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Review

The retinal ganglion cell axon's journey: Insights into molecular mechanisms of axon guidance

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Abstract

The developing visual system has proven to be one of the most informative models for studying axon guidance decisions. The pathway is composed of the axons of a single neuronal cell type, the retinal ganglion cell (RGC), that navigate through a series of intermediate targets on route to their final destination. The molecular basis of optic pathway development is beginning to be elucidated with cues such as netrins, Slits and ephrins playing a key role. Other factors best characterised for their role as morphogens in patterning developing tissues, such as sonic hedgehog (Shh) and Wnts, also act directly on RGC axons to influence guidance decisions. The transcriptional basis of the spatial–temporal expression of guidance cues and their cognate receptors within the developing optic pathway as well as mechanisms underlying the plasticity of guidance responses also are starting to be understood. This review will focus on our current understanding of the molecular mechanisms directing the early development of functional connections in the developing visual system and the insights these studies have provided into general mechanisms of axon guidance.

Keywords: Retinal ganglion cell; Optic pathway; Visual system; Axon guidance; Growth cone

Introduction

Visual information is relayed from the eye to the brain via the axons of retinal ganglion cells (RGCs). These connections are established during development by the extension of RGC axons along a precise path to recognise and form synapses with appropriate target cells. This is no mean feat as the distance travelled by individual axons is considerable with a multitude of potential targets presented on route.

The ability of developing axons to navigate unerring through the embryo is mediated by a specialised structure at their distal tip: the growth cone (Fig. 1). Neuronal growth cones are highly motile, sensory structures that constantly extend and retract two types of processes: thin, finger-like filopodia and flat, veil-like lamellipodia. By responding to cues arrayed in the extracellular environment, growth cones control the rate and direction of axon extension. These guidance cues can either be attractive, promoting growth towards a specific region, or repulsive, preventing growth in a particular direction (Fig. 1). Both attractive and repulsive signals can be associated with cell surfaces or the extracellular matrix, or be more diffusible and act at a distance from their source. Originally it was thought that individual guidance cues functioned either as attractants or repellents but not both. However we now know that the same cue can elicit different responses from distinct neuronal subpopulations or even from the same neuron at different points along its pathway (e.g. Shewan et al., 2002). The distance over which individual cues exert their effects also is variable (Kennedy, 2000).

Growth cones respond to extrinsic guidance cues via receptors on their surface which, by activating down-stream signalling pathways, induce changes in cytoskeletal organisation and, consequently, the rate and direction of axon outgrowth (reviewed by Gordon-Weeks, 2000). In recent years several conserved families of ligand–receptor signalling systems have been identified including the Ephrins/Ephs (Cheng et al., 1995; Drescher et al., 1995, reviewed by Klein, 2004) netrin/Dcc/

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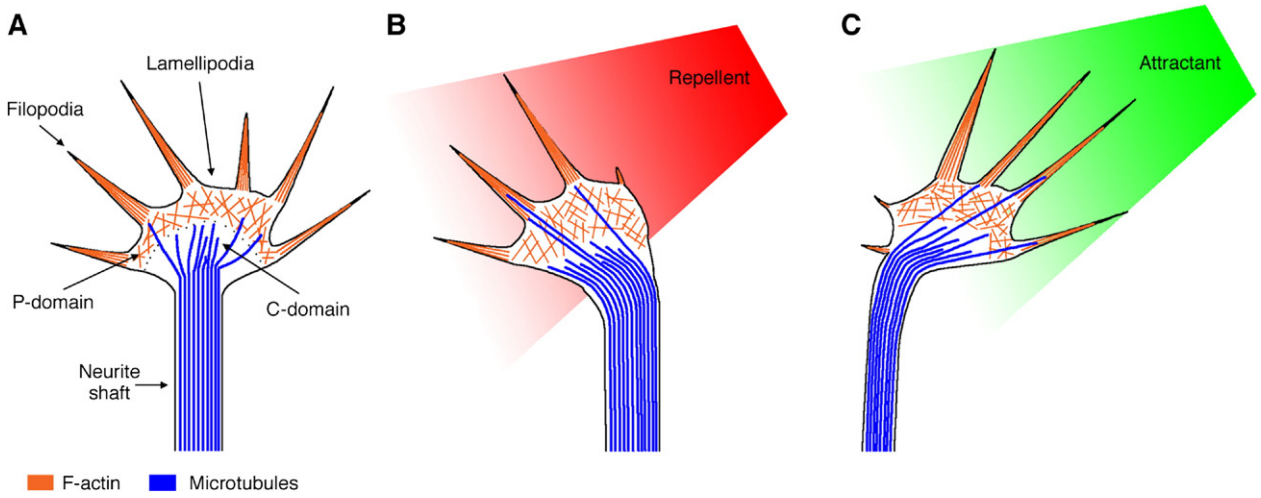

Fig. 1. Schematic diagram of growth cones growing in the absence (A) or presence (B, C) of guidance cues. A: Growth cones extend dynamic filopodia and lamellipodia. These structures are formed from F-actin, which is organised as bundles in filopodia and a meshwork in lamellipodia. This actin-rich region forms the thin peripheral (P) domain of the growth cone with the thicker central (C) domain being composed of microtubules and organelles. Microtubules are tightly bundled together in the neurite shaft but splay out in the growth cone. In a gradient of a repellent (B) filopodia and microtubules are lost selectively on the side of the growth cone facing the gradient resulting in repulsive turning. In a gradient of an attractant (C) filopodia and microtubules are stabilised selectively on the side facing the gradient resulting in turning towards the signal.

UNC5 (Hedgecock et al., 1990; Serafini et al., 1994; Keino-Masu et al., 1996; reviewed by Barallobre et al., 2005), Slits/Robos (Seeger et al., 1993; Kidd et al., 1998, 1999; Brose et al., 1999; Li et al., 1999; reviewed by Brose and Tessier-Lavigne, 2000) and Semaphorins/Neuropilins/Plexins (Luo et al., 1993; Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Winberg et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999; reviewed by Fujisawa, 2004). It also has become clear that secreted factors best known for their role as morphogens, such as Sonic hedgehog (Shh; Trousse et al., 2001; Charron et al., 2003; Bourikas et al., 2005), FGFs (McFarlane et al., 1995, 1996), BMPs (Augsberger et al., 1999) and Wnts (Lyuksyutova et al., 2003; Yoshikawa et al., 2003), are key regulators of axon guidance decisions (reviewed by Bovolenta, 2005; Charron and Tessier-Lavigne, 2005). Much work is being done currently to relate the function of these molecules to the formation of specific axonal pathways.

