Retinal Axon Growth at the Optic Chiasm: To Cross or Not to Cross

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Key Words

axon guidance, binocular vision, growth cone, Ephs/ephrins, patterning, transcription factors

Abstract

At the optic chiasm, retinal ganglion cell axons from each eye converge and segregate into crossed and uncrossed projections, a pattern critical for binocular vision. Here, we review recent findings on optic chiasm development, highlighting the specific transcription factors and guidance cues that implement retinal axon divergence into crossed and uncrossed pathways. Although mechanisms underlying the formation of the uncrossed projection have been identified, the means by which retinal axons are guided across the midline are still unclear. In addition to directives provided by transcription factors and receptors in the retina, gene expression in the ventral diencephalon influences chiasm formation. Throughout this review, we compare guidance mechanisms at the optic chiasm with those in other midline models and highlight unanswered questions both for retinal axon growth and axon guidance in general.

Contents

Optic chiasm: X-shaped commissure on ventral brain surface where fibers from both eyes converge and decussate before projecting to higher visual targets

RGC: retinal ganglion cell

VT: ventrotemporal

Line of decussation: invisible demarcation in the retina of binocular species that separates ipsilaterally and contralaterally projecting retinal ganglion cells

INTRODUCTION

During development, growth cones must navigate through diverse cellular environments and decision regions, simultaneously integrate multiple cues along their pathway, then identify target regions and form synapses with appropriate target cells. Despite significant progress over the past several decades, axon guidance remains one of the most complex problems in developmental neuroscience (reviewed in Yu & Bargmann 2001). Axon pathfinding at the neuraxis midline constitutes an ideal model for studying growth cone behavior in response to guidance cues because a growth cone's decision to cross or not to cross the midline is crucial for establishing proper neuronal connectivity and circuitry of both sensory and motor pathways.

Divergence of retinal axons at the optic chiasm midline implements binocular vision—stereopsis and depth perception—in higher vertebrate species. To establish binocular pathways, information from the visual field of each retina must be transmitted to centers in the thalamus and the cerebral cortex on both sides of the brain. In binocular species, axons from the nasal retina of each eye project contralaterally, whereas axons from temporal retina project ipsilaterally. Animals with eyes near the front of their head have a higher degree of binocular overlap (thus binocular vision) and a higher percentage of ipsilaterally projecting retinal ganglion cells (RGCs) compared with animals whose eyes are displaced more laterally. Humans are highly binocular, with ~40% uncrossed RGC axons. Ferrets have ~15% uncrossed fibers, whereas mice are a poor binocular species with ~3%–5% uncrossed axons, arising exclusively from the most peripheral ventrotemporal (VT) crescent. In most fish and birds, the lateral location of the eyes does not provide for overlap in visual space, and thus the projections from both retinae are entirely crossed (**Figure 1**).

A distinctive aspect of the optic chiasm is that this pathway consists of axons from only one type of sensory neuron, RGCs, whose functions and targets are known. Within the retina, the uncrossed and crossed RGC populations are not intermingled and are separated by a rather sharp line of decussation. These aspects, combined with the accessibility of the retina and optic chiasm for both in vitro assays and in vivo analysis, make the retina-to-optic chiasm pathway a compelling model for analyzing the cellular and molecular mechanisms that guide axons at decision regions.

Here we discuss the development of retinal axon projections as the optic chiasm forms, highlighting older work on the spatiotemporal aspects of retinal axon growth and growth cone dynamics during divergence at the optic chiasm midline. We review guidance mechanisms for the uncrossed retinal projection during repulsion at the chiasm midline and candidate systems that might facilitate traversing

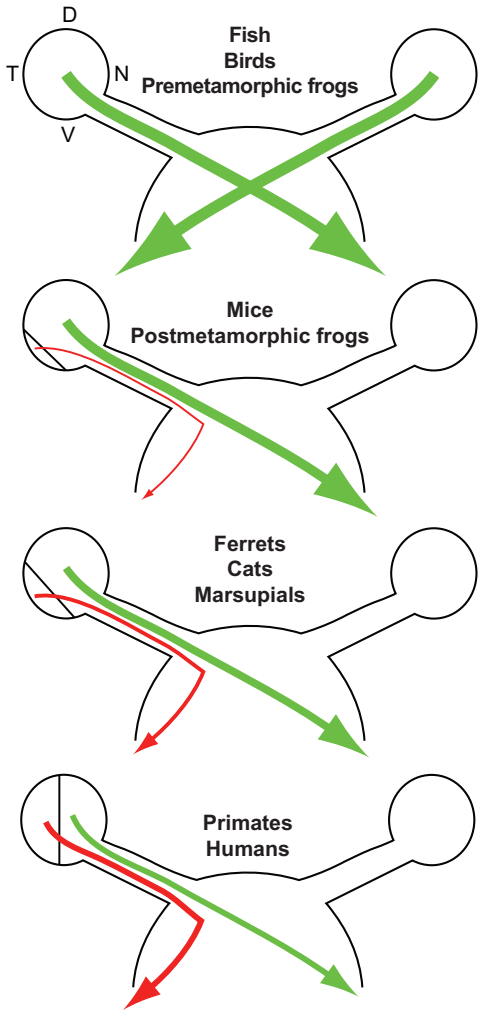

Figure 1

Retinal axon divergence at the optic chiasm of different vertebrate species. Depiction of the origins and relative proportions (but not the precise fiber trajectory) of the crossed and uncrossed retinal projections.

the midline. We then discuss transcription factors that specify RGC identity with respect to the laterality of projection and the development of the cellular terrain of the optic chiasm. Throughout this review, we discuss unresolved issues pertinent to RGC axon guidance at the optic chiasm, many of which are applicable to other midline models and axon guidance in general.

TIME COURSE OF OPTIC CHIASM FORMATION

RGC axon growth during the establishment of the optic chiasm can be divided into three stages. The earliest-born RGCs arise from the dorsocentral (DC) retina and enter the ventral diencephalon at E12–13.5, where they form both a crossed and an uncrossed projection (Colello & Guillery 1990, Godement et al. 1987b, Marcus & Mason 1995) (**Figure 2*a***). Rather than orienting toward the midline, the uncrossed DC fibers extend directly into the ipsilateral optic tract. This early uncrossed projection is transient, but the fate of the uncrossed DC cells is unknown. These initial projections are considered pioneer axons because they appear to demarcate the future site of the chiasm and provide scaffolding for later-born axons, as observed in the zebrafish postoptic commissure (Bak & Fraser 2003). How the pioneer axons from DC retina penetrate and traverse the cellular terrain before the chiasmatic path is established remains unclear (Trousse et al. 2001).

During the peak phase of axon growth in mice from E14 to E17, axons from VT retina approach the chiasm midline and turn back to the ipsilateral optic tract, while axons from all other retinal regions (non-VT retina) traverse the chiasm and project into the contralateral optic tract (Guillery et al. 1995) (**Figure 2*b***).

