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Axon guidance receptors direct growth cone pathfinding: rivalry at the leading edge

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ABSTRACT One of the earliest steps in the development of the central and peripheral nervous systems is the initiation of axon outgrowth from newly born neurons. Nascent axons then navigate towards their specific targets to establish the intricate network of axon projections found within the mature central nervous system. In doing so, the projecting axons must continually reassess their spatial environment and accurately select the correct pathways among the maze of possible routes. A variety of molecular navigational systems governing axon pathfinding have now been identified. Understanding how these individual molecular guidance systems operate at the level of a single axon, and, how these different systems work in concert to initiate and steer axonal migration is a major goal in developmental neurobiology.

KEY WORDS: *Axon guidance, receptor cross-talk, guidance cue distribution, receptor activation*

Introduction

Directionality in axonal migration is determined by the response of the growth cone to the local environment through which it travels. Long-range guidance cues, secreted from intermediate or final targets, form chemotactic gradients along the pathway of the exploring growth cone. Membrane-bound, or secreted short-range guidance cues are also employed to affect changes in the direction of growth cone migration along axon pathways and at specific choice points (Tessier-Lavigne and Goodman, 1996). The intracellular signalling cascade initiated upon detection of the guidance cue by the axon-bound receptor triggers dynamic rearrangements of the actin cytoskeleton within the growth cone, promoting cycles of extension and retraction of filopodia at the leading edge (reviewed in Song and Poo, 2001). This allows continual reassessment of the immediate environment by the growth cone. In the case of chemoattraction, movement along the desired trajectory is achieved by elongation of the actin cytoskeleton leading to the promotion of filopodia extension towards the source of the guidance cue. In contrast, chemorepulsion promotes actin depolymerisation and filopodia retraction resulting in growth cone collapse and ultimately migration away from the ligand source.

The known expression patterns of many families of guidance receptors reveal that projecting growth cones display an array of guidance receptors simultaneously on their surface. Moreover, a given guidance cue may be interpreted as either attractive or repulsive depending on the identity, or, the molecular environment of the receptor residing on the membrane of the growth cone. Therefore, to produce a synchronized biological response to the conflicting array of environmental signals encountered by the migrating growth cone, a multi-layered regulatory system has evolved to modulate receptor activity. The aim of this review is to explore the molecular mechanisms that (i) govern the dynamic temporal and spatial expression of the guidance cues and their receptors, and (ii) determine the biological consequences of receptor-ligand interactions. To set the scene, a brief summary of the biological relevance of the major guidance systems is given below.

The Netrin-DCC/UNC5 Guidance System

The DCC axon guidance receptor and its ligands, the Netrins, have been shown to play a pivotal role in the guidance of axonal projections toward the ventral midline throughout the developing nervous system (Fig. 1 A,B). The interaction of Netrin-1with DCC results in a chemoattractive response (Hedgecock *et al.*, 1990; Keino-Masu *et al.*, 1996) while interaction with the UNC5 family of Netrin receptors results in chemorepulsion (Hedgecock *et al.*, 1990; Leung-Hagesteijn *et al.*, 1992; Leonardo *et al.*, 1997; Przyborski *et al.*, 1998). Mice lacking DCC or Netrin-1 exhibit severe defects in commissural axon extension towards the floorplate

Abbreviations used in this paper: A/P, anterior/posterior; CAM, cell adhesion molecule, CNS, central nervous system; Comm, Commissureless; CST, corticospinal tract; DRG, dorsal root ganglion; D/V, dorsal/ventral; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; Np, Neuropilin; PNS, peripheral nervous system; RGC, retinal ganglion cell; Robo, Roundabout; Sema, Semaphorin.

