Glia, neurons, and axon pathfinding during optic chiasm development

Carol A Mason* and David W Sretavan†

The importance of vision in the behavior of animals, from invertebrates to primates, has led to a good deal of interest in how projection neurons in the retina make specific connections with targets in the brain. Recent research has focused on the cellular interactions occurring between retinal ganglion cell (RGC) axons and specific glial and neuronal populations in the embryonic brain during formation of the mouse optic chiasm. These interactions appear to be involved both in determining the position of the optic chiasm on the ventral diencephalon (presumptive hypothalamus) and in ipsilateral and contralateral RGC axon pathfinding, developmental events fundamental to binocular vision in the adult animal.

Addresses
*Departments of Pathology, and Anatomy and Cell Biology, Center for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, New York 10032, USA; e-mail: cam4@columbia.edu
†Departments of Ophthalmology and Physiology, University of California, San Francisco, 10 Kirkham Street, San Francisco, California 94143, USA; e-mail: dws@itsa.ucsf.edu

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Abbreviations

E	embryonic day
GFAP	glial fibrillary associated protein
MAP	microtubule-associated protein
POC	postoptic commissure
RGC	retinal ganglion cell
Shh	Sonic hedgehog
TPOC	tract of POC

Introduction

In the mammalian visual system, retinal ganglion cell (RGC) axons from the two optic nerves grow towards one another to meet at the ventral midline of the diencephalon where they establish an X-shaped axonal pathway intersection called the optic chiasm. In animals with binocular vision (such as mammals), but not in lower vertebrates (such as birds, amphibia and fish), RGC axons originating from the nasal retina cross the midline to project into the contralateral optic tract, while a population of RGC axons from the temporal retina do not cross, but project away from the midline into the ipsilateral optic tract. In mouse, ipsilaterally projecting RGCs are found in a ventral–temporal crescent of the retina, whereas contralaterally projecting RGCs are found throughout the retina [1–3].

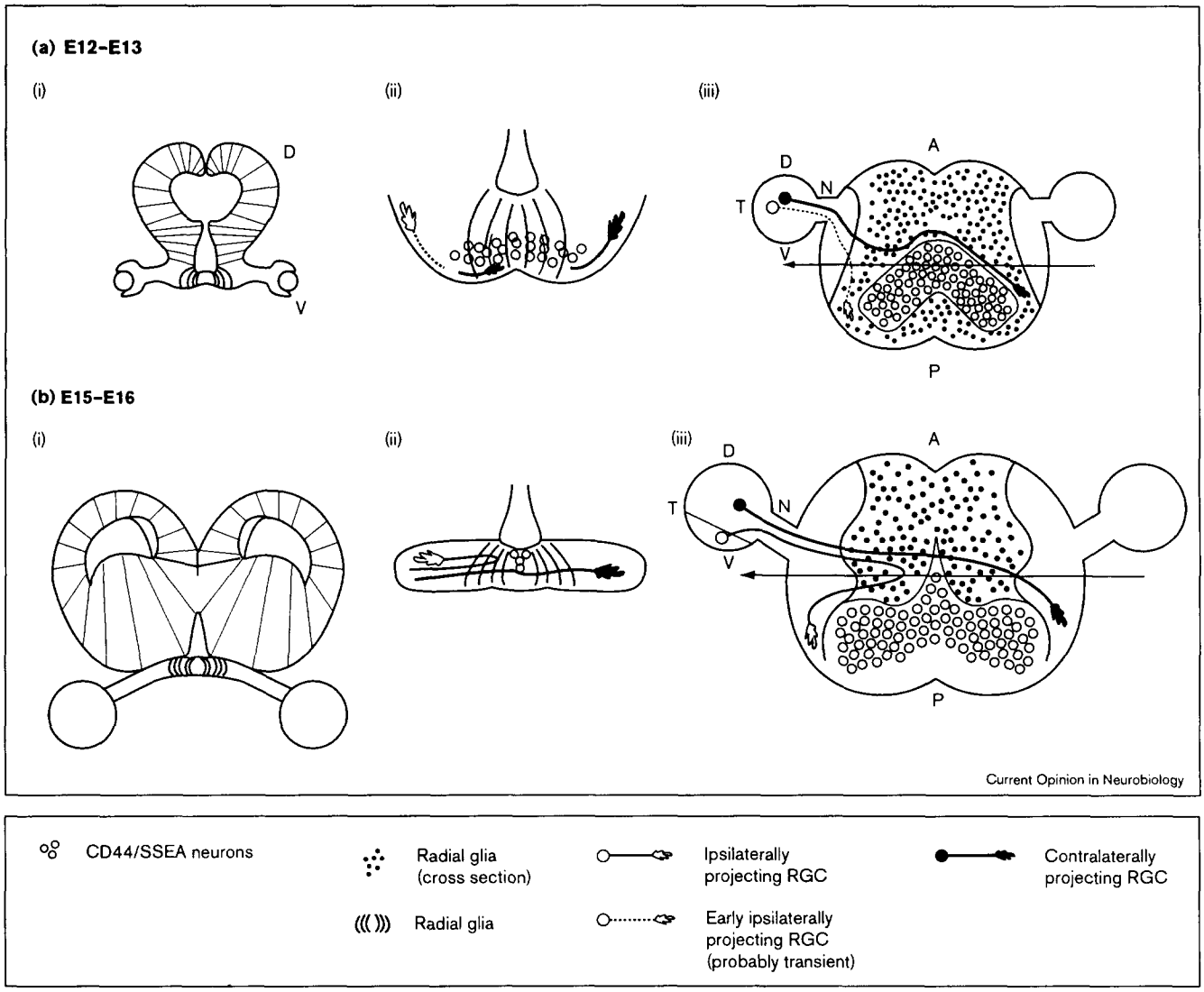
In this review, we discuss the functions of both radial glia and early generated neurons located in the developing ventral diencephalon (the presumptive hypothalamus) during RGC axon ingrowth to form the optic chiasm. The emphasis will be on studies examining the role of these resident cells, principally in mammals, particularly in rodents. The anatomical features of these cells, coupled with knowledge of the trajectory and behavior of retinal axons within the chiasm, make this a good model system with which to study cues for axon guidance and neuron–glia interactions. We will also discuss new evidence for support of neurite growth by radial glia, the role of early born neurons in patterning the optic chiasm, and regulatory gene expression during the establishment of domains in the ventral forebrain. In addition, recent studies from *Drosophila* and zebrafish allow us to draw parallels between systems in these species and the mouse optic chiasm, so as to elucidate the function of specialized midline glia and early neurons in the development of commissural projections.