Crossed RGC projections arise from the expanding peripheral retina until birth. However, during this late phase of RGC axon extension (E17.5–P0), most newborn RGCs in the VT crescent project contralaterally rather than ipsilaterally (Drager 1985) (**Figure 2*c***).

RETINAL AXON INTERACTIONS AND CELLULAR COMPONENTS AT THE OPTIC CHIASM

The insight that midline cues direct retinal axon divergence during optic chiasm formation came from observing the shape and trajectory of growth cones labeled with DiI during the peak growth phase. Whereas axons

DC: dorsocentral

Ventral diencephalon: ventral region of forebrain that includes the hypothalamus, where the optic chiasm forms

Ipsilateral: projecting to the same side of the midline

Pioneer axons: earliest-growing axons that navigate without the aid of preexisting tracts

Contralateral: projecting to the opposite side of the midline

DiI: 1,1′-dioactadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

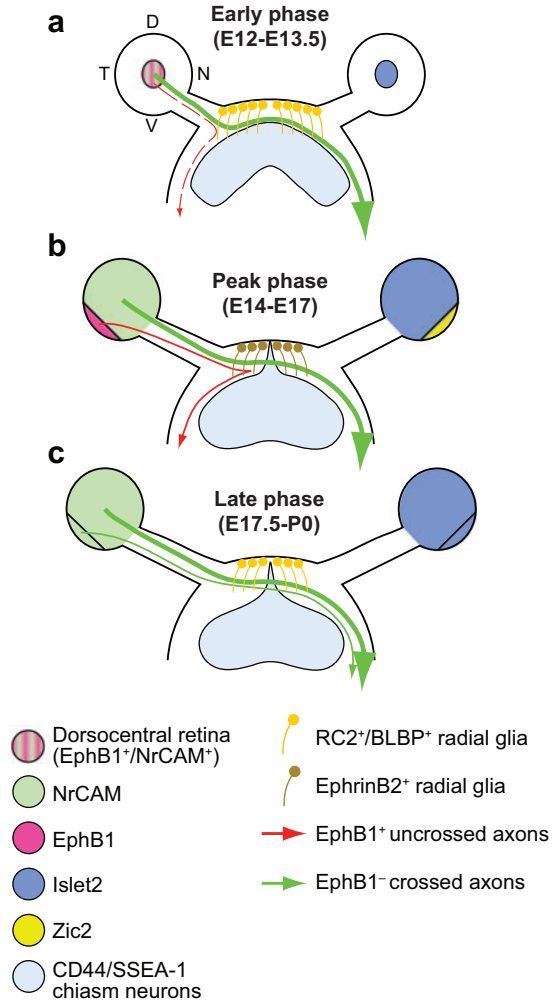

Figure 2

Phases of retinal axon extension during optic chiasm formation. View of the optic chiasm in approximate horizontal plane. Guidance molecules are indicated in the left retina; transcription factors are indicated in the right retina. (*a*) During the early phase (E12–E13.5), retinal fibers originating from the DC retina express *EphB1*, *NrCAM*, and *Islet2* (not *Zic2*) and project to both sides of the brain. Radial glial cells straddle the midline and express RC2 and BLBP. Note that crossing axons traverse the midline glia zone, but the transient uncrossed axons (*dotted line*) do not enter the glial palisade and instead turn directly into the ipsilateral optic tract. Both crossed and uncrossed axons follow the border of the CD44/SSEA-1 neurons. (*b*) During the peak phase (E14–E17), the radial glia cell palisade is more restricted than in earlier ages. Whereas ephrinB2 is weakly expressed in radial glia cells during the early and late phases, it is strongly upregulated in this peak phase. *Islet2* and *NrCAM* are expressed in non-VT (crossed) RGCs, whereas *Zic2* and *EphB1* are expressed in VT (uncrossed) RGCs. At this age, VT axons extend close to the midline before turning ipsilaterally. (*c*) During the late phase (E17.5–P0), *EphB1* and *Zic2* are downregulated in VT retina, and *NrCAM* and *Islet2* expression expands into the VT retina. Note that most late-born RGCs from VT retina project contralaterally.

arising from non-VT retina traverse the midline, uncrossed VT axons extend within several hundred microns of the chiasm midline and then turn sharply toward the ipsilateral optic tract (Godement et al. 1990). Video microscopy of DiI-labeled growth cones revealed the striking behaviors of ipsilaterally- and contralaterally-projecting retinal growth cones at the chiasm midline (Godement et al. 1994, Mason & Wang 1997, Sretavan & Reichardt 1993). All growth cones undergo saltatory growth in the optic nerve but extend rapidly when they advance. Upon entering the midline zone, RGC growth cones undergo cycles of spreading, pausing, and retraction, often lasting for several hours (Godement et al. 1994, Mason & Wang 1997) (**Figure 3**). While growth cones from non-VT retina quickly traverse the midline, the large spread growth cones of VT axons emit filopodia that resemble growth cone protrusions at the border of non-permissive substrates in vitro (Godement et al. 1990, Mason & Erskine 2000). Eventually, a backward-directed filopodium is consolidated, the main growth cone is progressively pruned, and the axon extends in the direction of the consolidated filopodium toward the ipsilateral optic tract (Godement et al. 1994) (**Figure 3**).

In the early 1990s, the advent of cell-specific markers made it possible to visualize and identify glial and neuronal cells at the chiasm midline. Radial glia cells at the base of the third ventricle extend processes that drape the midline of the chiasm (Marcus et al. 1995). Like radial glia in other regions of the immature brain (Lindwall et al. 2007), the chiasmatic midline radial glia express glial markers such as RC2, BLBP, and GLAST (but not GFAP) during the period of RGC axon growth, from E12 to P0 (Williams et al. 2003). In addition to these glial cells, a population of early-born neurons develops caudal to the chiasm and extends a raphe into the midline. The early-born neurons express epitopes such as SSEA-1, expressed by stem cells (Capela & Temple 2002), and CD44, expressed by cells of the immune system (Marcus & Mason 1995, Sretavan et al. 1994).

These immature glia and neurons are analogous to midline cells in the insect midline and vertebrate spinal cord floor plate (reviewed in Edenfeld et al. 2005, Mason & Sretavan 1997).

Colocalization of chiasm cells with DiI-labeled RGC axons revealed that both crossed and uncrossed RGC growth cones enter the glial palisade and intimately intertwine with glial processes (Marcus et al. 1995) (**Figure 3**). Uncrossed axons turn back at the outer edge of the palisade, and crossed axons traverse the palisade and midline raphe of the SSEA-1/CD44 neurons; both axon groups then extend caudally along the border of the chiasm neurons (Marcus et al. 1995, Mason & Sretavan 1997) (**Figure 2**). RGC axons cross the midline through the glial palisade at more dorsal levels, whereas the uncrossed fibers turn more ventrally (Colello & Coleman 1997, K.Y. Chung & C. Mason, unpublished observations). The interaction of axons with midline cells is thought to be important for crossing in other midline models, including forebrain commissures (Lindwall et al. 2007), but the precise cellular and molecular basis for this neuron-glial interaction has not been established.