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and also lack several major commissures within the forebrain, including the corpus callosum, and the hippocampal commissure (Serafini *et al.*, 1996; Fazeli *et al.*, 1997). In addition, examination of *Dcc* null embryos has also revealed that DCC is crucial for the migration of some neuronal populations (Serafini *et al.*, 1996; Bloch-Gallego *et al.*, 1999). DCC is also required for the dorsal migrations of some circumferential axons away from the ventral midline (the Netrin source) in the nematode *Caenorhabditis elegans* (*C.elegans*) (Hedgecock *et al.*, 1990), probably by participating in a receptor complex with UNC5 (Hong *et al.*, 1999). In the developing nematode, loss-of-function mutations in the *unc5* gene results in aberrant dorsal migrations for both axons and mesodermal cells

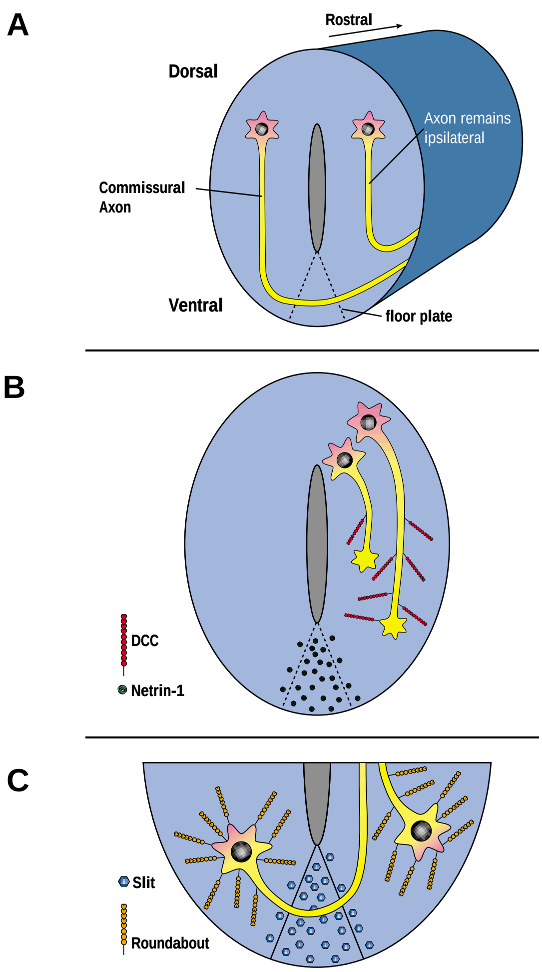

Fig. 1. Axon navigation in the developing mammalian neural tube. (A) *Commissural neurons in the dorsal neural tube project their axons ventrally and cross the floorplate to the contralateral side before projecting rostrally. Axons from some subpopulations of dorsal neurons project ventrally, but remain on the ipsilateral side of the ventral midline.* **(B)** *The Netrin receptor, DCC, guides commissural axons along a Netrin gradient (originating in the floorplate) towards the ventral midline.* **(C)** *The chemorepulsive guidance cue, Slit, is expressed by the floorplate. It is proposed that the Slit receptor, Robo, is expressed at high levels on those axons that never cross the midline. Axons destined to cross the midline express very low levels of Robo when projecting on the ipsilateral side. Once on the contralateral side, Robo protein is up-regulated on the axonal membrane and these axons never cross the midline again.*

(Hedgecock *et al.*, 1990). In the mouse, UNC5 has also been shown to drive neural cell migration within the developing cerebellum (Przyborski *et al.*, 1998). A third Netrin receptor, Neogenin, has been described in mammals (Keeling *et al.*, 1997; Meyerhardt *et al.*, 1997). This receptor is closely related to DCC and can bind the Netrins with high affinity, however, as yet, no insights into the function of this receptor have been gained.