Development of RGC axon projections at the optic chiasm

During mouse development, 8–10 days are required for the first to the last of over 100,000 RGC axons from each eye to grow into the developing brain. The formation of the optic chiasm appears to occur in two separate phases (see Figure 1). In the first phase, early generated RGC axons originating from dorsal–central retina reach the developing ventral diencephalon at embryonic day E12–E12.5 and grow across the ventral midline to establish the correct position of the X-shaped optic chiasm [4–6]. A number of these early axons, instead of crossing the midline, project into the ipsilateral side of the brain, forming a transient ipsilateral projection. RGC axons from more peripheral parts of the retina enter the chiasm later, at E13–E14, and make specific pathfinding choices such that the adult-like pattern of chiasmatic axon routing into the ipsilateral and contralateral optic tracts is established by E15–E16 [4–8]. Given that the essential pathfinding features of the optic chiasm are established quite early, studies have focused on RGC axon behavior from E12.5 to E16, when RGC axon interaction with local cells of the developing ventral diencephalon are presumably taking place.

The embryonic ventral diencephalon region in which RGC axons form the optic chiasm becomes part of the hypothalamus later in the adult. During initial RGC axon ingrowth, some hypothalamic neurons have undergone final mitosis, but the hypothalamus is not fully formed

Figure 1



Diagrams showing the resident cell types in the ventral diencephalon and the trajectories of RGC axons during formation of the optic chiasm. **(a)** Early phase of RGC axon ingrowth (E12–E13). **(b)** Later phase of RGC axon ingrowth (E15–E16). **(a,b)** (i) Schematics of frontal sections through the brain at the level of the developing optic chiasm at E12–E13 and at E15–E16, respectively. (ii) Higher magnification views of the region of the ventral diencephalon where RGC axons establish the optic chiasm. (iii) Horizontal views of the region of the ventral diencephalon where RGC axons establish the optic chiasm. Arrows here indicate the approximate planes of sections for the views depicted in (ii). (a) During E12–E13, the earliest generated RGC axons originating from dorsal-central retina (thick lines and filled growth cones) grow in close relationship to the inverted V-shaped array of CD44/SSEA neurons (circles) at the developing ventral diencephalon to grow contralaterally across the midline. However, a population of RGC axons, thought to be transient – dotted lines in (a) parts (ii) and (iii) – turn lateral to the early neurons and do not cross the midline. Note, the pathfinding mechanisms that direct these early transient 'ipsilaterally' projecting RGC axons from dorsal central retina are probably different from those that govern the specific ipsilateral pathfinding of RGC axons originating from ventral temporal retina (see below). Overall, the period of E12–E13 is characterized by the establishment of the nascent X-shaped optic chiasm at its correct location on the ventral surface of the developing diencephalon. (b) By E15–E16 an adult-like pattern of ipsilateral and contralateral RGC axon pathfinding is present. A group of RGC axons originating from ventral-temporal retina have turned away from the midline to project into the ipsilateral optic tract, whereas axons originating from all regions of the retina have crossed the midline to project into the contralateral optic tract. RGC axons appear to make pathfinding decisions to cross or not cross the midline as they grow within a palisade of RC2-positive radial glial cells: short lines in (a) parts (i) and (ii) and in (b) parts (i) and (ii); dots in (a) part (iii) and (b) part (iii). This glia palisade is positioned on both sides of the midline. These glia are specialized compared to most radial glial cells elsewhere (thinner long lines). Note the absence of RC2-positive glia in optic nerves and regions lateral to where the chiasm forms. At the later phase, note the anterior extension of CD44/SSEA neurons at the midline of the developing optic chiasm. Data compiled from [4–6,8,12,36].

and hypothalamic nuclei cannot yet be identified in their final location [9,10]. Despite the relative immaturity of this region, embryonic RGC axons do not simply encounter an undifferentiated neuroepithelium. Rather, the site in the developing hypothalamus at which the chiasm will form contains radial glia and early generated neurons, both of

which appear to present guidance information to RGC axons.

Radial glia and optic chiasm development

One of the cellular specializations localized to the site at which the chiasm will form is a palisade of radial glia draped along either side of the midline, occupying the midline zone at which retinal axon divergence occurs [8,11,12] (Figure 1). The palisade was revealed in mouse brain by monoclonal antibody RC2, a marker for radial glia throughout the embryonic murine CNS [13]. The dense concentration of the radial glia around the midline is in striking contrast to the more sparse, RC2-positive process-bearing or interfascicular glia in the lateral portions of the chiasm and optic nerves. The architecture of the glial palisade and its location at the ventral midline are reminiscent of the floor plate in the spinal cord, a major midline locus mediating commissural or crossing fiber trajectories [14].

In contrast to other radial glia at this level in the brain, the radial glial palisade, like the floor plate, is unique in that these glia express a number of molecules such as annexin, a substrate for the epidermal growth factor receptor tyrosine kinase [15,16]. In addition, several members of the family of Eph receptor tyrosine kinases are localized to the midline glial palisade but not to surrounding radial glia (RC Marcus *et al.*, *Soc Neurosci Abstr* 1996, 22:970; RC Marcus *et al.*, unpublished data).

Function of the chiasmatic midline glia

Because retinal growth cones segregate into crossed and uncrossed components within the midline glial palisade [8], we tested whether this site elicited differential retinal axon growth in a dissociated cell culture model [17]. In co-cultures of retinal explants and a mixed population of midline glia and early neurons (described below), crossed RGC axons grow more extensively than uncrossed neurites upon contacting these cells. Similar results were observed on membranes isolated from this region [18]. However, the glial cells themselves do not appear to signal RGC divergence; instead, they appear to support growth of both crossed and uncrossed retinal neurites. Analysis of the response of RGC growth cones to glia and neurons from the optic tectum [19•] also showed that the posterior tectal cue underlying the collapse of temporal retinal growth cones is present on tectal neurons, while the RGC response to neuroepithelial cells (presumably including radial glia) is less dependent on topographic origin, suggesting that glia present general cues. Further, consistent with previous studies on neuronal–glial interactions [20,21], RC2-positive glia are radial only when positioned in clusters of other neurons and glia, whereas lone glia have flat shapes (see also DP Sullivan, JSH Taylor, *Soc Neurosci Abstr* 1995, 21:1295).