In vitro studies in which retina explants were cocultured with cells from the chiasm midline indicated that the chiasmatic neurons and glia provide cues for axon divergence. When cocultured with chiasm explants in collagen gels, all retinal neurites display a reduction in outgrowth (Wang et al. 1996), indicating that chiasm cells express diffusible cues that are inhibitory to retinal axons. In addition, upon contacting chiasm cells, VT axons are repulsed while dorsotemporal (DT) axons extend relatively uninhibited, indicating that cells from the chiasm midline have differential contact-dependent effects on RGCs from specific retinal regions (Wang et al. 1995), modeling the divergent growth patterns in vivo. These results set the stage for investigating which family of guidance cues could underlie this differential response of crossed and uncrossed axons.

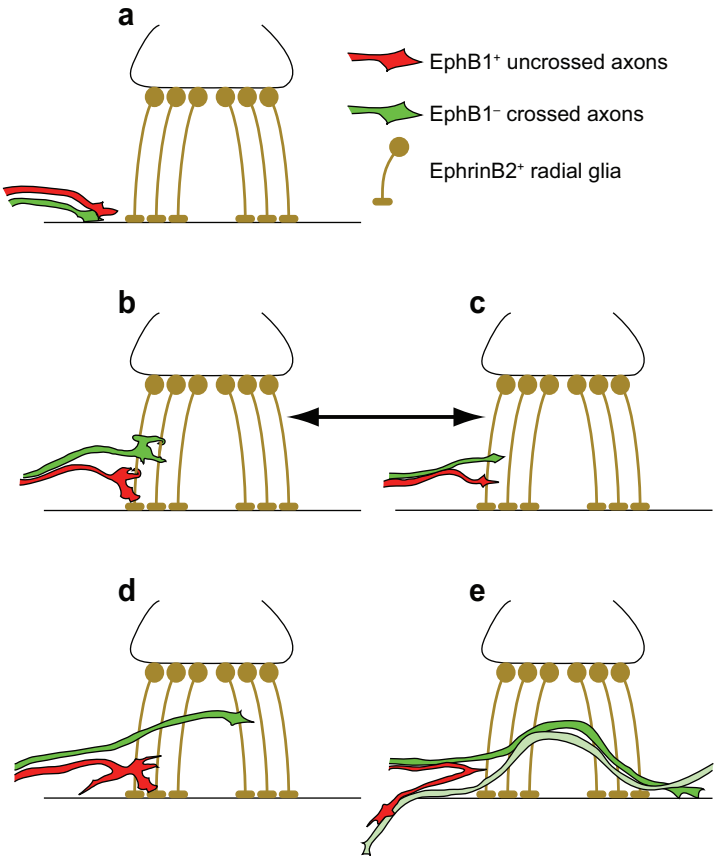

Figure 3

Retinal growth cone behavior at the optic chiasm midline. Frontal view of the optic chiasm during the peak phase (**Figure 2*b***). (*a*) After exiting the optic stalk, uncrossed and crossed retinal axons approach the radial glia palisade near the pial surface of the chiasm. (*b*) Both types of retinal axons interact with the midline glial processes and develop complex growth cones that wrap around midline glia. (*c*) All retinal axons withdraw their growth cone, then repeatedly extend, expand, and retract before advancing (*black double-headed arrow*). (*d*) Uncrossed growth cones spread extensively and emit protrusions in different axes. Crossed axons traverse the midline more dorsally within the radial glia palisade to the contralateral side. (*e*) Uncrossed axons consolidate a backward-oriented filopodium and the axon advances into the ipsilateral optic tract, possibly fasciculating with crossed axons from the contralateral retina (*light green*). Crossed axons descend toward the pia and enter the contralateral optic tract.

GUIDANCE FACTORS IMPORTANT FOR RETINAL AXON DIVERGENCE

The Usual Suspects

Soon after the behaviors of RGC growth cones and the character of the midline cells were

Filopodia: small, finger-like protrusions that extend from growth cones and probe the environment

Floor plate: an array of neuroepithelial cells at the ventral midline of the developing spinal cord; site through which commissural axons project

Fasciculation: axons contact each other and extend together in bundles

uncovered, the field witnessed the rapid identification of guidance factor families. Many of these cues were implicated in other midline-crossing models and were thus good candidates for retinal axon divergence at the chiasm midline (reviewed in Williams et al. 2004). Netrin-1 is a diffusible protein secreted by midline cells and acts as an attractant for axons expressing the deleted in colorectal cancer (DCC) receptor in the *Drosophila* ventral nerve cord and floor plate of the vertebrate spinal cord (reviewed in Kaprielian et al. 2001). However, *netrin-1* is not expressed at the optic chiasm, but is found at the optic disc where it is important for retinal fiber exit from the eye (Deiner et al. 1997). Although retinal axons in *netrin-1* and *DCC* mutant mice enter the chiasm at an altered angle, RGC divergence is not affected (Deiner & Sretavan 1999). Notably, although midline cells in other systems express an attractant cue, an analogous signal has not been identified at the optic chiasm.

Morphogens are now recognized to direct axon navigation (reviewed in Sanchez-Camacho et al. 2005). Sonic hedgehog (Shh) is expressed by floor plate cells where it induces cell differentiation in the vertebrate spinal cord (reviewed in Jessell 2000). Shh later plays a dual role in the floor plate, first as a chemoattractant for commissural axons toward the floor plate (Charron et al. 2003) and then as a repulsive cue for postcrossing axon projections into longitudinal tracts (Bourikas et al. 2005). Shh is expressed at the site of the future optic chiasm and appears to define the site of the chiasm (Torres et al. 1996), either by acting as a repulsive cue to retinal fibers (Trousse et al. 2001) and/or by regulating glial cell development and expression of guidance cues at the chiasm midline (Barresi et al. 2005). However, Shh appears to have no effect on axon divergence.

An obvious candidate for guiding RGC divergence is the Slit family of guidance cues and their receptors, Robos. Slits and Robos regulate the pathfinding of commissural axons across the midline in both the invertebrate ventral nerve cord and vertebrate spinal cord, where Slits act as repulsive guidance cues to Robo-expressing axons (reviewed in Dickson & Gilestro 2006). *Robo2* is expressed in RGCs, and *Slit1* and *Slit2* are present in the ventral diencephalon (Erskine et al. 2000). Whereas *Slit1* or *Slit2* single mutant mice show normal axon pathfinding at the optic chiasm, *Slit1/Slit2* double knockout mice display ectopic chiasm formation rostral to the normal chiasm, and retinal axons are misguided into the contralateral optic nerve and ventral diencephalon (Plump et al. 2002). However, even with these aberrations, a normal ipsilateral projection forms in these mice.

Similarly, in the zebrafish *robo2* mutant *astray*, RGC axons that are normally completely crossed display severe guidance errors at or after the midline. *astray* mutants also show ectopic chiasm formation and defasciculated axon growth (Fricke et al. 2001, Hutson & Chien 2002, Karlstrom et al. 1996), resembling the chiasm phenotype in *Slit1/Slit2* double knockout mice (Plump et al. 2002). Thus, Slit-Robo interactions channel RGC axons into the proper path and may regulate fasciculation rather than direct retinal axon divergence.