This review will focus on our current understanding of the mechanisms directing axon guidance in the developing visual system. Due to its relatively simple anatomy, ease of analysis and stereotypical projection pattern the developing optic pathway has proven to be one of the most useful models for studying axon guidance decisions. Much of our understanding of the actions of specific guidance cues has come from studies of this system. Here we will highlight recent progress in unravelling the precise repertoire of guidance signals required for optic pathway development. The transcriptional regulation of these guidance signals and factors that modulate the response of growth cones to specific cues also will be discussed.

The developing optic pathway

Following their differentiation RGCs extend their axons into the optic fibre layer (OFL) at the inner surface of the retina where they grow in a highly direct, radial fashion towards their exit point from the eye, the optic nerve head/disc. From here, they enter the optic nerves and extend towards the ventral midline of the diencephalon (developing hypothalamus) where the two nerves meet at an invariant position along the anterior–posterior axis of the brain to form the optic chiasm, a major brain commissure. In species with eyes located laterally all axons cross the midline at the chiasm whereas in animals with binocular vision a proportion of axons originating in the temporal region of the retina do not cross but instead project ipsilaterally. Irrespective of their behaviour at the chiasm, the RGC axons then project dorsally within the optic tracts towards their targets in the midbrain and thalamus (Fig. 2). At the optic nerve head, chiasm and on reaching their target, RGC growth cones increase in size and adopt highly complex morphologies tipped with multiple filopodia and lamellipodia (Bovolenta and Mason, 1987; Holt, 1989; Godement et al., 1994; Hutson and Chien, 2002). This behaviour is indicative of growth cones encountering novel environments or faced with a choice of pathway selection (Mason and Wang, 1997). Thus, the developing optic pathway can be considered as a series of discrete segments intersected by these intermediate targets/decision regions. The mechanisms directing RGC axon pathfinding in each of these regions has been studied in a wide range of vertebrates ranging from fish through to mammals (Table 1). Despite differences in the overall organisation of the RGC axons as they navigate through the optic pathway in different organisms, particularly at the optic chiasm, these studies have revealed a high degree of conservation in the underlying guidance mechanisms (e.g. Nakagawa et al., 2000; Herrera et al., 2003; reviewed by Jeffery and Erskine, 2005). Information gathered from different species will therefore be considered as a whole in this review.

Guidance within the retina

Shortly after its final cell division, an axon arises from the basal surface of each RGC and extends directly into the OFL where it grows straight towards the optic disc (Hinds and Hinds, 1974; Holt, 1989; Zolessi et al., 2006). The initiation of axon extension is regulated by integrins and cadherins (Lilienbaum et al., 1995; Riehl et al., 1996) with the site at which the axon forms being controlled by extrinsic factors in the surrounding neuroepithelium (Zolessi et al., 2006).

As they extend to the optic disc, RGC axons are restricted to the OFL at the inner, vitreal surface of the retina. This restriction is driven by the balance of the growth promoting properties, mediated by factors such as NCAM, of the neuroepithelial endfeet present in this region and inhibitory guidance cues localised to the outer retinal layers (Brittis and Silver, 1995; Brittis et al., 1995; Stier and Schlosshauer, 1995). Slit–Robo signalling contributes to this process (Table 1). In mice, two members of the Slit family, Slit1 and Slit2 are expressed in the RGC and inner nuclear layers of the retina and Robo2, one of their known receptors, is present on RGCs (Fig. 3A; Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000). In vitro, Slit1 and Slit2 are potent inhibitors of RGC axon outgrowth (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000; Plump et al., 2002) and in mice lacking inhibitory Slit-signalling a subset of RGC axons stray away from the OFL into the outer layers of the retina (Thompson et al., 2006a). Surprisingly, although located in the outer retina, these ectopic axons still grow towards the optic disc, providing direct evidence that the signals that determine disc-directed growth are not localised exclusively to the OFL (Goldberg, 1977). Slit signalling also has been implicated in helping restrict RGC axons to the OFL of the chick retina. However, in this species Slit1 is expressed by cells that are contacted by RGC axons extending actively within the OFL suggesting that Slit1 also can act in a positive manner to regulate RGC axon pathfinding (Jin et al., 2003).

Inhibitory signals preventing growth into the retinal periphery and positive factors promoting growth centrally act in concert to ensure that once within the OFL the RGC axons extend directly towards the optic disc (Table 1). Slit2 is secreted by the lens and helps control the initial polarity of RGC axon extension such that growth is directed away from the retinal periphery and straight towards the optic disc (Thompson et al., 2006a). The retinal neuroepithelium peripheral to the forefront of RGC differentiation also is inhibitory to RGC axon extension (Halfter, 1996). In rodents, this correlates with a wave of chondroitin sulphate proteoglycan (CSPG) expression that, by directing axons away from regions of high CSPG expression, controls the timing and initial direction of RGC axon extension (Brittis et al., 1992; Brittis and Silver, 1995). Several cell adhesion molecules, for example, L1, Neurolin/DM-GRASP/BEN and NrCAM act in a positive manner to promote the directed growth of RGC axons towards the optic disc (Brittis et al., 1995; Ott et al., 1998; Weiner et al., 2004; Zelina et al., 2005). Shh, a secreted signalling molecule best characterised for its role as a morphogen in patterning developing tissues (Fuccillo et al.,

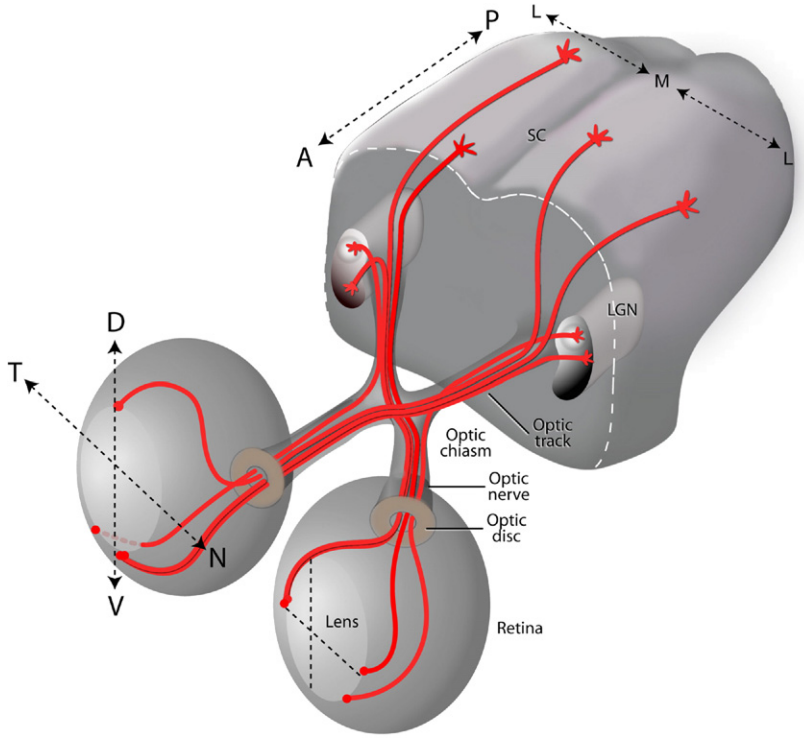

Fig. 2. General view of the visual system. RGC axons from different regions of the retina transverse the eye to exit at the optic disc. The axons then travel via the optic nerves to the optic chiasm where they cross or avoid the midline to project ipsilaterally or contralaterally to the main visual targets: the lateral geniculate nucleus in the thalamus (LGN) or the superior colliculus (SC) in mammals. D, dorsal, L, lateral, M, medial, N, nasal, T, temporal, V, ventral.