Together, these data suggest that cell–cell interactions between chiasmatic radial glia and the early neurons are important for presenting the signal(s) for pathfinding of crossed and uncrossed axons, as well as for maintenance of glial morphology (see Note added in proof). RGC growth cones contact the midline radial glia as optic axons segregate into the ipsilateral and contralateral components [8], suggesting that midline glia could provide information important for guidance. Such an interaction has been implicated in the *Drosophila* nervous system, where midline glia mediate commissure formation in the CNS [22]. Commissureless, a factor expressed in midline glia, is transferred from glia to axons during passage of axons through the midline [23••]. Evidence of cell–cell transference has also been observed between floor plate cells and commissural axons [24]. Whether such interactions between midline glia and RGC axons take place remains to be elucidated.

Where do the glia come from and where do they go?

Little is known about the lineage of the specialized radial glia of the chiasmatic midline. In *Drosophila*, genes that are key to a switch in neuronal versus glial fate have been identified. These genes, however, specify lateral and other glia, but not midline glial cells [25•], an additional indication that the midline glia in commissural pathways are unique [26].

The early development of the ventral diencephalon and presumptive hypothalamus is incompletely understood. Functional analogies and overlapping expression patterns of the chiasmatic midline and floor plate suggest that inductive events may be similar at these two sites. However, new analyses indicate that the floor plate and rostral diencephalic ventral midline are both induced by axial mesoderm: the floor plate by the notochord and the rostral ventral midline by the prechordal mesoderm. However, while Sonic hedgehog (Shh) is required for induction of the ventral midline at both poles of the neuraxis, bone morphogenetic protein 7 (BMP7) additionally drives the response of neural cells toward differentiation of rostral midline properties [27••]. Such differences in early development may account for later differences in epitope expression (C Mason, J Dodd, unpublished data).

In late embryonic and early postnatal stages, the midline radial glia, similar to radial glia elsewhere, appear to transit into stellate glial fibrillary associated protein (GFAP)-positive astrocytes [28]. One issue is whether the chiasmatic midline becomes a generative zone in the postnatal period for other types of glia, as proposed for the floor plate [29•]. In line with this, a possibility that remains to be tested is whether oligodendrocyte type 2 astrocyte (O2A) progenitors, known to derive from brain and to migrate into the optic nerve [30,31], originate from this locus.

Midline glia in the chiasm in other species

In other species, radial glia within the chiasm have been difficult to visualize because of the lack of antigen markers

for immature astroglia. Antibodies to GFAP have been utilized to study glia at other midline sites that serve as throughways [32] or act as barriers [33]. Of interest is that in the visual system, the site of segregation of ipsilateral- and contralateral-projecting RGC fibers is marked by the transition point between process-bearing, interfascicular glia in the optic nerve and radial glia in the brain [1]. In eutherians, this transition occurs in the midline glial palisade, whereas in marsupials, the segregation occurs distant to the midline within the prechiasmatic optic nerve [34].

Embryonic neurons and optic chiasm development

The region of the developing hypothalamus in which the optic chiasm will form also contains early differentiated neurons detectable prior to arrival of RGC axons. These cells are of interest because they are the first neurons to appear in this region [35,36], and, as a population, they are arranged in an inverted V-shaped array, which marks the posterior boundary of the X-shaped optic chiasm and the proximal optic tracts [12,36] (Figure 1). In addition to neuronal markers such as microtubule-associated protein 2 (MAP-2) and βIII tubulin, these neurons express L1, a cell adhesion molecule of the immunoglobulin superfamily, and CD44, a cell surface receptor of hematopoietic and endothelial cells [2,36], as well as SSEA-1 [8], a carbohydrate epitope on early stem cells. This epitope contains the Lewis X (Le^X) carbohydrate, recognized by the antibodies used in our studies as well as the FORSE-1 antibody that highlights other populations of early neurons in the forebrain [37,38]. Furthermore, the broad region in which the CD44/SSEA neurons are situated also express the glycosylphosphatidylinositol (GPI)-linked subclass of ligands of the Eph receptor tyrosine kinases (RC Marcus *et al.*, *Soc Neurosci Abstr* 1996, 22:970; RC Marcus *et al.*, unpublished data). The first RGC axons encountering these CD44/SSEA neurons do not grow through this early neuronal population nor do they turn to grow away from these cells; instead, they track along the anterior boundary of this inverted V-shaped neuronal array to establish the X-shaped optic chiasm [12,36].