The Ephs and ephrins, particularly in the A family, mediate retinotectal topographic projections (reviewed in Lemke & Reber 2005, McLaughlin et al. 2003). Multiple EphAs and ephrinAs are expressed in gradients in the retina and optic tectum, and they are also expressed in chiasm midline cells. Whereas perturbation of EphA signaling in vitro blocks the normal inhibition caused by chiasm cells (Marcus et al. 2000), VT and non-VT axons show little difference in their response to ephrinAs. In chick, ephrinA2 or ephrinA5 overexpression increases the transitory ipsilateral projection (Dutting et al. 1999), but abnormalities in chiasm divergence have not been described in EphA or ephrinA mutant mice. So far, no experiments indicate that EphA-ephrinA signaling plays a significant role in retinal axon divergence at the chiasm midline.

The Uncrossed Retinal Projection

Studies on *Xenopus laevis* yielded the first hints of which molecular cues mediate retinal axon

divergence at the optic chiasm. In *Xenopus* tadpoles, all retinal axons cross the midline, but during metamorphosis a subpopulation of RGCs from VT retina projects ipsilaterally, coinciding with the expression of ephrinBs at the optic chiasm (Nakagawa et al. 2000). Premature ectopic expression of ephrinB2 in the chiasm leads to a precocious ipsilateral projection, pointing to ephrinB as a cue for axon avoidance at the chiasm midline. Furthermore, EphB2 receptors are expressed in the ventral retina during metamorphosis. Because ephrinBs are not expressed at the optic chiasm in animals lacking an ipsilateral projection such as chick and fish, but are present in mice (Nakagawa et al. 2000), we asked whether EphB/ephrinB interactions might be important for retinal divergence in mice.

In situ hybridization experiments confirmed that ephrinB2 is indeed expressed at the mouse optic chiasm midline, specifically in the midline radial glia and most intensely during the peak growth phase of the ipsilateral projection (Williams et al. 2003) (**Figure 2*b***). Moreover, ephrinB2 is selectively inhibitory to VT retinal axons in vitro, and blocking ephrinB2 in semi-intact chiasm preparations reduces the ipsilateral projection. Among the several EphB receptors that interact with ephrinB2, only EphB1 is expressed early in DC retina and then exclusively in VT retina at the time of peak midline divergence (**Figure 2*b***). After E17.5, EphB1 expression is downregulated in VT RGCs, concurrent with the development of the late-born crossed projection from VT retina (**Figure 2*c***). *EphB1*$^{-/-}$ mice show a decreased ipsilateral projection, with VT RGC axons ectopically crossing the midline, indicating that VT axons in *EphB1*$^{-/-}$ mice are not repelled by ephrinB2-expressing radial glial cells at the optic chiasm midline (Williams et al. 2003) (**Figure 4**).

Thus, EphB1 in the VT retina and ephrinB2 at the chiasm midline have emerged as the receptor-ligand system unique to the uncrossed RGC projection. However, several important questions remain. One curious finding is that EphB2 is highly expressed in the ventral retina in both mice and *Xenopus* (Williams et al. 2003, Nakagawa et al. 2000). Even though EphB2 and EphB1 have a similar affinity to ephrinB2 (Flanagan & Vanderhaeghen 1998), EphB2

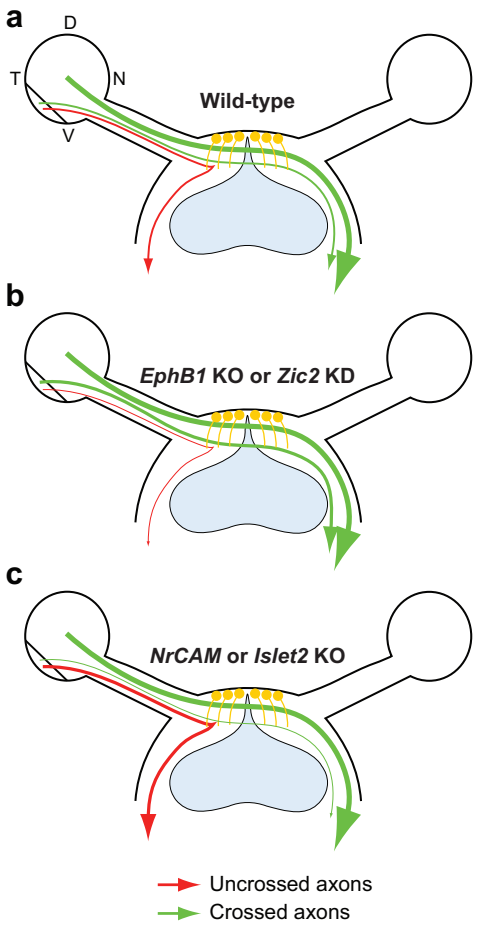

Figure 4

Abnormal divergence at the optic chiasm. (*a*) In wild-type mice, 3%–5% of retinal fibers project ipsilaterally, with uncrossed fibers arising from the VT retina during the peak phase and crossed fibers originating from the VT retina during the late phase. (*b*) In *EphB1* knockout (KO) and *Zic2* knockdown (KD) mice, the ipsilateral projection is reduced, with most VT retinal axons now projecting contralaterally. (*c*) In *NrCAM* KO and *Islet2* KO mice, the ipsilateral projection is increased because most late-born RGCs in VT retina project ipsilaterally rather than contralaterally. In all these mutants, note that the contralateral projection from non-VT retina is unchanged.

Commissural neurons: neurons whose axons cross the midline and project to targets on the opposite side of the organism

does not appear to play a role in directing the uncrossed projection (Williams et al. 2003). It will be interesting to determine why EphB1 is unique in its ability to induce repulsion upon contacting ephrinB2 at the chiasm midline.

An intriguing paradox of EphB1-ephrinB2 binding at the chiasm, as with all Eph-ephrin interactions, is that the receptor-ligand binding must be terminated for the growth cone to turn away from the midline and project ipsilaterally. Cleavage of the receptor-ligand complex is one mechanism to disengage the growth cone from the inhibitory cue. In *Xenopus*, matrix metalloproteinases are required for proper axon guidance at the optic chiasm (Hehr et al. 2005). Furthermore, both ephrin ligands (Hattori et al. 2000, Janes et al. 2005, Pascall & Brown 2004) and Eph receptors (Litterst et al. 2007) can be cleaved. Additionally, EphB/ephrinB complexes can be endocytosed bidirectionally by the receptor- or ligand-expressing cell (Mann et al. 2003, Marston et al. 2003, Zimmer et al. 2003), leading to cell detachment and axon retraction. EphrinAs and Sema3s can induce endocytosis (Fournier et al. 2000, Jurney et al. 2002), indicating that endocytosis may be a general mechanism for guidance cue regulation.