Table 1
Role of specific guidance cues in directing RGC axon pathfinding

Molecule	Expression	Role in retina	Role at chiasm	Role in optic tract/visual targets
CSPGs (Direct effect or modulation of other cues?)	High peripheral–low central wave in retina; bordering chiasm and optic tract.	Timing and polarity of RGC outgrowth (r; Brittis et al., 1992).	Path followed by RGC axons (m; Chung et al., 2000).	Inhibit outgrowth restricting axons to optic tract (c; Ichijo and Kawabata, 2001).
EphBs (reverse signalling)	High V–low D gradient in retina; Low M–high L gradient in tectum/SC.	Targeting of dorsal RGC axons to optic disc (m; Birgbauer et al., 2000).		Mapping along M–L axis of tectum (x; Mann et al., 2002).
EphrinAs	High N–low T gradient in retina; low A–high P gradient in tectum/SC; low M–high L gradient in LGN.			Mapping along A–P axis of tectum/SC and L–M axis of LGN. Organisation of eye specific layers (e.g. Frisen et al., 1998; Feldheim et al., 1998).
EphrinBs	High D–low V gradient in retina; chiasmatic midline; High M–low L gradient in tectum/SC.		Establishment of binocular visual pathways (x; Nakagawa et al., 2000; m; Williams et al., 2003).	Mapping along M–L axis of SC (m; Hindges et al., 2002).
netrin-1	Optic disc	Exit into the optic nerve (m; Deiner et al., 1997; x; Höpker et al., 1999).		
NrCAM	Contralaterally projecting RGC axons; chiasmatic midline.	Promotes growth towards and out of the optic disc (c; Zelina et al., 2005).	Promotes midline crossing of late-generated RGC axons (m; Williams et al., 2006).	
Sema3D	Midline of ventral diencephalon; Low M–high L gradient in tectum.		Promotes midline crossing and growth into contralateral optic tract (z; Sakai and Halloran, 2006).	Mapping along M–L axis of tectum (z; Liu et al., 2004).
Shh	High central–low peripheral wave in retina; midline of CNS except chiasm.	Promotes growth towards optic disc (c; Kolpak et al., 2005).	Inhibits growth; absence from midline required for crossing (c; Trousse et al., 2001; m; Torres et al., 1996; z; Macdonald et al., 1997).	
Slits	Lens; inner region of the retina; bordering chiasm and tract	Polarity of RGC axon outgrowth (m). Restrict axons to OFL via inhibitory (m; Thompson et al., 2006a) or attractive (c; Jin et al., 2003) signalling.	Inhibit outgrowth controlling position of chiasm and path followed by RGC axons (m; Plump et al., 2002).	Inhibit outgrowth restricting axons to optic tract (m; Thompson et al., 2006b).
Wnt3	Low M–high L gradient in tectum			Mapping along M–L axis of tectum (c; Schmitt et al., 2006).

A, anterior; D, dorsal; L, lateral; M, medial; N, nasal; P, posterior; T, temporal; V, ventral; c, chick; m, mouse; r, rat; x, Xenopus; z, zebrafish.

2006), also is involved in directing this process. In the developing retina, Shh is expressed in a dynamic high central–low peripheral gradient that, by promoting growth of RGC axons centrally, is essential for normal disc-directed growth (Kolpak et al., 2005). Little however is known about the signalling mechanism relaying the guidance response of RGC axons to Shh. RGCs express Patched (Ptc), a Shh receptor, and indirect evidence has implicated canonical Ptc-Smoothened (Smo) signalling in directing RGC axon pathfinding (Trousse et al., 2001). Recently two other receptors, Hedgehog interacting protein (HiP) and Boc, have been identified as key effectors of Shh-induced axon guidance (Bourikas et al., 2005; Okada et al., 2006). However, whether either of these molecules is expressed by RGCs and, if so, their role in directing optic pathway development has not been established (Fig. 3A).

Once RGC axons reach the optic disc they make their first major change in direction of growth to exit the eye. A key player in this process is the secreted signalling molecule netrin-1 (Table 1; Fig. 3A). Netrin-1 is expressed strongly by the glial cells surrounding the optic disc with its receptor DCC (deleted in colorectal carcinoma) expressed by RGC axons (Deiner et al., 1997; de la Torre et al., 1997; Lauderdale et al., 1997). In mice lacking either netrin-1 or DCC, RGC axons navigate normally to the optic disc but then fail to exit the eye resulting in optic nerve hypoplasia (Deiner et al., 1997). Thus, in contrast to the spinal cord where netrin-1 is secreted by the floorplate and acts at long-range to influence commissural axon outgrowth (Kennedy et al., 1994), in the retina netrin/Dcc signalling functions locally to direct axon outgrowth. Whether this reflects differences in the ability of netrin protein to diffuse in the retina and spinal cord or

