and in late fetal development in association with astrocytes (Vogel et al., 1992). Although its function during neuronal development is completely unknown, its presence at a major pathfinding region during initial formation of axon pathways in the brain raises the possibility that it may function in axon guidance. Although the molecular basis of retinal axon pathfinding has yet to be identified, it seems likely that retinal axon routing at the optic chiasm will also prove to involve cell surface molecules capable of mediating specific cellular recognition.

It should be noted that although the present work demonstrates an inhibitory role for CD44 in retinal axon outgrowth under in vitro conditions, it is not known for certain that CD44 exerts such an effect in vivo. Given evidence from the immune system that CD44 appears to modulate a number of cell surface recognition events and interactions, it would be important to understand the molecular context in which CD44 exists on the cell surface in vivo. Thus, an understanding of how CD44 might act in retinal axon guidance may well require examination of its potential interactions with other cell surface molecules within the developing retinal pathways.

A Posterior Boundary for Formation of X-Shaped Retinal Pathways at the Chiasm

Following the arrival of retinal axons at the ventral diencephalon midline, two sets of pathfinding events need to occur in order to form the adult pattern of X-shaped chiasmatic retinal pathways. First, retinal axons arriving at the ventral diencephalon at E12–E13 must not project in a widespread manner or grow along the midline. Instead, axons must somehow be routed in a specific fashion to form the X-shaped pathways we recognize as the optic chiasm. Second, retinal axons arriving at the midline region at E13–E14 must use pathfinding cues associated with local cells to decide whether to cross the midline and project contralaterally or to stay on the same side and project into the ipsilateral optic tract.

The presence at E12 of embryonic L1/CD44 neurons, which define the posterior boundary of the future optic chiasm, raises the possibility that this neuronal population may be involved in the first of these guidance events. An X-shaped set of retinal pathways forms as arriving retinal axons do not penetrate, but instead grow along, the anterior margin of the inverted V-shaped array, forming a cap anterior to the embryonic chiasm neuron array. One possible explanation for the observed behavior of retinal axons is that neurons at the chiasm express a molecule that inhibits growth cone penetration, a possibility consistent with our finding that CD44 is inhibitory for embryonic retinal axon outgrowth *in vitro*. According to this model, CD44 or another inhibitory molecule expressed by the inverted V-shaped array of chiasm neurons serves to define the shape and location of the optic chiasm on the ventral surface of the embryonic mouse diencephalon.

Inhibitory cues are thought to be involved in the formation of orderly topographic patterns of axon projection within target nuclei (Cox et al., 1990; Walter et al., 1987), and specialized regions such as the midbrain roof plate have also been proposed to contain inhibitory cues and serve as barriers restricting axon growth (Snow et al., 1991). The location of the inverted V-shaped array of chiasm neurons directly in the path of retinal axons at the ventral diencephalon is similar to the positioning of the posterior sclerotome in the path of developing motor axons growing out of the chick spinal cord (Keynes and Stern, 1984; Stern and Keynes, 1987; Tosney, 1988). In the spinal cord, embryonic motor axons avoid growing through the posterior sclerotome, but instead are routed around this population and grow through the anterior sclerotome cells. Evidence suggests that this feature of motor axon pathfinding is in part due to an inhibitory influence present on the cell surface of the posterior sclerotome cells (Oakley and Tosney, 1993). This routing of motor axons by the posterior sclerotome cells is highly reminiscent of the diversion of retinal axons posteriorly and laterally upon encountering the inverted V-shaped array of chiasm neurons.

Is CD44 Involved in the Decision to Cross or Not to Cross the Midline?

The presence of CD44 at the ventral midline at E14, during the time when retinal axons must decide to cross or not to cross the midline, raises the additional possibility that this cell surface molecule might be involved in the ipsilateral versus contralateral pathfinding process. Published studies have shown that an adult-like pattern of axon routing can be detected by the time retinal axons have entered the optic tracts, at E15–E16 (Colello and Guillery, 1990; Godement et al., 1990; Sretavan, 1990; Sretavan and Reichardt, 1993), implying that retinal axons made correct pathfinding decisions as they grew through the optic chiasm earlier at E13–E14. Direct time-lapse analyses of axon pathfinding in live retina–optic chiasm preparations at these ages (Sretavan and Reichardt, 1993) have shown that these ipsilateral and contralateral pathfinding cues are located within a 100 μ m wide region centered on the chiasm midline. Immunohistological evidence here shows that CD44 is present at the midline of the developing optic chiasm at E14 (Figures 5 and 8).