In addition to how an Eph/ephrin interaction is terminated, recent evidence indicates that ephrins can regulate Eph receptor signaling in *cis* (on the same cell) as well as in *trans* (Carvalho et al. 2006, Marquardt et al. 2005). Because RGCs express many Eph receptors and ephrins, *cis* interactions comprise a possible mechanism for adjusting the responsiveness of growth cones to various ephrins in the projection pathway. Along with *cis* interactions between members of the same guidance family, there can be receptor crosstalk between different guidance families. An example of this receptor crosstalk also comes from the vertebrate spinal cord midline, where Robo receptors are thought to silence the receptor DCC to netrin-1, such that axons that have crossed the midline are no longer attracted to netrin-1 (Stein & Tessier-Lavigne 2001).

Along with these forms of Eph receptor regulation, mRNA levels may be regulated by local translation of proteins necessary for guidance at the growth cone. There is ample evidence that mammalian axons contain mRNA and translational machinery (reviewed in Koenig & Giuditta 1999), and recent studies indicate that inhibiting local protein translation can alter growth cone response to guidance cues (Campbell & Holt 2001, Ming et al. 2002), likely by regulating the translation of downstream effectors such as β-actin or RhoA (Leung et al. 2006, Wu et al. 2005, Yao et al. 2006). However, only one study has shown that guidance receptors might be locally translated in the growth cone; EphA2 may undergo local translation in chick commissural neurons, but only after they cross the spinal cord midline (Brittis et al. 2002). The ability of growth cones to translate proteins locally on demand is a mechanism that allows for rapid response to guidance cues and represents a major advance in the field of axon guidance (reviewed in Lin & Holt 2007).

One important goal is to determine if and how these cellular mechanisms (cleavage and/or endocytosis, *cis-trans* Eph-ephrin interactions, and local translation) function in the context of the optic chiasm. Of note, EphB1 is expressed in the temporal retina and ephrinB2 at the optic chiasm in human fetal brain (Lambot et al. 2005), which suggests that EphB1-ephrinB2 may be an evolutionarily conserved mechanism for RGC guidance at the optic chiasm. Thus, unraveling how EphB1-ephrinB2 interactions lead to formation of the ipsilateral projection should illuminate how the binocular projection is patterned in higher vertebrates.

The Crossed Retinal Projection

The guidance factors described above are related to the uncrossed retinal projection. Which mechanisms underlie retinal axon crossing of the chiasm midline? One hypothesis proposes

that traversing the chiasm occurs by default: Retinal axons project straight through the midline zone into the contralateral optic tract because they lack receptors to inhibitory cues. However, the suppression of neurite extension by chiasm cells (Wang et al. 1995, 1996) and the pausing and extension/retraction behaviors of all precrossing axons (Godement et al. 1994, Mason & Wang 1997) argue against this hypothesis.

To identify cues that mediate crossing, we focused on the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) because NrCAM plays a role in commissural axon midline crossing in chick spinal cord (Lustig et al. 1999, Stoeckli et al. 1997). Since NrCAM is expressed at the chiasm midline (Lustig et al. 2001), and CAMs can interact homotypically, we examined NrCAM expression in the retina. NrCAM is expressed in all retinal regions except for the VT crescent from E12 to E17 (**Figure 2*a*,*b***). NrCAM expression then expands into the VT region from E17 to birth, corresponding to the time when crossing axons arise from VT retina (Williams et al. 2006) (**Figure 2*c***). Thus, NrCAM is a strong candidate for mediating midline crossing. Indeed, blocking NrCAM function results in the decreased ability of fibers to cross the chiasm, and *NrCAM*$^{-/-}$ mice have an enhanced uncrossed RGC projection (**Figure 4**). Surprisingly, this increase arises strictly from the late-born VT RGCs. In contrast, the crossed projections from non-VT retina are unaltered despite their NrCAM expression profile (Williams et al. 2006).

The curious restriction in NrCAM function to the late-born crossed VT RGCs could be explained by other CAMs acting redundantly or in concert with NrCAM. Members of the CAM family such as *L1*, *neurofascin*, and *TAG-1* are strongly expressed in non-VT retina during the peak phase of the crossed RGC projection. During the late phase, *neurofascin* and *TAG-1* are upregulated in VT RGCs, similar to *NrCAM*. The exception is that *TAG-1* is strongly downregulated in non-VT retina at late stages (Williams et al. 2006). Thus, the L1 family of CAMs is expressed in all RGCs that cross the midline. While other CAMs may compensate for NrCAM function in non-VT retina, they cannot solely explain the restricted function of NrCAM to the late-born crossed VT projection because *neurofascin* and *TAG-1* are also upregulated in VT during the late phase. There are likely other as-yet-unidentified guidance factors or signaling molecules that make late-born VT RGCs distinct from crossed projection arising from non-VT retina.

Recently, the Semaphorins have been identified as playing a role in the formation of the crossed projection. In zebrafish, Sema3d is expressed around the chiasm in a pattern similar to Slit1 and Slit2 in mice, and perturbing Sema3d by overexpression or knockdown impairs crossing (Sakai & Halloran 2006). In this system, Sema3d is thought to act as a repulsive cue that guides retinal axons out of the chiasm into the contralateral optic tract. In mice, Sema5A channels RGC axons through the optic nerve (Oster et al. 2003), but the expression and function of other Semaphorins have gone unstudied. Of note, interactions between the L1 family of Ig CAMs and the semaphorin receptors, plexins and neuropilins, can change Semaphorin-induced repulsion to attraction (reviewed in Bechara et al. 2007). This interaction is critical for axon guidance in other commissural pathways (Falk et al. 2005). A role for semaphorins and their receptors, including potential interactions with CAMs, remains to be established in the mouse optic chiasm.

Ig: immunoglobulin

CAM: cell adhesion molecule

NrCAM: Ng(neuron-glia)-CAM related cell adhesion molecule

AXON ORGANIZATION AND FASCICULATION

A poorly understood aspect of RGC axon extension to central targets is how uncrossed and crossed fibers are organized when they enter, course within, and exit the optic chiasm. In eutherian species, RGC axons are organized in a grossly topographic manner as they exit the retina, but this topographic organization is lost as they approach the chiasm (reviewed in Guillery et al. 1995, Jeffery 2001). At the optic nerve–chiasm junction, RGC axon bundles are

no longer surrounded by glia and they defasciculate. As they progress through the chiasm, uncrossed axons separate from crossing axons, yet each component enters the optic tract again with crude retinotopic and age-related order (Jeffery 2001, Plas et al. 2005). This plan differs from that in marsupials, where the retinotopy and fasciculated bundles in the optic nerve are maintained as axons traverse the chiasm (Dunlop et al. 2000) and uncrossed fibers project directly into the optic tract, similar to the course of the first uncrossed RGC axons from DC retina in mice (reviewed in Guillery et al. 1995).