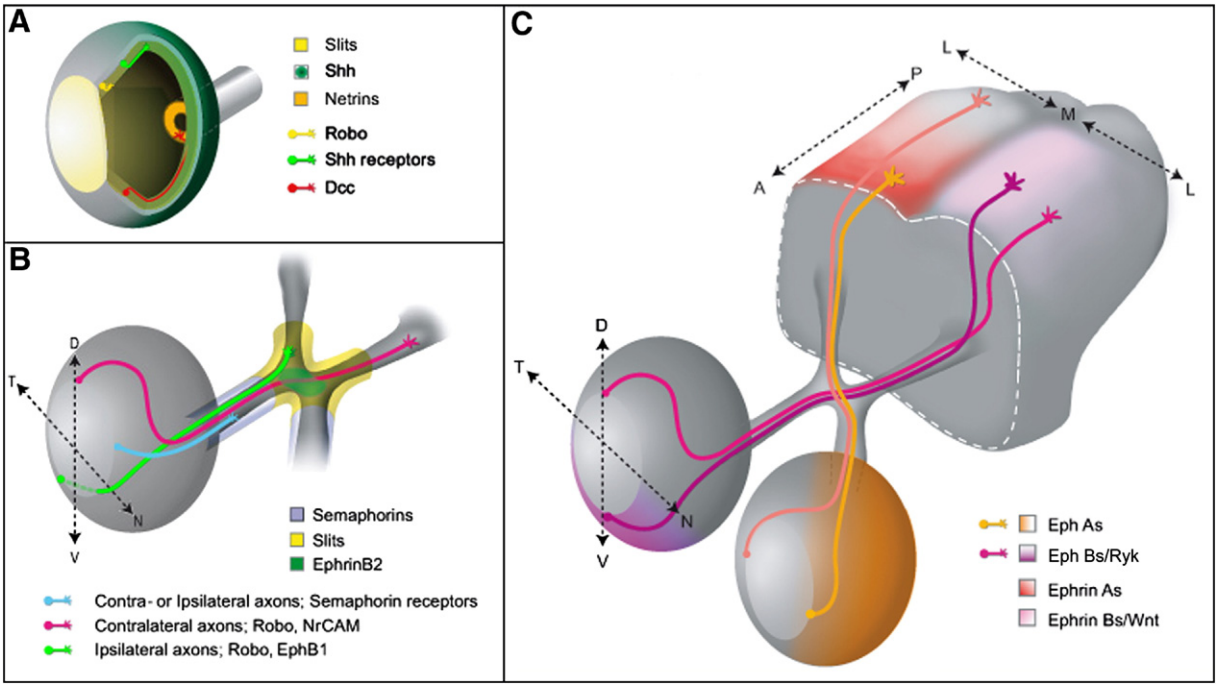

Fig. 3. Key families of guidance cues encountered by RGC axons as they navigate through the mouse optic pathway. (A) Guidance within the retina. Slits acting via Robo2, Shh and cell adhesion molecules (not shown) direct growth to the optic disc where netrin-1 acts locally to guide the RGC axons out of the eye. (B) Guidance at the chiasm. Growth is constrained to the optic nerve through repulsive signalling mediated by Sema5A and at the chiasm, by inhibitory Slits and Shh. At the midline, RGC axons from the VT region of the retina that express EphB1 are repelled by ephrinB2 generating an ipsilateral projection with crossing of the midline being facilitated in part by NrCAM. (C) Topographic mapping of RGC axons in the superior colliculus (SC). Relative levels of EphA receptors on the RGCs and gradients of ephrinAs in the SC determine topographic mapping of the RGC axons along the A–P axis of the SC. The balance of attractive EphBs/ephrinBs and repulsive Wnt/Ryk signalling regulates mapping along the M–L axis. D, dorsal, L, lateral, M, medial, N, nasal, T, temporal, V, ventral. See text and Table 1 for details.

the responsivity of the axons to netrin remains to be determined. Several other factors, including reverse-signalling by EphBs acting as guidance cues, BMP receptor 1B and NrCAM also contribute to the targeting of RGC axons to the optic disc and subsequent exit from the eye (Birgbauer et al., 2000, 2001; Liu et al., 2003; Zelina et al., 2005).

Guidance at the optic chiasm

After leaving the eye, RGC axons run into the developing optic stalk, where they are curtailed to the optic pathway by inhibitory Sema5A signalling (Oster et al., 2003; Fig. 3B), and grow toward the brain where they enter at the ventral-most aspect of the diencephalon. There, axons from the two eyes cross over each other to form a characteristic X-shape known as the optic chiasm (Fig. 2). The factors determining the invariant place at which the chiasm forms are beginning to be elucidated (Table 1).

Shh, in addition to be expressed at the retina, is present along the entire axial midline of the chick embryo prior to the arrival of the RGC axons. As the first RGC axons approach this area, Shh is downregulated specifically at the optic recess level, a spatiotemporal change that, by alleviating a block on RGC axon extension, is critical for chiasm formation (Trousse et al., 2001). In *Pax2* null mice and the equivalent *noi* zebrafish mutant, the chiasm fails to develop and this is associated with persistent expression of Shh in the optic recess (Torres et al., 1996; Macdonald et al., 1997). This suggests that, in contrast to the retina (see above) Shh acts in the chiasmatic region as an inhibitor of RGC axon extension. In vitro, Shh has a dual effect on RGC axons depending on its concentration (Kolpak et al., 2005). Thus, one possibility for this differential responsivity of RGC axons in the retina and chiasm is different levels of Shh expression. Alternatively, intrinsic changes in the RGC axons as they navigate through the optic pathway, for example in cyclic nucleotide levels (see below) or receptor expression (Bourikas et al., 2005) could modulate the response. Further studies will be required to elucidate the basis for this change in Shh function in the retina and chiasm. Slit molecules also are expressed in the diencephalic area and, through inhibitory signalling via Robo2, outline the precise position along the midline neuroaxis at which the optic chiasm develops (Erskine et al., 2000; Fricke et al., 2001; Hutson and Chien, 2002; Plump et al., 2002) (Fig. 3B).

In binocular species, once RGC axons are in the right position and approaching the midline, they have to decide whether to cross or remain uncrossed. Work in *Xenopus* indicated that ephrin-Bs play an important role in inducing divergence at the midline (Nakagawa et al., 2000). Further studies in mice expanded upon these findings and demonstrated that ephrinB2/EphB1 signalling is crucial for the formation of the ipsilateral projection (Table 1). EphrinB2 is expressed by chiasmatic radial glia at the time ipsilateral axons are turning at the midline and is not only sufficient but also necessary for the formation of the ipsilateral projection (Williams et al., 2003; Fig. 3B). A receptor for ephrinB2, EphB1, is expressed highly

by ipsilateral RGCs and *EphB1* null mice show a markedly reduced ipsilateral projection (Williams et al., 2003).

Much less is known about the molecular basis that mediates crossing. So far, only two guidance molecules have been implicated in this process: the cell adhesion molecule, NrCAM and a secreted Semaphorin (Sema3D; Table 1). NrCAM is expressed by RGCs that project contralaterally and at the chiasmatic region. Blocking NrCAM function in vivo causes an increase in the size of the ipsilateral projection and, in vitro, reduces contralateral axon outgrowth on chiasm cells (Williams et al., 2006). Sema3D is expressed at the zebrafish chiasm region when axons are crossing the midline and is involved in guiding RGC axons across the midline and into the contralateral optic tract (Sakai and Halloran, 2006). Signalling by other Semas is mediated by receptor complexes formed by neuropilins and CAMs (Castellani et al., 2000, 2002, Falk et al., 2005) suggesting that perhaps NrCAM and Sema3D interact functionally to promote midline crossing. However this, as well as many other aspects about the role of these molecules in crossing, still need to be investigated. Nonetheless, these studies have demonstrated clearly that crossing the midline is not a default mechanism but an active process and that the midline tissue is a mixed source of permissive, attractive and repulsive cues, which together help to determine the laterality of RGC projections.