In vitro experiments show that the midline pathfinding cues are most likely membrane-associated molecules that may act to inhibit selectively the growth of ipsilaterally projecting axons from ventral–temporal retina, without affecting contralaterally projecting axons from nasal retina (Wisemann et al., 1993). In the current study, we have found that CD44 can influence embryonic retinal axon growth and does so through an inhibitory mechanism. Although such an inhibitory molecule might function well in helping set up a posterior boundary for the X-shaped retinal pathways, the fact that all axons at E12.5–E13.0 (both contralaterally and ipsilaterally projecting) appear to avoid this CD44-expressing region would make it difficult to envision a simple mechanisms in which CD44 itself also serves as a selective midline cue allowing contralaterally projecting axons to pass while turning away ipsilaterally projecting axons. One possibility, however, is that CD44 present in the elongated strip at the

midline coexists and interacts with different cell surface molecules compared with CD44 in the more posteriorly located inverted V region and thus exhibits modified effects on retinal axons. An alternative is that CD44 at the midline serves to slow down retinal axons, allowing interaction with a separate pathfinding cue that then directs axons to pass through or to turn away from the midline.

Possible Combination of Growth-Promoting and Growth-inhibiting Mechanisms on One Cell Population

Studies of axon growth have provided evidence for a large number of cell surface molecules that promote axon growth (Harrelson and Goodman, 1988; Jessell, 1988; Reichardt and Tomaselli, 1991; Rutishauser and Jessell, 1988; Takeichi, 1991) and diffusible factors capable of attracting axons at a distance (Heffner et al., 1990; Lumsden and Davies, 1983; Tessier-Lavigne et al., 1989). In addition to growth-promoting mechanisms, attention has been directed toward cell surface repulsive cues that inhibit the progress of developing axons (Kapfhammer and Raper, 1987a; Oakley and Tosney, 1993; Raper and Kapfhammer, 1990; Snow et al., 1991; Walter et al., 1987). A recent study has also raised the possibility of secreted factors that act at a distance to inhibit axon growth (Pini, 1993). It seems likely that these mechanisms, placed individually at different positions along a developing pathway, may sequentially act to direct axons along highly specific pathways within the nervous system. Our current study demonstrates that an additional feature of axon guidance may be the coexistence of both axon growth-inhibiting (CD44) and growth-promoting (L1) cues on the same cell surface and suggests that an understanding of how such positive and negative cues interact with one another may help bring about a clearer understanding of axon pathfinding mechanisms.

Experimental Procedures

Animals

Timed pregnant C3H or C57/B16 mice were obtained from an in-house breeding colony or from commercial vendors (Simonsen, Crl:NIH-3T3, CA; Charles River Labs, NY). The morning of vaginal plug detection was counted as E0. Pregnant mice were anesthetized by intraperitoneal injections of sodium pentobarbital (4 mg) for cesarian sections. Following delivery of embryos, adult animals were euthanized by an overdose of sodium pentobarbital delivered intraperitoneally.

Antibodies

The antibodies used in this study include an anti-MAP2 polyclonal antiserum (a gift from S. Halpain and P. Greengard); HM-2, a mouse MAb against MAP2 (Sigma M4403); TuJ1, a mouse MAb against β III neuron-specific tubulin (Lee et al., 1990; Moody et al., 1989; a gift from A. Frankfurter); a rabbit polyclonal antiserum against L1 (Chung et al., 1991; a gift from C. Lagenaur). This antiserum binds major protein bands of 200K and 140K from embryonic mouse retina (D. W. S. and L. F. R., unpublished data), consistent with it recognizing mouse L1 (Rathjen and Schachner, 1984). Anti-CD44 antibodies used included rat MAbs KM201 (IgC₁; Trowbridge et al., 1982), IM7 (IgC_{2b}; Miyake et al., 1990a), and IRAW 14.4 (IgG; Lesley et al., 1992).

Immunostaining of Cryostat Sections

Embryos delivered via cesarian section were dissected in 4°C artificial cerebrospinal fluid (ACSF; 150 mM NaCl, 5 mM KCl, 1.15 mM KH₂PO₄, 1.15 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, 25 mM NaHCO₃, 10 mM D-glucose) to remove cranial cartilage and expose the underlying brain tissue to 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.35) at 4°C. After fixation for 1–2 hr, tissues were infiltrated with 30% sucrose in 0.1 M sodium phosphate