Which cellular and molecular mechanisms underlie this reorganization of fibers, and do such rearrangements play a role in axon guidance? For example, at the insect midline, commissural axon growth cones fasciculate with their contralateral homolog as they cross the midline, a rearrangement thought to ensure proper pathway formation (Myers & Bastiani 1993). Whether a similar interaction occurs in the vertebrate optic chiasm is not known. Another question is whether uncrossed axons fasciculate with new partners after executing the turn away from the midline back toward the ipsilateral optic tract. Insight into this issue comes from monocular enucleation experiments in eutherian species, in which a decrease in the uncrossed pathway is observed. One hypothesis for this decrease is that the uncrossed axons from one eye need to fasciculate with postcrossing axons from the other eye to project correctly into the ipsilateral optic tract (Chan et al. 1999, Godement et al. 1987a).

Genetic and cellular analyses of the fasciclins in the insect nervous system illustrate the importance of CAMs in axonal pathway formation (reviewed in Goodman 1996, Van Vactor 1998), but the molecular mechanisms directing axon reorganization (e.g., fasciculation and defasciculation) in commissural pathways are not yet understood. In the chick optic tract, enzymatic removal of polysialic acid (PSA) from neural CAM (NCAM) produces significant defasciculation of RGC axons (Yin et al. 1995). Changes in CAM expression on crossing axons within the spinal cord midline were identified 20 years ago, with commissural neurons downregulating TAG-1 and upregulating L1 after crossing the midline (Dodd et al. 1988), although the function of these changes remains unknown. In the visual system, PSA-NCAM is similarly downregulated as axons enter the optic chiasm and upregulated in a subset of axons upon entering the optic tract (Chung et al. 2004). One function of these transitions may be to implement defasciculation, resorting, and subsequent refasciculation of axons at sites of decussation, potentially in collaboration with other guidance factors. For example, Sema3D can promote fasciculation by modulating L1 CAM receptor levels (Wolman et al. 2007). However, many CAM mutants do not display severe defects in fasciculation (Demyanenko & Maness 2003, Van Vactor 1998). Overlapping expression patterns and possible redundant function could explain this phenotype but also complicate unraveling the effects of CAMs on axon reorganization.

Other possible modifiers of guidance factors such as chondroitin sulfate (CSPGs) and heparan sulfate proteoglycans (HSPGs) are expressed in cells adjacent to the optic nerve–chiasm and chiasm–tract junctions, loci for axon fasciculation and defasciculation (Leung et al. 2003, Pratt et al. 2006, Reese et al. 1997). In support of this notion, heparan sulfate biosynthesis is perturbed in zebrafish mutants (*dak* and *box*) that display axon missorting in the optic tract (Lee et al. 2004). The well-documented interaction between CSPGs and CAMs (reviewed in Grumet et al. 1996) suggests a role for these families of molecules in axon rearrangement at the optic chiasm that may be independent of receptor-ligand signaling that mediates RGC divergence.

TRANSCRIPTION FACTORS THAT PATTERN THE RETINAL AXON PROJECTION

The link between transcription factor designation of neuron subpopulations and axon trajectory, through control of guidance cue

expression, has been demonstrated in a number of models (reviewed in Butler & Tear 2007, Polleux et al. 2007). This has been especially well illustrated in the control of ephrins and netrins in motor neurons (Kania & Jessell 2003, Labrador et al. 2005). Here we describe regulatory genes that have been uncovered in the retina and ventral diencephalon that relate to optic chiasm formation.

Genes in the Retina

Zinc finger transcription factors (Zic1-5) are critical for early neural patterning and the midline of the body plan (reviewed in Merzdorf 2007). *Zic2* is expressed in the retina during eye cup formation (Nagai et al. 1997) and is then downregulated. Strikingly, *Zic2* is subsequently upregulated in the VT crescent between E14.5 and E17.5, precisely at the time when VT axons project ipsilaterally (**Figure 2**). Zic2 is required for formation of the uncrossed pathway, as demonstrated in *Zic2* knockdown mice in which the ipsilateral projection is significantly reduced (Herrera et al. 2003) (**Figure 4**). In addition, overexpression of *Zic2* in DT retinal explants induces these axons to be repulsed by chiasm cells, similar to the response of VT axons. *Zic2* expression in uncrossed RGCs appears to be evolutionarily conserved, as its expression in RGCs of animals such as *Xenopus* and ferrets correlates precisely with the proportion of ipsilaterally projecting RGCs (Herrera et al. 2003). The finding that Zic2 directs the uncrossed retinal projection heralded the first transcriptional program specifically directing an ipsilateral course of axon pathfinding at the midline in vertebrates. For the mouse retina, immediate questions arise: As both Zic2 and EphB1 are expressed in VT RGCs and are necessary for axon divergence at the optic chiasm, does Zic2 regulate EphB1, and are Zic2 and/or EphB1 sufficient to induce changes in the crossing behavior of non-VT RGCs? Recent findings indicate that ectopic expression of Zic2 in RGCs is sufficient to elicit avoidance of the midline, and this repulsion is largely EphB1-dependent (García-Frigola et al. 2008). Irrespective of whether Zic2 directly or indirectly regulates EphB1 expression, these findings strengthen the notion that Zic2 and EphB1 are in the same pathway.

In contrast with Zic2 expression in VT retina, the LIM homeodomain transcription factor *Islet2* is expressed in non-VT retina and is absent from uncrossed VT RGCs from E13.5 to E15.5 (**Figure 2*a*,*b***). However, *Islet2* is upregulated at E17.5 in late-born VT RGCs that cross the midline (Pak et al. 2004) (**Figure 2*c***). Although Islet2 is not expressed in every RGC, the retinal domains containing Islet2^{+} cells give rise exclusively to the crossed projection. Furthermore, the Islet2 expression pattern is remarkably similar to that of NrCAM, suggesting that Islet2 might regulate NrCAM, but this relationship has not been demonstrated. In *Islet2*^{tauLacZ} knockin mice, Islet2 is expressed only in contralateral projecting RGCs, and *Islet2*$^{-/-}$ mice display an increase in ipsilateral fibers. As with *NrCAM*$^{-/-}$ mice, this aberrant ipsilateral projection arises strictly from the VT retina. The increase in uncrossed axons in *Islet2*$^{-/-}$ mice is concurrent with an increase in *Zic2* and *EphB1* expression in the VT crescent (Pak et al. 2004) (**Figure 4**), but whether Islet2 suppresses Zic2 and/or EphB1 within the VT crescent remains to be established.

The differential expression of these transcription factors and guidance receptors in the retina points to one major difference in the patterning and implementation of axonal decussation at the optic chiasm midline compared with other midline models. In the retina, *Zic2* and *EphB1* are expressed solely in the uncrossed RGC population in the VT retina and are necessary for the formation of the ipsilateral pathway. A different set of genes, *Islet2* and *NrCAM*, are expressed in crossed RGC projections from non-VT and late-born VT retina. In contrast, at the vertebrate spinal cord midline or the *Drosophila* ventral nerve cord midline, both uncrossed and crossed axons express the same receptor, Robo, and the decision to cross or avoid the midline is achieved through fine regulation of this receptor at the growth cone membrane

(Dickson & Gilestro 2006). Crossing axons traverse the Slit$^+$ zone because the Commissureless protein prevents Robo trafficking to the membrane by sequestering it into endosomes (Keleman et al. 2005). In uncrossed axons, Robo expression remains elevated at all times, leading to avoidance of the midline. These findings have been extended to the vertebrate spinal cord commissural neurons, where Rig-1 causes Robo$^+$ axons to be insensitive to Slit prior to crossing the floor plate (Sabatier et al. 2004).