From chiasm to targets: guidance in the optic tract

The growth of RGC axons from the chiasm into the optic tracts requires cell autonomous GAP-43 function to overcome inhibitory signals within this region (Kruger et al., 1998; Sretavan and Kruger, 1998; Zhang et al., 2000). Once in the tracts, the axons extend adjacent to the telencephalon but do not normally invade this tissue and display a regional-specific organisation pattern that correlates with the presence of secreted inhibitory factors within the diencephalon (Tuttle et al., 1998). To date, only a few molecules essential for guidance in the optic tracts have been identified (Table 1).

As with other regions of the optic pathway, Slit–Robo signalling is required and serves a barrier function preventing the axons from straying away from their normal pathway into the telencephalon and inappropriate regions of the diencephalon such as the epithalamus and pineal (Ringstedt et al., 2000; Fricke et al., 2001; Hutson and Chien, 2002; Thompson et al., 2006b; Fig. 2). CSPGs also help prevent RGC axons from extending aberrantly across the diencephalic/telencephalic boundary (Ichijo and Kawabata, 2001) and cell–cell and cell–matrix interactions mediated by *N*-cadherin and β-1 integrin play a key role in restricting the RGC axons to the optic tract (Stone and Sakaguchi, 1996). Interestingly, there is genetic and biochemical evidence of interactions between Slits and both *N*-cadherin and β-1 integrin suggesting that cross-talk between these signalling systems may be important for normal development of the optic pathway (Rhee et al., 2002; Stevens and Jacobs, 2002). Inhibitory signalling by Tenascin-R and SFRP1 (secreted frizzled related protein 1) plays an important role in define the precise path followed by the RGC axons as they navigate through the optic tract (Becker et al., 2003; Rodriguez et al., 2005) with entry into the tectum requiring FGF signalling (McFarlane et al., 1995, 1996). In vitro assays and expression studies also have implicated Sema3A in regulating optic tract development (Campbell et al., 2001). However, a functional requirement for this molecule during optic tract development in vivo has not yet been demonstrated. Clearly further work is required to unravel not only the role of Sema3A but also the precise repertoire of signals underlying RGC axon guidance in this poorly studied region of the optic pathway.

Axon guidance at the visual targets

Vertebrate nervous systems carry multiple representations or maps of the external world. These representations tend to organise topographically and translate stimulus features into a coherent neural code, allowing the interpretation of sensory information. The study of map development in the two main visual targets, the lateral geniculate nucleus (LGN) and the superior colliculus (SC) in mammals (optic tectum among non-mammals) has been one of the most productive models to understand general mapping processes.

In the visual system, the cartesian coordinates of the eye are mapped onto those of the tectum/SC. Axons that arise from RGCs in the nasal retina project to targets at the posterior end of the tectum/SC, axons from the temporal region project to the anterior end and the dorsal–ventral axis of the retina is mapped with equal precision onto the medial (dorsal)–lateral (ventral) axis of the tectum/SC (Fig. 2). The work of many groups has proven definitively the chemoaffinity theory formulated by Sperry almost 50 years ago. Sperry proposed that RGCs carry stable positional chemical labels disposed in gradients to determine their tectal termination (Sperry, 1963). It is well accepted now that the role of these chemical "labels" is played mainly by the Eph/ephrin family (Table 1; Fig. 3C). Genetic gain- and loss-of-function experiments with EphA receptors (Brown et al., 2000; Feldheim et al., 2004) together with analyses of mice carrying targeted deletions of the ligands ephrinA5, ephrinA2 or both, show that EphA/ephrinA signalling is required for proper mapping along the anterior–posterior axis of the tectum/SC (Frisen et al., 1998; Feldheim et al., 2000). EphrinAs are also expressed in a high-to-low gradient along the lateral–medial axis of the LGN and disruption of EphrinA5 causes loss of topographic precision in the LGN (Feldheim et al., 1998). It seems however that during the establishment of the retinotopic map there is a period when neuronal activity modulates, non synaptically, the repellent action of ephrin-As. Neuronal depolarisation acts synergistically with the ephrin signal to enable a retraction response of the growth cone, involving the downstream second messengers calcium and cAMP (Nicol et al., 2007).

In parallel, EphBs/ephrinBs pattern the mapping along the medial–lateral axis of the tectum/SC (Table 1; Fig. 3C). EphB receptors show a high ventral–low dorsal expression gradient in the retina and low lateral–high medial gradient in the tectum/SC. Ephrin-Bs show complementary patterns in each

structure. In mice, deletion of EphB2 or EphB3 receptors leads to defects in topographic mapping of ventral RGC axons demonstrating a key role for classical Eph forward signalling in mediating this process (Hindges et al., 2002). In Xenopus, alterations in ephrinB2 expression in the retina also result in mistargeting along the medial–lateral axis of the tectum/SC but this time through reverse signalling (Mann et al., 2002). Based on the expression patterns, in vitro assays and these loss-of-function experiments it appears that EphBs/ephrinBs serve an attractive function within the tectum/SC (Hindges et al., 2002; Mann et al., 2002). Counterbalancing this is a gradient of inhibitory Wnt/Ryk signalling (Table 1). In the tectum/SC one member of the Wnt family of secreted signalling molecules, Wnt3, is expressed in an essentially identical gradient to ephrinBs with the expression of Ryk in the retina matching that of the EphBs (Fig. 3C). Over-expressing Wnt3 in the tectum results in RGC axons avoiding these areas whereas expression of a dominant-negative Ryk in dorsal RGCs results in a shift in RGC axon termination zones to the medial area of the tectum (Schmitt et al., 2006). In zebrafish a high lateral–low medial gradient of Sema3D also has been implicated in regulating medial–lateral mapping in the tectum through inhibitory signalling (Liu et al., 2004). Thus medial–lateral mapping in the tectum/SC is dependent on opposing functional gradients of attractants and repellents that co-operate to induce precise map formation.