The Slit/Roundabout Guidance System

Studies in *Drosophila melanogaster* have identified a guidance system which prevents axons from crossing the ventral midline inappropriately. It is presumed that a similar mechanism operates in the vertebrate neural tube (Fig. 1 A,C). In *Drosophila*, Slit is expressed by glia at the ventral midline where it acts as a chemorepulsive guidance cue (Rothberg *et al.*, 1990). The Slit receptor, Roundabout (Robo), is expressed at high levels on those axons that never cross the midline (Kidd *et al.*, 1998a). In contrast, axons destined to cross the midline express very low levels of Robo when projecting on the ipsilateral side. Once on the contralateral side, Robo protein is greatly up-regulated on the axonal membrane and these axons never cross the midline again. *Robo* loss-of-function mutations result in both the commissural and non-commissural axons crossing the midline multiple times (Kidd *et al.*, 1998a; Kidd *et al.*, 1998b). Thus, Robo acts as a "gatekeeper" for midline crossing. Three Slit and three Robo orthologues have been identified in mammals (for review see Brose and Tessier-Lavigne, 2000). The ability of mammalian Slits to act as chemorepulsive guidance cues has been clearly demonstrated for a variety of axon populations including olfactory bulb, hippocampal, and spinal motor axons (see Brose and Tessier-Lavigne, 2000). In addition, the chemorepulsive activity of the Slits has been implicated in the targeted migration of neuroblasts within the rostral migratory stream towards the olfactory bulb (Hu, 1999; Wu *et al.*, 1999) and GABAergic neurons from the ganglionic eminence into the cortex (Zhu *et al.*, 1999). Unexpectedly, Slit2 has also been shown to induce axon branching in sensory neurons (Wang *et al.*, 1999). Although there has been no direct demonstration that the Robos are the Slit receptors in the mammal, the ability of the mammalian Slits and Robos to interact biochemically (see Brose and Tessier-Lavigne, 2000) along with their complementary expression patterns during embryogenesis, suggest that this will be the case in many instances.

The Semaphorins and their Receptors

The Semaphorins were identified as chemorepellent axon guidance cues in the developing nervous system of the fly, and the rodent (Kolodkin *et al.*, 1993; Luo *et al.*, 1993) and have now also been implicated in a variety of other biological processes, including angiogenesis, cardiac, bone, and skeletal development, and in the immune response (reviewed in Raper, 2000). In vertebrates, Semaphorins may be transmembrane proteins (classes IV to VI), attached to the membrane surface via a phosphatidylinositol linkage (class VII), or secreted (class III). Sema3A, a secreted Semaphorin, has been shown *in vitro* to behave as a chemorepulsive guidance cue for hippocampal and olfactory axons, and pontocerebellar mossy fibres from the central nervous system (CNS) as well as for sensory, motor and sympathetic axons in the peripheral nervous system (PNS) (reviewed in Raper, 2000). Semaphorins have also been found to act as chemoattractive guidance cues for cortical dendrites (Polleux *et al.*, 2000) and olfactory bulb axons (de

Castro *et al.*, 1999). The first Semaphorin receptors to be identified were the Neuropilins (Np-1 and Np-2) (Chen *et al.*, 1997; He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997) which recognize only the secreted Semaphorins. Gene targeting of the *Sema3A* and *Np-1* loci have demonstrated that Sema3A-Np-1 interactions are required for the fasciculation of the peripheral fibres of the trigeminal and vagal projections (Kitsukawa *et al.*, 1997; Taniguchi *et al.*, 1997). The interpretation of this phenotype is that Sema3A surrounding the projecting axons forces them to fasciculate rather than remain exposed to the repulsive activity of the Semaphorin. Gene targeting of the *Np-2* gene has demonstrated that Np-2 (the Sema3F receptor) is required for the organization and fasciculation of several cranial and spinal nerves (Chen *et al.*, 2000; Giger *et al.*, 2000). In addition, several major fibre tracts in the CNS are either severely disorganized or missing. It is also believed that the Np-1-Np-2 hetrodimer is the receptor complex that recognizes Sema3C. More recently, a large family of transmembrane proteins, the Plexins, has been uncovered. Plexins directly associate with the Neuropilins but cannot interact with the Semaphorins (Winberg *et al.*, 1998; Tamagnone *et al.*, 1999). It is believed that the Plexins are the signalling receptors for all Semaphorin classes.