Which genes regulate Zic2 and Islet2? *Vax2* is expressed in a high-ventral to low-dorsal gradient (Barbieri et al. 2002, Mui et al. 2002). The effect of mutations in *Vax* genes in chiasm formation has been examined, but the expression of *Islet2* and *Zic2* in these mutants has not been reported. The ventral retina is further partitioned by *Foxd1* (previously known as *BF2*), which is confined to the VT quadrant in a zone extending more centrally than *Zic2*, whereas *Foxg1* (previously known as *BF1*) is expressed throughout the nasal retina (Herrera et al. 2004, Pratt et al. 2004). *Zic2* and *EphB1* expression are downregulated in *Foxd1* mutants, implicating *Foxd1* as a gene upstream of Zic2 (Herrera et al. 2004).

Zic2 and EphB1 comprise a guidance program for the uncrossed RGC projection from the VT retina. In mouse retina, as in other higher vertebrates, the line of decussation demarcates the sectors containing ipsilaterally and contralaterally projecting RGCs. Several questions then arise: Which molecules demarcate this distinct boundary between crossed and uncrossed RGCs? How is the VT crescent specified to have a unique spatial and temporal gene expression pattern for the uncrossed projection (*Zic2*, *EphB1*) during the peak phase and the crossed projection (*Islet2*, *NrCAM*) during the late phase? As outlined in the *Xenopus* retina, does the uniqueness of the VT region arise from the unusual embryonic derivation of these cells (Jacobson & Hirose 1978) or by differential regulation of cell division in this part of the retina at later stages (Marsh-Armstrong et al. 1999)? Is the uncrossed population of RGCs, and thus the line of decussation, specified very early in development by cues from nonretinal tissues acting exogenously on the developing eye (Lambot et al. 2005), or is the line of decussation specified later by repulsive interactions, possibly between Zic2 and Islet2 and/or between Foxd1 and Foxg1?

Albinism is a unique condition that may provide clues to answer these questions on the development of the line of decussation. It is the only known mutation in which a decrease in the proportion of uncrossed axons is accompanied by a shift in the line of decussation toward the periphery (Guillery et al. 1995, Hoffmann et al. 2003, Jeffery & Erskine 2005). Albino mice show a reduction in the number of *Zic2*$^+$ cells, in agreement with the diminished ipsilateral projection (Herrera et al. 2003). It is unclear how perturbations in melanin biosynthesis and trafficking affect retinal gene expression and divergence at the midline, but one possibility is that factors in the melanin pathway could affect cell proliferation, perturbed in the albino (Rachel et al. 2002, Tibber et al. 2006), and in turn affect cell fate. Further studies are needed on the early stages of eye development to understand how the line of decussation is established.

Patterning of the Optic Chiasm Terrain

Previous studies have outlined regulatory gene expression in the ventral diencephalon where retinal axons converge and form the optic chiasm (Marcus et al. 1999, Wilson et al. 1993). Some genes are expressed in areas flanking the midline (e.g., *Foxd1*, *Dlx2*), whereas other genes are rostral or caudal to the chiasm with a raphe extending into the midline (e.g., *Foxg1*, *Nkx2.2*) (Herrera et al. 2004, Marcus et al. 1999) (**Figure 5*a***). Strikingly, at the early stages of chiasm formation, the earliest-growing retinal axons follow the borders of these subdivisions (Marcus et al. 1999).

Many of these transcription factors are expressed in both the retina and the ventral diencephalon (e.g., *Foxd1* and *Zic2*), and mice lacking these genes can have severe