Ephs/ephrins also may be involved in controlling the other level of organisation in the patterning of RGC projections: the eye-specific layering. In species with binocular vision in addition to mapping topographically in the LGN and tectum/SC, RGC axons are organised in an eye-specific manner. Early in development, the projections from the two eyes overlap, but then segregate and form eye-specific layers postnatally (Rakic, 1976; Linden et al., 1981; Shatz, 1983). Inhibiting activity in the retina prevents this segregation and thus this phenomenon was thought to depend purely on neural activity (Penn et al., 1998; Shatz and Stryker, 1988; Chapman, 2000; Stellwagen and Shatz, 2002). However, the projections from each eye terminate in stereotyped locations within the LGN and this regularity cannot be explained easily by activity-dependent mechanisms alone. Both loss of EphrinA function or overexpression of EphAs induces eye-specific targeting errors in the LGN, demonstrating a key role for EphA/ephrinA signalling in directing this process (Huberman et al., 2005; Pfeiffenberger et al., 2005). Together with previous studies (Huberman et al., 2002, 2003) this suggests that activity and EphAs–ephrinAs signalling act in parallel to construct eye-specific maps, with activity segregating the axons from the two eyes and EphAs/ephrinAs dictating the shape, size and position of the eye-specific territories. Thus, multidisciplinary studies combining electrophysiology and cellular and molecular approaches as well as in vivo and in vitro assays, all applied to investigate the formation of projection patterns in both the LGN and the SC, have revealed the importance of synergistic interactions between neuronal depolarisation and ephrin signalling in the establishment of retinotopic maps.

Regulatory genes controlling the RGC axon pathway

Many studies using the vertebrate visual system have reinforced the general idea that axonal trajectories are determined initially by sets of transcription factors that in turn regulate the expression of specific axon guidance molecules (Table 2). A number of regulatory genes expressed in the developing retina are essential for axon guidance along the optic pathway and in a few cases some of their target molecules have been described.

Mutant mice for several transcription factors involved in retinal differentiation such as *Vax1*, *Vax2*, *Pax2*, *FoxG1* or *FoxD1* exhibit different defects in RGC axon trajectory. In *Pax2* mutants all the axons turn prematurely at the hypothalamus and extend aberrantly into the ipsilateral tract (Torres et al., 1996). *Vax1* mutants show defects in RGC axons penetrating the brain (Bertuzzi et al., 1999) whereas a reduction in the ipsilateral projection is observed in *Vax2* KO mice (Barbieri et al., 2002; Mui et al., 2002). Loss of the forkhead transcription factor *FoxG1* causes pathfinding defects along the optic pathway including exiting the retina and misrouting at the optic chiasm (Pratt et al., 2004), and absence of *FoxD1* leads to axons extending aberrantly at the optic chiasm and tracts (Herrera et al., 2004). However, since all these genes are expressed at early stages of retinal differentiation more studies are needed to differentiate between their role in general patterning as opposed to regulation of axon guidance cues specifically.

In contrast, a small number of regulatory genes have been shown to directly alter axonal trajectories. Misexpression of the zinc finger transcription factor *Zic3* (Zhang et al., 2004) or gain- or loss-of-function of the homeobox gene *Irx4* within the retina (Jin et al., 2003), affects intraretinal pathfinding. Both genes are expressed in the RGC layer and one of the targets for *Irx4* is Slit1, a guidance signal known to be critical for RGC axon pathfinding (Plump et al., 2002; Jin et al., 2003; Thompson et al., 2006a, b). Several members of the Pou family of transcription factors are expressed in postmitotic RGCs and are key regulators of RGC axon pathfinding. RGC axons from single mutants lacking *Brn3b* (*Pou4f2*) as well as in double *Brn3b/Brn3c* (*Pou4f3*) mutants show defects in axon navigation along the entire optic pathway (Erkman et al., 2000; Wang et al., 2002). Transcription factors, such as Brn3a, Dlx2, Dlx3, Irx4, Irx6, Isl2, Olf-1/2, Gli1, Gfi1, have been described as targets for Brn3b, as well as axon guidance molecules including Shh, L1 and Hermes and regulators of the cytoskeleton, such as GAP-43 and abLIM (Erkman et al., 2000; Mu et al., 2004, Pan et al., 2005). This suggests that retinal expression of Brn3b may control a complex developmental program that activates expression of specific axon guidance receptors at distinct points along the optic pathway.

Perhaps the clearest case of a transcription factor in determining directly axon guidance in the RGC axon pathway is the zinc finger transcription factor Zic2. Zic2 is transiently expressed in RGCs that project ipsilaterally and is crucial in specifying the ipsilateral RGC axon projection at the optic midline (Herrera et al., 2003). Zic2 expression in ventral

Table 2
Regulatory genes controlling RGC axon pathfinding

Gene	Expression	Function
Brn3b	Majority of RGCs	Controls intrinsic transcriptional network required for guidance in retina, optic chiasm and tract and mapping in SC (m; Erkman et al., 2000).
En-1/2	Low A–high P gradient in the tectum/SC	Control mapping in the tectum by regulating ephrinA expression (c; Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996).
FoxD1	VT quadrant of retina, developing chiasmatic region.	Patterning of retina and ventral diencephalon required for normal chiasm formation (m; Herrera et al., 2004) and A–P mapping in tectum (c; Yuasa et al., 1996).
FoxG1	Nasal RGCs; ventral diencephalon anterior to chiasm.	Patterning of retina and ventral diencephalon underlying establishment of binocular visual pathways (m, Pratt et al., 2004) and A–P mapping in tectum (c; Takahashi et al., 2003; Yuasa et al., 1996).
GH6	High N–low T gradient in retina	Controls mapping in tectum by regulating negatively EphA3 expression (c; Schulte and Cepko, 2000)
Irx4	Subset of cells in RGC layer of retina.	Controls RGC axon fasciculation and guidance within the retina by regulating negatively Slit1 expression (c; Jin et al., 2003).
Islet2	Contralaterally projecting RGCs	Specification of contralaterally projecting RGCs by repressing Zic2 expression (m; Pak et al., 2004).
Lhx2	Retina; ventral diencephalon including pre-optic area	Controls expression of guidance cues in preoptic area of diencephalon required for chiasm formation (z; Seth et al., 2006).
Pax2	Proximal region of retina; glial cells of optic stalk; midline of ventral diencephalon.	Represses Shh from the chiasmatic midline enabling formation of the crossed chiasmatic projection (m; Torres et al., 1996; z; Macdonald et al., 1997).
SOHo	High N–low T gradient in retina	Controls mapping in tectum by regulating negatively EphA3 expression (c; Schulte and Cepko, 2000)
Tbx5	Dorsal retina	Controls mapping in the tectum by regulating EphB/ephrinB expression (c; Koshiba-Takeuchi et al., 2000).
Vax1	Glial cells of optic nerve; CNS midline.	Controls expression of attractive guidance cues required for growth of RGC axons into the brain (m; Bertuzzi et al., 1999).
Vax2	Ventral retina	Patterning of retina underlying establishment of binocular visual pathways and mapping in tectum/SC (m; Mui et al., 2002; Barbieri et al., 2002; c; Schulte et al., 1999).
Zic2	Ipsilaterally projecting RGCs	Specification of ipsilaterally projecting RGCs (f, m, x; Herrera et al., 2003).
Zic3	Receding high peripheral — low central gradient in the retina	Controls expression of inhibitory guidance cue(s) required for intraretinal RGC axon pathfinding (c; Zhang et al., 2004).