(pH 7.35) for 12–24 hr at 4°C. Sections (10 µm thick) were cut on a cryostat and mounted on gelatin-coated glass slides for immunostaining. Tissue sections were preincubated with 10% normal goat serum in 0.1 M sodium phosphate buffer (with 0.1% Triton X-100 for MAP2 and β III tubulin staining) for 30 min at room temperature and then incubated with primary antibody in 1% normal goat serum for 1–2 hr at room temperature. Sections were then washed four times for 5 min each in 0.1 M sodium phosphate buffer and incubated with secondary antibody for 1 hr at room temperature. As secondary antibodies, goat anti-rat IgG conjugated to FITC was used at 1:100, and goat anti-mouse IgG conjugated to Texas Red was used at 1:100. Immunoreactivity detection using diaminobenzidine (1 mg/ml in 0.05 M Tris, pH 7.35) was accomplished using a biotinylated goat anti-mouse antibody (Vector Labs) at 1:200 for 30 min at room temperature, followed by incubation with the avidin–biotin–peroxidase complex at 1:100 for 45 min at room temperature.

Immunostaining of Ventral Diencephalon Whole Mounts

The region of the ventral diencephalon together with the optic nerves was dissected out from 4% paraformaldehyde-fixed tissues and incubated in 10% normal goat serum, 0.5% Triton X-100 in 0.1 M phosphate buffer for 1 hr at 25°C on an orbital shaker. Whole mounts were then immunostained for 14–16 hr at 25°C with primary antibodies (anti-L1, 1:500; anti-CD44, 10 µg/ml) diluted in 1% normal goat serum. After four 20 min washes with 0.1 M PBS, whole mounts were incubated with appropriate biotinylated secondary antibodies (Vector Labs) for 1–2 hr at 25°C. Immunoreactivity was then visualized using the avidin–biotin–peroxidase method and diaminobenzidine.

Dil and DiO Labeling of Embryonic Tissues

Embryos delivered via cesarian section were dissected in 4°C ACSF to remove cranial cartilage and expose the underlying brain tissue to fixative and fixed for 1–3 days in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.35) at 4°C. To label retinal axons anterogradely, the cornea was removed and Dil crystals (D282, Molecular Probes) picked up by the tip of glass micropipettes were placed into the central retinal region to label all axons exiting through the optic disc (Sretavan, 1990). To label the axons of the embryonic optic chiasm neurons anterogradely, Dil was implanted at the ventral midline of the diencephalon in the vicinity of the chiasm neuron cell bodies. In separate experiments, Dil or DiO crystals were implanted in the region of the lateral wall of the diencephalon in the vicinity of chiasm neuron axons to label chiasm neurons retrogradely.

Following dye labeling, tissue was stored in 4% paraformaldehyde at 37°C for approximately 1 week to allow for the diffusion of dye. Embryonic tissue was then embedded in 3% agarose and sectioned at 50 µm using a vibratome. Vibratome sections were wet mounted onto a microscope slide in 0.1 M phosphate buffer. Dil-labeled cellular elements were visualized using rhodamine fluorescence optics; DiO-labeled material was visualized using fluorescein optics.

Protein Immunoblots

E12–E18 mouse embryos were dissected in ACSF at 4°C; postnatal mice were anesthetized using hypothermia before dissection. With the aid of a dissection microscope, the diencephalon was isolated and cut into dorsal and ventral halves (E12–E14 embryos) or into dorsal and ventral thirds (E15–P0 animals). The ventral-most diencephalon pieces from 10–25 embryos were collected and homogenized in Ca^{2+} -, Mg^{2+} -free PBS, 1 mM EDTA, with the protease inhibitors leupeptin (0.1 mg/100 ml), N-ethylmaleimide (2.5 mg/ml), phenylmethylsulfonyl fluoride (0.2 mg/ml), and pepstatin (0.1 mg/100 ml). The homogenate was then centrifuged at $10,000 \times g$ for 30 min, and the supernatant was discarded. The pellet was then extracted in homogenizing buffer containing 0.5% deoxycholate and subjected to centrifugation at $10,000 \times g$ for 30 min. The supernatant was collected, and the amount of protein extracted was

quantified using the Amido-Schwartz method (Schaffner and Weismann, 1973). Approximately 50 µg of protein was loaded for each lane in 7.5% nonreducing SDS gels. After electrophoresis, protein bands were transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked using 5% non-fat dry milk for 30 min at 25°C and incubated with 10 µg/ml primary antibody (KM2011 in 0.1 M sodium phosphate buffer for 1 hr at 25°C. Following four 5 min washes using 5% non-fat dry milk, secondary antibody (1:1000, anti-rat IgG conjugated to alkaline phosphatase; Promega) was applied for 1 hr at 25°C. After four 5 min washes, nitrocellulose membranes were placed into alkaline phosphatase buffer (5 mM MgCl₂, 0.1 M NaCl, 0.1 M Tris-HCl [pH 9.0]), and alkaline phosphatase was visualized using 5-bromo-4-chloro-3-indolyl phosphate (15 mg/ml) and nitro blue tetrazolium (30 mg/ml).