Positioning of the optic chiasm

Studies of the cell surface molecules expressed by CD44/SSEA neurons and the function of these neurons both *in vitro* and *in vivo* suggest that the interactions of RGC axons with these cells may be complex. For example, these neurons express both L1, which promotes axon outgrowth *in vitro* [39], and CD44, which, in addition to postulated roles in hematopoietic cell–cell interactions [40,41•], appears to be inhibitory for RGC axon growth *in vitro* [36]. Studies of MAP-2-positive embryonic neurons isolated from the developing hypothalamic region show that these neurons inhibit the growth of RGC axons in culture [17], supporting the idea that one function of this neuronal population may be to serve as a posterior boundary during optic chiasm development [36]. However, *in vivo* ablation studies show that in the absence of these neurons, RGC axons fail to grow into the ventral hypothalamus to form an optic chiasm, suggesting that CD44/SSEA neurons may also serve to promote ingrowth [42].

It is not known whether functional heterogeneity exists such that CD44/SSEA neurons in the anterior portion of the inverted V-shaped array promote growth while neurons located posteriorly are inhibitory. The tracking of RGC axons along the anterior border of the neuron array is reminiscent of axons growing at boundaries between two differing substrates *in vitro* [39,43], suggesting that RGC axons sense a relative substrate difference between the CD44/SSEA neurons and the adjacent neuroepithelium. Thus, it is possible that by playing the dual roles of supporting RGC axon growth at its anterior boundary and preventing retinal axons from growing posteriorly, these cells participate in precisely positioning the X-shaped optic chiasm on the developing hypothalamus.

Where do these neurons come from and where do they go?

The expression patterns of several genes, including Nkx 2.2 and Shh, in the embryonic ventral hypothalamus show an inverted V-shaped pattern similar to the inverted V-shaped array of CD44/SSEA neurons (RC Marcus *et al.*, unpublished data). In chick, Shh has been shown to induce Islet-1-positive ventral forebrain neurons [44], which may correspond to the CD44/SSEA neurons in mouse. Although it is not known whether Shh induces CD44/SSEA neurons in mice, the observation that mouse Shh is also expressed in an inverted V-shaped domain in the ventricular zone near these neurons is consistent with this possibility. Of note, studies in zebrafish show that Shh influences the Nk2.2 expression pattern (zebrafish homolog of Nkx 2.2), which in turn demarcates early generated neurons in the nucleus of the tract of the postoptic commissure (TPOC) [45]. Given the spatial correspondence between the CD44/SSEA neurons in mouse with Nkx 2.2 expression (RC Marcus *et al.*, unpublished data), a similar event may occur in mice. If so, then Shh, a signalling molecule involved in the differentiation of the ventral aspect of the nervous system, may also govern the position of the optic chiasm by influencing the correct spatial and temporal induction of neurons carrying guidance information.

The fate of the CD44/SSEA neurons remains unknown at present. They may represent a transient population of neurons similar to subplate cells of the developing cortex [46] or Cajal–Retzius cells in the hippocampus [47]. Indeed, CD44/SSEA neurons may correspond to neurons of the anterobasal nucleus described in the embryonic rat brain [48], which are thought to be transient and eventually disappear, although definitive proof of cell death is lacking. Given the dispersive cell migration patterns in the chick diencephalon [49•], it is possible

that the early generated CD44/SSEA neurons in mouse hypothalamus do not die but migrate away. Alternatively, it is also possible that CD44/SSEA neurons survive and become one of the many hypothalamic nuclei, thus serving both a developmental role and a second adult function. BrdU (5-bromo-2′-deoxyuridine) birthdating shows that CD44/SSEA neurons are generated sometime around E9.5 (L Feng, D Sretavan, unpublished data), suggesting that candidate hypothalamic nuclei include those generated early in development [10]. The answer to this question of cell fate will require the use of appropriate markers to follow these cells.

Chiasm development in mouse and zebrafish

Major differences exist in the interactions of RGC axons with glia and neurons during the formation of the optic chiasm in mouse compared to other species such as zebrafish. Although studies in both mouse [12,36,42] and chick [44] have identified early developing neurons at the ventral hypothalamic midline, the cellular organization of this region in zebrafish appears somewhat different. In zebrafish, neurons apparently do not exist at the ventral midline; instead, there are axons forming the postoptic commissure (POC), whose cell bodies presumably originate from the nucleus of the TPOC located in more lateral parts of the brain [50,51•]. Thus, unlike mouse RGC axons, which seem to use guidance information provided by CD44/SSEA neurons at the midline, zebrafish RGC axons entering the ventral hypothalamus do not have access to such cues nor is it clear whether they interact with POC axons [52].