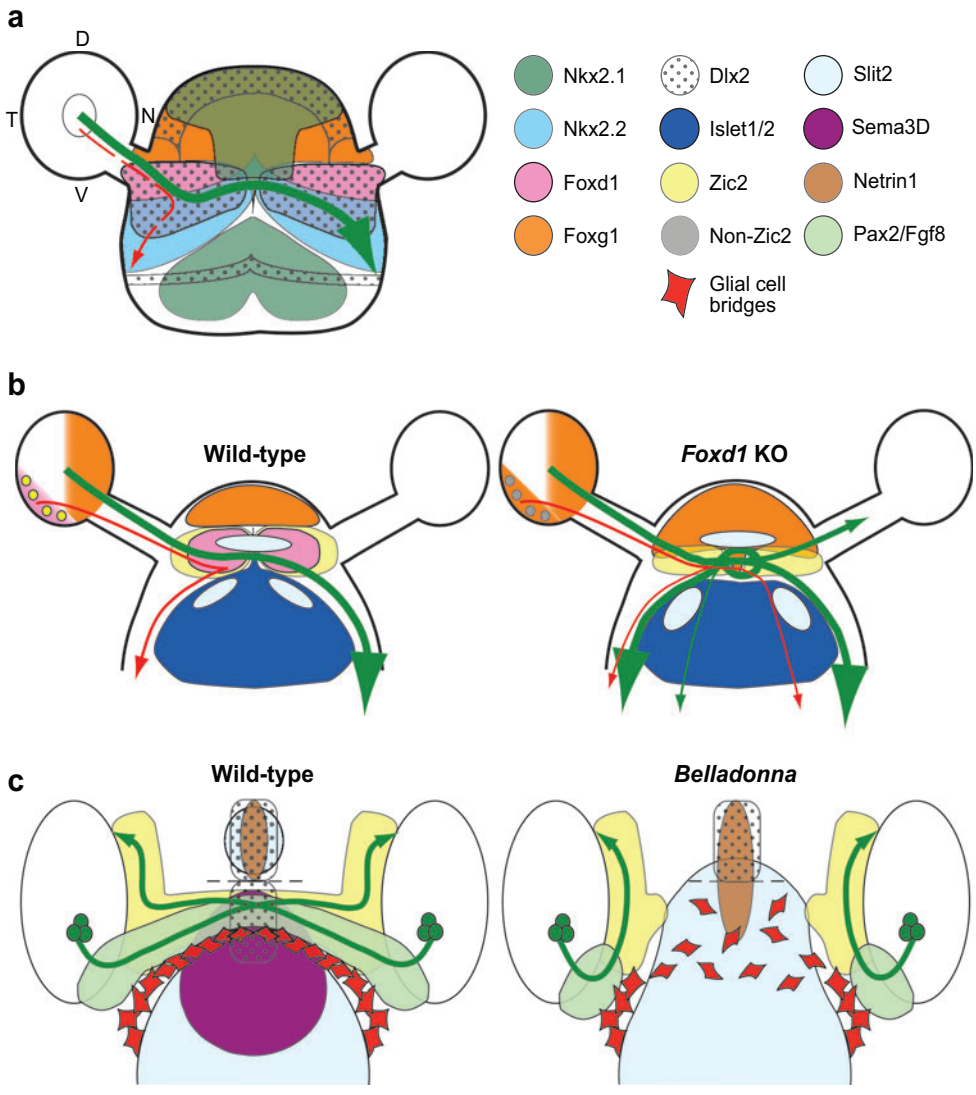

Figure 5

Patterning of the optic chiasm terrain. (*a*) At early stages of mouse optic chiasm formation (E11–E13), transcription factor expression delineates subregions within the ventral diencephalon. Retinal axons avoid *Nkx2.1* and *Foxg1* regions but project through the *Nkx2.2*, *Foxd1*, and *Dlx2* regions. Adapted from Marcus et al. 1999. (*b*) The retinal axon pathway with respect to patterns of gene expression in wild-type and *Foxd1* KO mice at E15.5. In wild-type mice, *Foxg1* is expressed in the nasal retina, whereas *Foxd1* is expressed in the VT quadrant, which includes the smaller $Zic2^+$ zone (VT crescent). In *Foxd1* KO mice, *Foxg1* expression expands into the area normally occupied by *Foxd1* in both the retina and the ventral diencephalon, and *Zic2* is no longer expressed in the VT retina and is diminished near the chiasm. *Foxd1* KO mice display numerous projection errors at the optic chiasm due to the abnormal patterning of the ventral diencephalon, with an increased ipsilateral projection arising from all retinal regions. Adapted from Herrera et al. 2004. (*c*) In wild-type zebrafish, all retinal axons are crossed, extending through *Pax2-*, *fgf8-*, and *Zic2.1*-expressing regions to reach the contralateral optic tectum. *Belladonna* mutants are achiasmatic with an entirely uncrossed projection. *Sema3D* expression is completely abolished, *Pax2/fgf8* expression is strongly diminished, and *Slit2* and *netrin1* expression expands. In addition, the glial bridge is disrupted at the midline. These perturbations of the ventral diencephalon make this terrain refractory for all retinal axons. Adapted from Seth et al. 2006.

defects both in retinal axon trajectory and in the cellular organization of the optic chiasm (Bertuzzi et al. 1999, Herrera et al. 2003, 2004, Marcus et al. 1999, Pratt et al. 2004). In the retina of *Foxd1*$^{-/-}$ mice, *Zic2* and *EphB1* are missing, and *Foxg1* expression extends into the temporal retina (Herrera et al. 2004) (**Figure 5*b***). The absence of *Zic2* and *EphB1* in *Foxd1*$^{-/-}$ mice predicts a decrease in uncrossed projections. Unexpectedly, the uncrossed retinal projection increases dramatically, and these fibers arise from all regions of the retina. Such imbalances in RGC divergence result from a distortion in the architecture and regionalization of the ventral diencephalon: *Foxg1* expands toward the *Foxd1* region, *Zic2* and *Islet1* expression is diminished, and the *Slit2* zone is extended and enhanced, resulting in abnormalities in chiasm shape (Herrera et al. 2004) (**Figure 5*b***). Thus, alterations in the ventral diencephalon override defects in the retina to produce abnormalities in chiasm formation and retinal axon projection. Similar findings are observed in the *circletail*, *loop-tail*, and *Pax3* mutants (Rachel et al. 2000).

The zebrafish mutant *Belladonna* provides an additional example of the crucial role of molecular and cellular composition of the optic chiasm terrain. Like the Belgian sheepdog (Williams et al. 1994) and *Pax2*$^{-/-}$ mice (Torres et al. 1996), the *Belladonna* mutant is achiasmatic, having a totally uncrossed rather than the normal completely crossed retinal projection (Karlstrom et al. 1996) (**Figure 5*c***). The mutated gene is *Lhx2*, and transcription factor and guidance cue expression is perturbed in the chiasm (Seth et al. 2006). *Zic2* and *Dlx2* zones are diminished medially and caudally, *netrin-1* and *Slit2* expand, and *Sema3D*, implicated in axon crossing (Sakai & Halloran 2006), is missing (**Figure 5*c***). In addition, the glial bridge is disrupted, likely hindering crossing of the entire retinal axon cohort. This expansion of *Slit* expression and perturbed chiasm terrain is also observed in the Gli2 zebrafish mutant *you-too*, where Shh signaling is perturbed (Barresi et al. 2005). These examples illustrate the difficulty of unraveling a mutation's effect simply by examining the phenotypes of retinal axon trajectory alone.

CONCLUSIONS AND PERSPECTIVES

The specification of retinal ganglion cells and the receptor system for the uncrossed and late-forming crossed pathways from VT retina have begun to be uncovered. The program that regulates the crossed trajectory of RGCs that reside outside of the VT crescent remains to be identified. It will be especially challenging to dissect how various guidance families implement crossing through an apparently refractory midline and to illuminate the underpinnings of fasciculation during fiber reorganization in the chiasm. The field can look forward to the discovery of yet more regulatory genes that control navigation at the midline, and more importantly, explication of precisely how transcription factors regulate guidance molecule expression. Addressing these unsolved issues of axon guidance, with the optic chiasm and other midline scenarios as models, should enlighten the next discovery period.

SUMMARY POINTS

1. Retinal axon growth to the optic chiasm can be divided into three phases, with each cohort of retinal ganglion cell projections displaying different divergence patterns at the optic chiasm.
2. The transcription factor Zic2 and the guidance receptor EphB1 are expressed in the ventrotemporal retina during the peak phase of retinal ganglion cell axon outgrowth and regulate the uncrossed projection.

3. All growth cones enter and pause within the chiasm midline region, but only EphB1-expressing axons from ventrotemporal retina are repelled by ephrinB2-expressing midline radial glial cells and turn ipsilaterally.
4. The transcription factor Islet2 and the L1-family member NrCAM are expressed in non-ventrotemporal retina during the peak phase and are upregulated in the ventrotemporal retina during the late phase of retinal ganglion cell development, but Islet2 and NrCAM are required only for the late-born crossed projection from VT retina.
5. Transcription factors such as Foxd1 and Zic2 are crucial for patterning the ventral diencephalon as well as the retina and, in turn, affect the expression of cues for divergence at the optic chiasm.

FUTURE ISSUES

1. How is the retina patterned to produce two distinct sectors containing retinal ganglion cells that project ipsilaterally and contralaterally, and how is the line of decussation established?
2. Which molecular program(s) direct retinal ganglion cell projections arising from outside the ventrotemporal retina to cross the midline?
3. Which downstream signaling cascades become activated upon EphB1-ephrinB2 interaction, especially with respect to cytoskeletal reorganization during growth cone repulsion and turning?
4. What are the cellular interactions between growth cones and midline glia that instigate receptor trafficking, local translation, and termination of the receptor-ligand interaction during midline crossing and repulsion?
5. How do transcription factors regulate guidance factor expression in the retina and ventral diencephalon?
6. How do guidance programs for crossing or avoiding the midline relate to proper innervation of target regions in the thalamus and superior colliculus/tectum, and further distally, in the cortex?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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