Isolation of Peripheral Blood Lymphocytes

Blood from adult mice was collected into heparinized tubes and diluted with 1 vol of PBS. Four milliliters of diluted blood was then layered onto 3 ml of Ficoll-Paque (Pharmacia) and centrifuged at 400 × g for 40 min at 20°C. Peripheral blood leukocytes at the plasma/ficoll-Paque interphase were collected and subjected to centrifugation on a discontinuous sucrose density gradient (4%–20% [w/v]) layered onto Ficoll-Paque to separate lymphocytes and platelets. After centrifugation at 200 × g for 15 min at 20°C, lymphocytes were collected from the sucrose/Ficoll-Paque interphase. Membrane protein fraction were obtained from isolated lymphocytes after extraction using 0.5% deoxycorticosterone as described above. Protein quantitation, SDS electrophoresis, and immunoblotting were carried out as described.

Neuraminidase and Chondroitinase ABC Enzymatic Digests

Membrane protein fractions obtained from embryonic mouse ventral diencephalon tissues were subjected to digestion with 0.01 U of neuraminidase (from *Vibrio cholerae*; Boehringer Mannheim #1080725) for 8 hr at 37°C or with chondroitinase ABC (from *Proteus vulgaris*; Seikagaku #100332) in 0.2 M Tris, 0.06 M sodium acetate (pH 8.0) for 8 hr at 37°C. Following digestions, proteins were separated using SDS gel electrophoresis, and immunoblotting was performed as described.

Reverse Transcription PCR Analysis

RNA was isolated from embryonic ventral diencephalon tissue using the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Following dissection, ventral diencephalon tissue was immediately placed into 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Total cellular RNA was then extracted using phenol-chloroform and precipitated using isopropanol. Poly(A)⁺ mRNA was selected from total cellular RNA using oligo(dT)-cellulose (Micro-Fast Track, Invitrogen). The downstream 3' reverse primer designed for CD44 PCR analysis (see below) was used as the primer to direct reverse transcription cDNA synthesis from isolated mRNA. Reverse transcription was carried out using 1 µl of a 20 mM solution of reverse primer at 42°C for 2 hr with addition of new reverse transcriptase after the end of 1 hr.

PCR was carried out using cDNA template from 2 µl of the reverse transcription reaction, 1.5 mM Mg²⁺, the downstream reverse primer, 5'-CCCAATCTTCATGTCCACAC-3', and one of the following upstream primers: (1) 5'-CGGACCGTGGAAGACACCAA-3'; (2) 5'-CTCATCATCTTGCCATCTCT-3'; (3) 5'-AGAGATCCAGACTCATCCAA-3'; (4) 5'-GCCTCCACCATCGACAAGAG-3'; (5) 5'-TCCCATCTCACAGAAGGGAC-3'; (6) 5'-ACAACCACCACCATGACTGA-3'. The number designation of each primer corresponds to the numbers indicated in Figure 7B. PCR was initiated with a hot start using a denaturing temperature of 94°C for 1 min, annealing at 60°C for 1 min, and extending at 72°C for 3 min for a total of 35 cycles. PCR products were analyzed after electrophoresis using a 1% agarose gel in electrophoresis buffer containing 0.5 µg/ml ethidium bromide.

Generation of Cell Lines

Full-length cDNA insert encoding the short (hematopoietic) form of mouse CD44 was inserted into the HindIII and NsiI sites of the pCDNA/neo plasmid vector (Invitrogen). K562 erythroleukemia cells were transfected with electroporation using 2.5×10^6 cells in 250 ml of serum-free RPMI in the presence of 10 μ g of plasmid DNA. Twenty-four hours after electroporation, transfected cells were placed into selection medium containing G418 (0.65 mg/ml bioactive). After 2 weeks, individual colonies were identified, isolated, and expanded. For some cell lines, transfected cells were also first selected for the surface expression of CD44 by passage through a fluorescence-activated cell sorter (Becton-Dickinson). Single cells were then sorted into individual wells of 96 well plates for expansion. Individual cell lines were screened for expression of CD44 using protein immunoblots with detection with KM201, a rat MAb recognizing mouse CD44.