The Ephs and Ephrins

The most intensely studied of all guidance receptor families is the Eph receptor tyrosine kinase family. Interaction of these receptors with their membrane bound ligands, the Ephrins, drives axon pathfinding throughout the developing CNS and PNS via a chemorepulsive mechanism (for comprehensive reviews see: Holder and Klein, 1999; Wilkinson, 2001). The Eph-Ephrin system plays a key role in establishing topographical maps within the CNS. The best characterized of these is the retinotectal map in the chick which determines the position of retinal axon terminations along both the anterior/posterior (A/P) and dorsal/ventral (D/V) axes within the tectum (see above reviews for details of the retinotectal mapping system). Briefly, axon navigation along the A/P pathway is driven by the graded response of retinal axons to overlapping gradients of EphrinA2 and EphrinA5 in the tectum (anterior-low to posterior-high). Axons extending from the temporal retina express high levels of EphA3 and are sensitive to low concentrations of Ephrins in the anterior tectum and are therefore effectively repulsed from this region. In contrast, axons deriving from the nasal retina express low levels of EphA3 and are less sensitive to higher Ephrin concentrations and can therefore penetrate through the anterior tectum into the posterior tectum. Several other Eph/Ephrin gradients overlay this EphA3-based guidance mechanism to create the intricate topographic relationship between the source of the projecting retinal axon (eg. nasal versus temporal) and their termination along the A/P and D/V axes of the tectum.

In addition to their pivotal role in axon guidance, the chemorepulsive activity of the Ephs and Ephrins has also been shown to play a key role in the essential developmental processes of tissue patterning and boundary formation by restricting intermingling between cells expressing the receptors and those expressing the ligand. Over recent years the Eph-Ephrin story has become further complicated with the demonstration that the Ephrins themselves are capable of initiating signal cascades (reverse signalling) (Lu *et al.*, 2001), and in some instances appear to be acting as the guidance receptor (Henkemeyer *et al.*, 1996). Intriguingly, evidence is now emerging that in some developmental systems (angiogenesis, vascular remodelling, and neural tube closure) the activation of Eph receptors leads to enhancement of cell adhesion (Wang *et al.*, 1998; Adams *et al.*, 1999; Holmberg *et al.*, 2000).

Spatial Distribution of Guidance Cues

Studies in both invertebrates and vertebrates have lead to a synergistic model of axon guidance in which distinct guidance cues cooperate to steer axons through complex microenvironments to their final target. The balance between repulsion and attraction is governed by the relative guidance cue concentrations, not absolute concentrations. Secreted guidance cues such as the Netrins and the Slits have been shown to acts as a long-range cues, secreted from intermediate or final targets and are presumed to form a chemotactic gradient along the pathway of the exploring growth cone (Serafini *et al.*, 1996; Kidd *et al.*, 1999). In other instances these factors can behave as short range guidance cues, where they act over a distance of only a few cell diameters to affect changes in the direction of growth cone migration at specific choice points (Deiner *et al.*, 1997; Wu *et al.*, 1999). Secreted guidance cues are unlikely to be freely diffusable in the extracellular environment, but most likely interact with components of the extracellular matrix (ECM) or moieties bound to the cell/axon membrane. The ability of a secreted guidance cue to act over a distance or in a restricted zone surrounding the point of synthesis, is likely to be governed by the molecular composition of the local environment. The molecular mechanisms that can influence the spatial distribution of guidance cues are (i) interaction with heparan sulfate proteoglycans (HSPGs), (ii) interaction with protein components of the ECM or axon-bound proteins, and (iii) selective proteolytic cleavage of the guidance protein.