A, anterior; D, dorsal; N, nasal; P, posterior; T, temporal; V, ventral; c, chick; f, ferret; m, mouse; x, Xenopus; z, zebrafish.

temporal retina matches the spatiotemporal expression of EphB1 that is necessary for the formation of the ipsilateral projection (Williams et al., 2003; see above). However the precise relationship between these two molecules has not yet been established. In particular it is not know if Zic2 is a direct regulator of EphB1 expression and this is the focus of ongoing work. The lim homeodomain transcription factor Islet2 also is involved in determining laterality at the chiasm. Islet2 is expressed specifically in contralaterally projecting RGCs and mice lacking *Islet2* show an increase in the number of axons that project ipsilaterally as a result of increased expression of Zic2 and EphB1 (Pak et al., 2004).

Transcriptional regulation of EphAs in retina also controls targeting to the tectum/SC. *SOHo*, *GH6*, *FoxG1* and *FoxD1* are all expressed in chick retina along the nasotemporal axes. Misexpression of any of these genes in the retina results in projection errors along the anterior–posterior axis of the tectum by altering the expression of EphA receptors (Yuasa et al., 1996; Schulte and Cepko, 2000; Takahashi et al., 2003). *Tbx5* is expressed in the dorsal retina repressing *Vax2*, which is restricted to the ventral retina (Koshiba-Takeuchi et al., 2000, Sakuta et al., 2001). Alteration of either of these two transcription factors causes mapping errors at the tectum/SC through regulation of EphB receptors and likely other, yet unidentified, factors (Schulte et al., 1999; Koshiba-Takeuchi et al., 2000).

Some regulatory genes are expressed in the tissues along the optic pathway as well as at the final targets. Lhx2, FoxD1 and Gli2 are all expressed at the developing ventral diencephalon. *Lhx2* zebrafish mutants lack Sema3D at the diencephalon and Slit2 expression is expanded across the ventral midline (Seth et al., 2006). Interestingly, genetic removal of FoxD1 in mouse also causes misexpression of Slit2 at the chiasm (Herrera et al., 2004) and disruption of Gli2 leads to reduction in the expression of Sema3D (Barresi et al., 2005).

Transcription factors expressed in the target tissues also contribute to mapping through regulation of Eph/ephrins. The homeobox genes engrailed-1 (En-1) and engrailed-2 (En-2) are expressed in a low-to-high gradient along the anterior–posterior axis, controlling ephrinA levels (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996). As in the retina, it is not clear how far back these regulatory genes are from directly regulating axon guidance proteins. Interestingly, En-2 may also function as an axon guidance cue, since when applied exogenously in culture, it acts as an attractant for nasal RGC axons and a repellent for temporal axons (Brunet et al., 2005). However, in vivo demonstration of this guidance ability for En-2 has not yet been reported.

Post-transcriptional regulation of guidance responses

Guidance of axons along particular pathways is not simply the result of expressing specific cues in the environment and the corresponding receptors on the growth cone. There is considerable plasticity in guidance responses that can be modulated by a range of post-transcriptional mechanisms, many of which are essential for axon guidance within the developing visual system. These regulatory mechanisms include modifiers of receptor–ligand interactions, endocytosis of receptor–ligand complexes, local translation and trafficking of stored mRNAs and changes in the intracellular concentration of cyclic nucleotides (Table 3).

Among the first group are heparan sulphate proteoglycans (HSPGs). These are extracellular glycoproteins that have long been known to play a role in RGC axon guidance (Halfter, 1993; Walz et al., 1997). HSPGs bind to Slits and Robos forming a ternary complex (Hussain et al., 2006) and are key regulators of Slit localisation and function (Hu, 2001; Inatani et al., 2003; Bülow and Hobert, 2004; Johnson et al., 2004, Steigemann et al., 2004; Piper et al., 2006). Mice lacking HSPGs, generated by genetic removal of the biosynthetic enzyme Exostsin1 (Ext1), display misrouting of axons at the optic chiasm with many axons invading the contralateral optic nerve (Inatani et al., 2003). This phenotype is similar to that seen in *slit1/2*-deficient mice (Plump et al., 2002) and *Ext1* and *slit2* show genetic interactions, suggesting that these molecules operate in the same pathway (Inatani et al., 2003). HSPGs are extremely diverse molecules due to the wide range of different sulphation and epimerisation patterns of their HS side chains, enabling potentially precise regulation of guidance cue function. Indeed, in the developing visual system, specific HS structures are synthesised at defined locations along the optic pathway and, in part by regulating Slit–Robo signalling, are required for distinct aspects of guidance in the optic chiasm and tracts (Walz et al., 1997; Lee et al., 2004; Pratt et al., 2006). Slits are not the only guidance cues dependent on HSPGs and specific HS structures also are obligate co-receptors for other factors, for example FGFs, essential for optic pathway development (McFarlane et al., 1995, 1996). CSPGs also are required for guidance in the developing visual system (Table 1; Brittis et al., 1992; Chung et al., 2000; Walz et al., 2002). However, whether this is the result of a direct effect on axons (Snow et al., 1991) or by modulating the function of other guidance signals (Kantor et al., 2004) remains to be tested. Nevertheless, together these studies have provided novel insights into the role of proteoglycans, in particular their carbohydrate side-chains, in directing axon guidance in vivo.

The chemokine stromal cell-derived factor-1 (SDF-1) has no guidance activity by itself but can attenuate the signalling of multiple chemorepellents including Slits. This anti-repellent activity is mediated via its receptor CXCR4 resulting in elevations in cAMP levels, a known modulator of guidance responses (Chalasani et al., 2003a; see below). In the developing visual system, CXCR4 is expressed by RGCs and SDF-1 at multiple points along the optic pathway (Chalasani et al., 2003b, 2007; Li et al., 2005). RGC axon guidance defects in zebrafish with decreased levels of Robo2 can be rescued by reducing SDF-1-signalling. This is likely an indirect effect and the result of increased effectiveness of Slit (Chalasani et al., 2007). Thus the anti-repellent activity of SDF-1 is an important mechanism by which the response of growth cones to specific guidance cues can be regulated to help direct the precise path followed by RGC axons.