Cell Surface Expression of CD44

The surface expression of CD44 in transfected K562 cell lines was verified by immunolabeling using live cells. CD44⁺ cells were harvested and incubated at 37°C for 20 min with anti-CD44 rat MAb KM201 at a concentration of 10 μ g/ml in RPMI containing 10% fetal calf serum (FCS). Labeled cells were then washed twice in RPMI plus 10% FCS for 5 min and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at 25°C. Fixed cells were then incubated with 10 μ g/ml goat anti-rat IgG secondary antibody conjugated to FITC for 20 min at 37°C. Following two 5 min washes in RPMI plus 10% FCS, propidium iodide (2 μ g/ml) was added to the cell suspension for 1 min to label all cell nuclei. Labeled cells were then gently pelleted, resuspended in Celvatol containing 5% n-propyl gallate, and placed on a glass slide for analysis. Untransfected K562 cells were stained in an identical manner to serve as controls for nonspecific immunostaining.

Collagen Gels Seeded with Membrane Fragments

Collagen gels were made using bovine collagen at 1 mg/ml (Vitrogen-100, Celtrix Pharmaceuticals) suspended in L15-CO₂ medium with 10% FCS. The pH of the collagen was adjusted to pH 7.35 using sterile filtered NaOH.

Membrane fragments from untransfected and transfected K562 cell lines were obtained by homogenization in Ca²⁺-, Mg²⁺- free PBS with 0.32 M sucrose, 1 mM EDTA, leupeptin (0.1 mg/100 ml), N-ethylmaleimide (2.5 mg/ml), phenylmethylsulfonyl fluoride (0.2 mg/ml), and pepstatin (0.1 mg/100 ml). Following homogenization, centrifugation was carried out at $100 \times g$ for 10 min at 4°C to remove any remaining intact cells. The supernatant was saved and subjected to centrifugation at $10,000 \times g$ for 10 min to pellet membrane fragments. Membrane fragments were then resuspended in collagen gel at 4°C with each milliliter of the collagen gel membrane fragment mixture containing fragments from about 5.5×10^6 cells, corresponding to a protein concentration of about 11 mg/ml.

Retinal Explant Outgrowth Assay

E12.5–E13.0 embryos delivered by cesarian section were placed in 4°C ACSF, and the corneas were removed by microscissors. After discarding the lens and vitreous material, the retina was separated from the scleral tissue and isolated. Explants were made by cutting the retina into equal pieces. E12.5 retinas were cut into four pieces, and E13 retinas were cut into eight pieces. Retina pieces were then maintained at 37°C in L15 medium, supplemented with 25 mM NaHCO₃ and 10% FCS, prior to placement in collagen gels.

Retinal explants were placed into individual wells of 96 well plates, each holding 40 μ l of collagen gel–membrane fragment mixture containing fragments from either transfected or

untransfected cell lines. Collagen gels containing retinal explants were then placed at 37°C in a 5% CO₂ incubator for 30 min to allow gelation to occur. Forty microliters of L15 medium supplemented with 25 mM NaHCO₃, 10% FCS, and penicillin–streptomycin was then added to each well. After 72 hr, retinal explants were fixed with 4% paraformaldehyde, and the total length of axon fascicles extending from the explants was measured.

Collagen gel explants were also cultured in the presence of anti-CD44 MAbs in order to block the effects of CD44 on retinal axon growth. Three MAbs, KM201, IM7, and IRAW 14.4, were used separately, each at a concentration of 25 µg/ml. Antibody was added to the collagen mixture prior to gelation and to the culture medium. One set of experiments contained a mixture of all three MAbs, each at a concentration of 25 µg/ml.

Hyaluronidase (20 TRU/ml from *Streptomyces hyalurolyticus*; Seikagaku #100740) was added to the collagen gel prior to gelation and was included in the culture medium. Hyaluronic acid (from human umbilical cord; Seikagaku #400724) at a concentration of 0.1 mg/ml was added to membrane preparations from CD44⁺ cells and incubated for 1 hr at 25°C. Membranes were then washed three times for 10 min each in culture medium prior to use in collagen gels.

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Figure 1. Schematic Diagrams of the Ventral Surface of the Embryonic Mouse Diencephalon at E11, E12–E13, and E15–E16, Showing the Relationship between the Retinas, Optic Nerves, and Optic Chiasm

RET, retina; ON, optic nerve; OC, optic chiasm. The shaded regions represent the approximate extent of the retinal axon projections into the mammalian brain at different ages. Concomitant with the formation of the X-shaped pattern of axon pathways on the ventral diencephalon, axons from nasal retina cross the midline to grow into the contralateral optic tract, whereas axons from a group of ganglion cells in ventral–temporal retina turn away from the midline to grow into the ipsilateral optic tract. The rectangular box in the E11 diagram outlines the approximate region shown in Figures 2A and 2B.