The relationship between RGC axons and glia in mouse and zebrafish is also quite different. In zebrafish, the two optic nerves approaching the brain are enwrapped by glia into separate bundles and enter the brain neuroepithelium in the region of the contralateral optic tract, their first encounter with radial glia [1,50,53]. In contrast, in mice, individual RGC axons course through the hypothalamic neuroepithelium of the ventral diencephalon to grow amongst the end-feet of radial glia, mixing with axons from the opposite eye. Thus, during optic chiasm development in mice, RGC axons are in a position, before crossing the midline, to interact with both the neuronal and radial glial cells in this region of the developing hypothalamus, while these interactions may not occur, or occur only to a limited extent, in zebrafish.

In addition to differences in RGC axon interactions with neurons and glia, the manner in which optic chiasm development is affected by genes that pattern the ventral hypothalamic region may also be somewhat different in these two species. For example, absence of *Pax2* gene function in mouse leads to a failure of RGC axons to cross the midline to form an X-shaped chiasm [54••], whereas a null mutation of *Noi*, the likely *Pax2* ortholog in zebrafish, leads to variable phenotypes, among which is abnormal RGC axon crossing of the midline into the opposite optic nerve [55••].

The consequences of the aforementioned differences between optic chiasm development in mice and zebrafish are not clear at present. One possibility is that some of these differences relate to the presence in mice of the specific sorting of RGC axons within the chiasm into the ipsilateral and contralateral optic tracts, a pathfinding event characteristic of mammalian visual system that does not exist in zebrafish, in which retinal fibers from each eye project only to contralateral targets.

Conclusions and future directions

Work in the past few years in mice has identified both neuronal and glial cell populations in the developing ventral hypothalamus that appear to provide guidance information to ingrowing RGC axons as they establish the optic chiasm. The evidence points to a role for the inverted V-shaped array of CD44/SSEA neurons in the positioning of the X-shaped optic chiasm on the brain surface and in cooperativity between specialized radial glia and CD44/SSEA neurons for proper ipsilateral and contralateral axon pathfinding.

Future areas of investigation include elucidation of the genes that govern the development of the radial glia and CD44/SSEA neurons and the eventual fates of these cells. Comparison of optic chiasm development between zebrafish and mice is also likely to be informative, and despite their differences, should lead to a deeper understanding of this process in both species. Of major importance are the identities of the axon guidance molecules that direct RGC pathfinding during optic chiasm formation. Efforts are being made to determine whether axon guidance molecules, or their related family members, known to play a role in pathfinding in other brain regions may also be involved in RGC axon guidance during pathfinding in the optic chiasm. Molecules of interest include the netrins (which are involved in commissural axon guidance to the floor plate at the ventral midline of the spinal cord), members of the semaphorin family (which are thought to be involved in pathfinding in a number of different systems), and receptor tyrosine kinases and their ligands (which are involved in axon pathfinding along pathways and in target tissues) [56,57•].

A survey of a limited number of candidates thus far show that netrin-1 and netrin-2 in mouse do not appear to be expressed at high levels in the developing hypothalamic midline region during RGC axon ingrowth (M Deiner, T Kennedy, M Tessier-Lavigne, D Sretavan, unpublished data). However, netrin-1 supports retinal axon growth *in vitro* [58•] and appears to be involved in RGC axon exit at the developing optic disc *in vivo* (M Deiner *et al.*, unpublished data). Likewise, mRNA for semaphorins sema III [59] and sema VIa [60] are not present in the developing hypothalamic region around the chiasm, but sema VIa mRNA is present in the developing mouse

optic nerve [60]. Perturbation of Eph ligands on the CD44/SSEA neurons renders chiasm cells supportive to RGC axon growth *in vivo* and *in vitro* (RC Marcus *et al.*, *Soc Neurosci Abstr* 1997, 23:324; RC Marcus *et al.*, unpublished data), consistent with a role for this family in directing axonal growth in CNS pathways [57•]. Ultimately, the formation of the optic chiasm is probably mediated by the coordinated actions of multiple guidance cues from neurons and glia populating the ventral aspect of the developing hypothalamus.

Note added in proof

A recent ultrastructural study analyzes the changing relationship between RGC growth cones and radial glial cells in the chiasm, optic nerve and entry to the optic tract in E16 mouse embryos [61].

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