Heparan Sulfate Proteoglycans

HSPGs are comprised of a core protein (membrane-bound or within the ECM) that can display a large array of modified heparan sulfate glycosaminoglycan side chains. The diversity in core proteins and the large potential for structural heterogeneity in side chain composition allows these molecules to selectively interact with many different molecules within the extracellular environment. HSPGs have been implicated in the formation of morphogenic gradients in the *Drosophila* embryo where the extracellular accumulation of key patterning morphogens such as Sonic Hedgehog, is dependent on their interaction with specific HSPGs (Tabata, 2001). A similar process is likely to influence the distribution of the secreted guidance cues. The HSPG, Glypican-1, has been shown to specifically bind to Slit1 and Slit2 with high affinity *in vitro* and is expressed in overlapping domains in the developing rat brain (Ronca *et al.*, 2001). Thus, the spatiotemporal expression pattern of Glypican-1 may be responsible, at least in part, for the establishment of Slit chemotactic gradients. In addition, it has been recently demonstrated that the removal of heparan sulfate from the axon or cell membrane results in the loss of Slit2 repulsive activity for olfactory bulb axons and olfactory interneuron precursors (Hu, 2001) suggesting that HSPGs are essential for Slit driven chemorepulsion. These HSPGs may be responsible for establishing effective local Slit concentrations and/or for presenting Slit to the receptor in an appropriate format. Interactions with HSPGs may also be a key factor in the establishment of Netrin-1 gradients since Netrins also bind to heparan sulfate (Serafini *et al.*, 1994).

Proteins in the Local Environment can influence Guidance Cue Distribution

Protein components of the ECM such as the Laminins may act to concentrate secreted factors within tight zones surrounding the cells where they are synthesized. Slit2 has been shown to bind Laminin-1 (Brose *et al.*, 1999) suggesting that localization of Slit2 to precise choice points such as the ventral floorplate may be due to direct interactions with the Laminin isoforms within the surrounding ECM. Intriguingly, Slit2 has also been shown to directly interact with Netrin-1 (Brose *et al.*, 1999). Both Slit and Netrin-1 are co-expressed in many regions of the embryonic brain including the floorplate of the neural tube (Fig. 1). Netrin attracts commissural axons toward the floorplate while Slit acts to repel axons from the floorplate. Once at the floorplate, the chemoattractive response to Netrin-1 is silenced by the direct coupling of the Netrin receptor, DCC, with the chemorepulsive Slit receptor, Robo, (Fig. 5) allowing the growth cones to escape the attractive forces of Netrin-1 and move away from the floorplate (Stein and Tessier-Lavigne, 2001). The direct interaction between Slit and Netrin would be expected to bring their receptors into close apposition on the growth cone membrane thereby promoting DCC-Robo heterodimer formation and subsequent silencing of the Netrin-DCC attractive response. In addition, since Slit and Netrin are likely to work in concert to steer axon trajectories, the co-localization of these cues may act to align and stabilize their respective chemotactic gradients.

Guidance Receptors can Capture and Redistribute Guidance Cues

Recently, a novel and unanticipated mechanism controlling the distribution of Netrin within the developing nervous system of the fly has been uncovered. The formation of the longitudinal tract in the fly nerve cord requires the correct targeting of dMP2 axons. Targeting of these axons is dependent on Netrin and Frazzled (the DCC orthologue). Unexpectedly, Frazzled is not found on the dMP2 neuron but in the cells underlying the choice point at which the dMP2 axons encounter the Netrin protein (Hiramoto *et al.*, 2000). It appears that Frazzled guides dMP2 axons by capturing and presenting Netrin to a second, as yet unidentified, Netrin receptor residing on the dMP2 axons. Moreover, Hiramoto and colleagues have also demonstrated that Frazzled within the axon membrane actively rearranges Netrin protein in a spatial pattern completely distinct from that of the cells producing Netrin mRNA. These studies have uncovered a unique mechanism in which guidance receptors transport their ligands along axons to new locations distant from their point of synthesis thereby determining their spatial distribution (Fig. 2). In addition, at a specific choice point, Frazzled can present Netrin in the appropriate format for recognition by a second signalling receptor residing on the growth cone.