Figure 2. Sections through the Future Optic Chiasm Region in E12 Mouse Embryos following Immunostaining with Neuron-Specific Markers or Labeling with Dil

(A) A horizontal section showing the pattern of immunoreactivity in the ventral diencephalon following staining with an anti-MAP2 antibody. Bar, 100 μ m. (B) A horizontal section showing the pattern of immunoreactivity following staining with an anti- β III tubulin antibody. Bar, 100 μ m. Note that at E12, prior to arrival of retinal axons, MAP2- and β III tubulin-immunopositive cells are distributed in an inverted V-shaped array on the ventral diencephalon surface straddling the midline, with the tip of the V pointing anteriorly and the legs of the V extending posteriorly (arrows in [A] and [B] indicate position of the anterior tip of the inverted V neuronal array). (C) A coronal section through the diencephalon following placement of a Dil crystal at the ventral midline to label the axon projections from embryonic chiasm neurons extending along the lateral walls of the diencephalon. Dorsal is toward the top; ventral is below; the midline is at the center. During their course within the diencephalon, the axons of the chiasm neurons run just underneath the pial surface. Bar, 250 μ m. (D–F) Coronal sections of the ventral diencephalon showing embryonic chiasm neurons in an E12.5 embryo that have been backfilled from their axons with Dil. Retrogradely labeled cells are found in the ventral diencephalon below the pial surface (dotted white lines) and are aligned with their axons

running in the medial-lateral direction. These cells have long axons and multiple dendrite-like short processes emanating from their cell bodies. The midline is not present in (D)–(F). Bar, 30 μm .

Figure 3. Whole Mounts of the Ventral Embryonic Diencephalon following Immunostaining with a Polyclonal Antibody Directed against the Cell Surface Molecule L1

Anterior is toward the top; the midline is at the center. OS, optic stalk (called the optic nerve after retinal axons have entered). The two eyes normally present at the ends of the optic stalks were not included in these preparations. (A) An immunolabeled whole mount from an E11 embryo. At this stage, retinal axons have yet to enter the diencephalon region. However, immunostaining with anti-L1 antibody reveals that L1-positive axons are already present in the future region of the optic chiasm arranged in an inverted V shape. Bar, 300 μ m. (B) Ventral diencephalon whole mount from an E12 embryo. At this age, retinal axons (arrowheads), which are also immunoreactive for L1, have begun to invade the ventral diencephalon but have yet to reach the chiasm neurons. Bar, 200 μ m. (C) Whole mount from an E13 embryo. By this stage, a large number of retinal axons have grown into the diencephalon and are intermixed with the cell bodies and processes of the embryonic chiasm neurons. Note that retinal axons leaving the optic nerve to enter the ventral diencephalon do not grow straight, but on average all turn about 45° posteriorly (see arrows). As a result of this turn, axons head toward the inverted V-shaped array of chiasm neurons and their processes. Bar, 200 μ m.

Figure 4. Horizontal Sections through the Ventral Diencephalon of E13 Embryos Showing the Relationship between Incoming Retinal Axons and Embryonic Optic Chiasm Neurons and Their Processes

The retinal axons from one eye have been anterogradely labeled from the retina with Dil, and in these photographs appear yellow-orange; chiasm neurons and their processes have been retrogradely labeled from their axons with DiO placed on both sides of the diencephalon and appear green. The dotted white lines represent the borders of the V-shaped array of embryonic optic chiasm neurons. Retinal axons, upon entering the ventral diencephalon, grow into and overlap with the anterior one-third of the inverted V-shaped array of chiasm neurons. Retinal axons, however, do not penetrate further into this array, but instead turn and either cross the

midline to head to the other side (A) or grow away from the midline to project ipsilaterally (B). The midline in (A) is in the center. In (B), the midline is toward the left edge. Bars, 50 μm (A); 25 μm (B).

Figure 5. Sections through the Ventral Diencephalon of E12 Embryos Showing Immunostaining of Embryonic Optic Chiasm Neurons with Anti-CD44 MAb and Anti- β III Tubulin Antibody (A–C) Horizontal section through the future optic chiasm region showing the pattern of immunoreactivity obtained with each of the anti-CD44 MAb: (A) KM201, (B) IM7, (C) 18C8. The staining pattern is similar with all three antibodies and appears as an inverted V shape, corresponding to the distribution of the embryonic chiasm neuron cell bodies as revealed by anti-MAP2 and anti- β III tubulin staining. (The plane of section in [C] is at a slight angle compared with the others.) The dotted line in (A) represents the plane of section for (D). Bars, 100 μ m. (D) A coronal section through the ventral diencephalon region showing embryonic chiasm neurons stained with MAb KM201. Glial and neuroepithelial cells are not stained. The