Another set of modifiers for ligand–receptors interactions are the metalloproteases. These enzymes regulate effective signalling by cleaving the ectodomains of specific guidance cues and/or their receptors (Galko and Tessier-Lavigne,

Table 3
Post-transcriptional regulators of RGC axon pathfinding

Regulator	Function	Role in optic pathway development
Cyclic nucleotides	Relative levels in growth cone determine sensitivity and response to individual guidance cues (Song et al., 1997, 1998).	Changes in cAMP levels triggered by composition of the extracellular matrix required for growth out of the eye (x; Höpker et al., 1999). Intrinsic changes in cAMP level induce age-related alterations in response to specific guidance cues (x; Shewan et al., 2002).
Endocytosis	Sensitivity and response of growth cones to guidance cues (e.g. Marston et al., 2003; Piper et al., 2005; Zimmer et al., 2003).	?
HSPGs	Co-receptors for several guidance cues including Slits and FGFs (e.g. Hussain et al., 2006).	Specific HS structures regulate distinct aspects of guidance in optic chiasm and tracts (x; Walz et al., 1997; z, Lee et al., 2004; m; Pratt et al., 2006).
Metalloproteases	Cleave guidance cues regulating signalling timing/efficacy and nature of response (Galko and Tessier-Lavigne, 2000; Hattori et al., 2000).	Required for guidance in optic chiasm and tracts (x; Webber et al., 2002; Hehr et al., 2005).
Protein synthesis in growth cones	Regulation of guidance responses induced by extracellular signals (Campbell and Holt, 2001).	?
SDF-1	Antagonises function of multiple guidance cues (Chalasani et al., 2003a).	Regulates pathfinding through modulation of Slit signalling (z; Chalasani et al., 2007).

m, mouse; x, Xenopus; z, zebrafish.

2000). Metalloproteases also are required to terminate the high affinity interaction that occurs between ephrinAs and their Eph receptors resulting in release of contact, thereby enabling growth cone retraction and termination of signalling (Hattori et al., 2000). In the developing visual system, addition of metalloprotease inhibitors results in severe RGC axon guidance defects at multiple points along the optic pathway (Webber et al., 2002; Hehr et al., 2005). The precise metalloproteases involved and the guidance cues affected are not known. However, these studies have provided direct evidence that regulation of guidance responses by metalloproteases is critical for the normal development of the visual system.

Growth cones exposed constitutively to guidance cues exhibit adaptation and cycle between consecutive phases of desensitisation and resensitisation. Both processes are ligand specific with desensitisation requiring endocytosis and resensitisation local protein synthesis within the growth cone (Ming et al., 2002; Piper et al., 2005). Endocytosis also regulates several other aspects of guidance cue responses and is required for the collapse response induced by several different guidance cues and, in the case of EphB/ephrinB interactions, to enable disengagement of the cells, turning adhesion into repulsion (Fournier et al., 2000; Mann et al., 2003; Marston et al., 2003; Zimmer et al., 2003; Piper et al., 2006). Protein synthesis that occurs locally in the growth cone also is a key mechanism downstream of many signalling molecules enabling guidance responses (Campbell and Holt, 2001; Brittis et al., 2002). One of the proteins synthesised locally in growth cones is β-actin. In growth cones exposed to a gradient of guidance information β-actin mRNA becomes asymmetrically localised and translated providing a polarised source of actin that, due to its key role in regulating growth cone motility, may underlie turning (Leung et al., 2006; Yao et al., 2006). Clearly, endocytosis and local protein synthesis are important regulators of both the acute and long-term adaptive responses of growth cones to guidance cues. However, the precise role of these processes in directing axon pathfinding in vivo has not been established and further studies will be required to investigate this key issue.

Intracellular second messengers, such as cyclic nucleotides and Ca^{2+} also modulate growth cone responses. Changing the ratio of cAMP: cGMP can switch attraction to repulsion and vice versa with some cues, for example netrin-1, being more dependent on cAMP and others, such as Sema3A and Slit on cGMP (Song et al., 1997, 1998; Nguyen-Ba-Charvet et al., 2001; Nishiyama et al., 2003). Decreasing the resting growth cone intracellular Ca^{2+} concentration also switches attractive turning to repulsion (Zheng, 2000). In Xenopus RGC axons netrin-1-induced attraction can be converted to repulsion by a substrate of laminin and the associated decrease in growth cone cAMP levels. Laminin is co-expressed at the optic nerve head with netrin-1 and adding exogenous laminin peptides to the retina results in RGC axon pathfinding errors and failure of some axons to exit the eye (Höpker et al., 1999). Intrinsic changes in growth cone cAMP levels also occur in RGC axons with increased age and correlates with a switch in responsiveness to netrin-1 from attraction, to no response, to repulsion (Shewan et al., 2002). Dynamic rather than static changes in cAMP levels have also been demonstrated recently to play a key role in modulating guidance cue function and provide a link between spontaneous neural activity and ephrinA-induced RGC axon retraction in the tectum/SC (Nicol et al., 2007). Thus cyclic nucleotides provide a mechanism by which guidance information can be integrated from multiple co-expressed signals and may underlie endogenous changes in the growth cone enabling the same cue to serve different functions in disparate regions of the pathway.

Conclusions and future directions

Studies of RGC axons, both in vivo and in vitro, have played a pivotal role in revealing the molecular basis of axon guidance. Investigations using this system have been instrumental in revealing the precise function of specific guidance cues and the mechanisms controlling their spatial–temporal expression as well as the plasticity of guidance responses. An emerging theme is that a small number of cues are used reiteratively along the pathway to serve different functions (Table 1). However, we have only just begun to scratch the surface of the mechanisms regulating optic pathway development and much remains to be discovered both in terms of the molecular nature of the guidance signals and the factors regulating their function. It is clear that multiple cues act in concert to control guidance decisions and a key challenge will be to unravel the complex co-operative and redundant interactions that occur between co-expressed signals and parallel signalling pathways. Additionally, although transcriptional regulation is important for guidance decisions, the precise relationship between this regulatory mechanism and the control of guidance responses is still an ongoing question. One possibility is that transcriptional regulation during neurogenesis provides the complete repertoire of receptors for the growth cone to navigate its entire pathway. Alternatively, transcriptional regulation may be more dynamic and function in a hierarchical manner to induce distinct signalling capabilities in the growth cone at specific points of the pathway. Furthermore, since transcriptional regulation cohabits with post-transcriptional mechanisms, such as local translation and post-translational modifications, the coordination of these disparate processes needs to be investigated. We have come a long way in deciphering the guidance code used by RGC axons on route to their target and continued studies of this system will undoubtedly result in further advances in our understanding of the general mechanisms underlying the precise wiring of the nervous system.

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