dotted white line indicates the ventral pial surface. Bar, 40 μm . (E and F) Coronal section through the ventral diencephalon of an E12 mouse embryo showing embryonic chiasm neurons following immunostaining with an anti- β III tubulin antibody (E) and anti-CD44 MAb KM201 (F). Bars, 20 μm . (C and H) Coronal section through the ventral diencephalon of an E14 mouse embryo showing embryonic chiasm neurons following immunostaining with an anti- β III tubulin antibody (G) and anti-CD44 MAb KM201 (H). Bars, 20 μm . The colocalization of β III tubulin and CD44 immunoreactivity on the same cells demonstrates the neuronal identity of CD44⁺ cells. At E14, β III tubulin is present in a large number of neurons, only some of which are CD44⁺.

Figure 6. CD44 Immunoblots

(A) Membrane fractions from the ventral diencephalon of E12 C57/B16 mouse embryos and membrane fractions from peripheral circulating lymphocytes of adult C57/B16 mice. Tissues from the ventral diencephalon contain a single diffuse immunoreactive band at 85K, a finding consistent with the presence of the low molecular weight isoform of CD44. Adult circulating lymphocytes have two immunoreactive bands, a major band at 95K and a minor band at 115K. (B) CD44 immunoblots using membrane fractions from the ventral diencephalon of E12–P0 mice. A single immunoreactive band at 85K is seen during these stages of embryonic development. Higher molecular weight forms were not detected. (C) CD44 immunoblots following enzymatic digestions. Lane 1, undigested membrane protein fraction. (CD44 immunoreactivity following incubation under conditions for digestions appear as a doublet.) Lane 2, neuraminidase digestion; lane 3, chondroitinase ABC digestion. The shift in the molecular weight of CD44 from 85K to 70K after neuraminidase treatment demonstrates the presence of sialic acid. Chondroitinase treatment did not result in a mobility shift of the CD44-immunoreactive band, indicating that CD44 in the embryonic diencephalon is not modified with glycosaminoglycan side chains, a finding consistent with the existence of a low molecular weight form of CD44 in the ventral diencephalon.

Figure 7. Reverse Transcription PCR Analysis of CD44 Isoforms in the Embryonic Ventral Diencephalon

(A) Diagram illustrating the domain structure and posttranslational modifications on both the long and short forms of CD44. Open circles, N-linked glycosylations; closed circles, O-linked glycosylations; closed triangles, glycosaminoglycan side chains; open triangles, cysteine; EX, extracellular domain; TM, transmembrane domain; CYTO, cytoplasmic domain. Alternative splicing of a 132–165 amino acid segment into the extracellular domain creates a long form of CD44 that promotes cellular metastasis (Gunthert et al., 1991). (B) Diagram showing the location of the PCR primers used in this study (see Experimental Procedures for nucleotide sequences of PCR primers). Primer 1 is located in exon 5, primer 2 in exon 15, primer 3 in exon 17 (transmembrane domain), and primer 4 in exon 19 (cytoplasmic tail). Primers 5 and 6 are located in the alternatively spliced extracellular region of the molecule and are located in exon 8 (also known as v4) and exon 10 (also known as v6), respectively. (Exon nomenclature is from Screaton et al., 1993, and Screaton et al., 1992.) (C) PCR products obtained using the primers identified in (B). In each lane, the PCR product was obtained using the reverse primer (RP) together with the upstream primer indicated by the number at the top of the lane. Results from this PCR analysis demonstrate the presence of mRNA encoding the short form of CD44 at the embryonic ventral diencephalon. There was no evidence for mRNA transcripts encoding longer forms of CD44 containing alternatively spliced inserts in the extracellular segment. Numbers at the left of (C) denote size in bases.

Figure 8. E14 Ventral Diencephalon Whole Mount following Immunostaining with Anti-CD44 Mab
Previous studies have shown that contralaterally projecting retinal axons arriving at the ventral diencephalon at E14 cross the midline over to the opposite side whereas ipsilaterally projecting axons do not cross, but instead turn away from the midline to grow into the ipsilateral optic tract (Colello and Guillery, 1990; Godement et al., 1990; Sretavan, 1990; Sretavan and Reichardt, 1993). At this age, CD44 immunoreactivity is present at the midline (arrow) and forms the posterior border of the optic chiasm (arrowhead). ON, optic nerve. Bar, 200 μ m.

Figure 9. CD44 Cell Surface Labeling and Immunoblots from CD44⁺ and CD44⁻ Parental Cell Lines

(A and B) CD44⁺ cells. (A) Staining of nuclei with 4,6-diamidino-2-phenylindole (DAPI) (B) Anti-CD44 immunostaining. (C and D) CD44⁻ parental cells. (C) DAPI staining. (D) Anti-CD44 immunostaining. CD44 is expressed on the surface of transfected cells, whereas untransfected cells demonstrate no immunoreactivity. Bar, 20 μm. (E) CD44 immunoblots. Lane 1, membrane fractions from E12 mouse ventral diencephalon; lane 2, CD44⁻ parental (K562) cells; lane 3, CD44⁺ cell line (high expressor); lane 4, CD44⁺ cell line (low expressor); lanes 5 and 6, transfected cell lines with no detectable expression of CD44. CD44 expressed on transfected cells appears as a diffuse 85K band, similar in size to CD44 present in the embryonic ventral diencephalon.

Figure 10. Montage Showing E13 Mouse Retinal Explants Growing in Collagen Gels under Different Conditions

(A) Retinal explants in the presence of membrane fragments from untransfected parental K562 cells. (B) Retinal explants growing in the presence of membrane fragments from a CD44⁺ cell line. (C) Retinal explants in the presence of CD44⁺ membranes and anti-CD44 rat MAb KM201. (D) Retinal explants in the presence of CD44⁺ membranes and anti-CD44 rat MAb IM7. (E) Retinal explants in the presence of CD44⁺ membranes and anti-CD44 rat MAb IRAW 14.4. Bar, 500 μ m. Retinal explants in collagen gels seeded with parental K562 membranes extended fascicles of axons in a radial fashion from the explant. In explants growing in the presence of CD44⁺ membranes, axon fascicles of similar thicknesses extended in a radial fashion but were on the average much shorter. This decrease in axon fascicle growth was not reversed by anti-CD44 MAb KM201 or IM7. It was, however, partially reversed after the addition of anti-CD44 MAb IRAW 14.4.

Figure 11. Total Length of Axon Fascicles from E13 Retinal Explants Grown in Collagen Gels Seeded with CD44⁺ Membranes in the Presence of AntiCD44Antibody IRAW and Hyaluronidase or Hyaluronic Acid

Each black dot represents the total fascicle length from one retinal explant. The numbers of explants (n), mean fascicle length ($\bar{x}$), and SD are given at the bottom. (A) Compared with explants in the presence of membranes from untransfected parental cells, explants in the presence of CD44⁺ membranes showed on average a 60% decrease in fascicle length ($p < .001$). The addition of hyaluronidase (HA ase) did not effect this inhibitory effect of CD44. (B) Fascicle length of retinal explants grown under the following conditions were compared and determined to be significantly different when analyzed using the two-tailed t test. CD44⁺ +

IRAW versus CD44⁺ ($p < .005$). CD44⁺ + HA ase + IRAW versus CD44⁺ ($p < .005$). Thus addition of hyaluronidase did not affect the ability of IRAW to reverse partially the inhibitory effects of CD44. The same statistical analysis revealed no significant difference in fascicle lengths between CD44⁺ versus CD44⁺ + hyaluronic acid (HA) or CD44⁺ versus CD44⁺ + HAase, indicating that the effects of CD44 on retinal axon growth are likely not to be mediated through its N-terminal hyaluronate-binding region.

Figure 12. Total Axon Fascicle Length from Retinal Explants Growing under Different Conditions
Fascicle length of retinal explants grown under the following conditions was compared and determined to be significant when analyzed using the two-tailed t test. CD44⁻ versus CD44⁺ ($p < .001$), CD44⁺ versus CD44⁺ + IRAW ($p < .01$), CD44⁺ versus CD44⁺ + antibody (Ab) mix ($p < 0.035$). The same statistical analysis revealed no significant difference in fascicle length between retinal explants under the conditions of CD44⁺ versus CD44⁺ + KM201 MAb or CD44⁺ versus CD44⁺ + IM7 MAb.

Figure 13. Summary Diagram of Findings

Top: at E11, a group of early generated CD44⁺ neurons is present as an inverted V-shaped array at the future region of the optic chiasm prior to the arrival of retinal axons. Middle: at E12–E13, L1-expressing retinal axons have begun to arrive and become closely associated with L1/CD44 embryonic chiasm neurons and their processes. Bottom: by E15/E16, retinal axons have established an X-shaped optic chiasm within which ipsilaterally and contralaterally projecting axons are specifically routed into the correct optic tracts. Throughout the period when initial retinal pathways are laid down at the ventral diencephalon, CD44⁺ neurons are present about the midline and define the posterior boundary of the developing optic chiasm. The X-shaped optic chiasm is established anteriorly, capping the inverted V array of chiasm neurons.