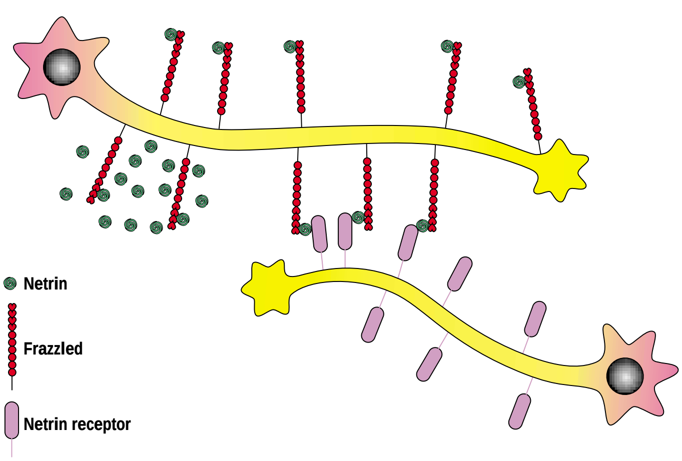

Fig. 2. Active transport of Netrin by its axon-bound receptor determines the spatial distribution of the guidance cue. *In* Drosophila, *Frazzled (the DCC orthologue) on the axonal membrane actively rearranges Netrin protein in a spatial pattern distinct from that of the cells producing* Netrin *mRNA. Frazzled can present Netrin to a second, as yet unidentified, Netrin receptor on an adjacent axon.*

Such a mechanism may explain the unusual distribution of Netrin-3 in the developing mouse PNS. Our immunohistochemical analysis of Netrin-3 protein localization has revealed that Netrin-3 is tightly associated with axons projecting from both sensory and sympathetic ganglia and is also present on the soma of these neurons (Fig. 3A and Seaman and Cooper, 2001). We have also demonstrated that in transfected cells, Netrin-3 is tightly associated with the cell surface and cannot be detected in the supernatant of these cultures as is the case for Netrin-1 (Seaman and Cooper, unpublished observations). Thus, it is unlikely that a diffusion-based mechanism could distribute Netrin-3 along the axon shaft. It is possible that the Netrin-3 protein is synthesized by these neurons and then transported along the length of the axonal processes. We have also observed significant levels of the Netrin receptor, Neogenin, on axons projecting from the sensory ganglia (Seaman and Cooper, unpublished observations). In addition, our biochemical studies indicate that the primary receptor for Netrin-3 is Neogenin (Cooper *et al.*, unpublished observations). Taken together these observations suggest that Netrin-3 may be actively transported along the length of the axon by its receptor Neogenin (Fig. 3C). Other membrane–bound moieties such as HSPGs could also fulfil this role.

Regulation of Guidance Receptor Levels on the Axonal Membrane

A second strategy to modulate the biological response of the projecting axon at a specific choice point is to control the density of guidance receptors at the tip of the exploring growth cone and regionally along the axon shaft.

Regulation of Robo Levels at the Midline

An unusual contact-dependent mechanism appears to tightly regulate Robo levels on commissural axons as they cross the midline in *Drosophila*. *Robo* loss-of-function mutations result in commissural axons aberrantly recrossing the midline of the nerve cord. Loss-of-function mutations at a second locus, *commissureless* (*comm*), lead to the opposite phenotype where the bi-symmetrical longitudinal tracts at the midline collapse into a single tract (Tear *et al.*, 1996). *Comm* gain-of-function mutants, however, display a *Robo*-like phenotype. These observations suggest that an inverse correlation exists between the expression of Robo and Comm. Immunohistochemical analysis revealed that Comm is a transmembrane protein expressed by the same population of midline glia that produce Slit (Tear *et al.*, 1996). When high levels of Comm are expressed at the midline, low levels of Robo are observed on

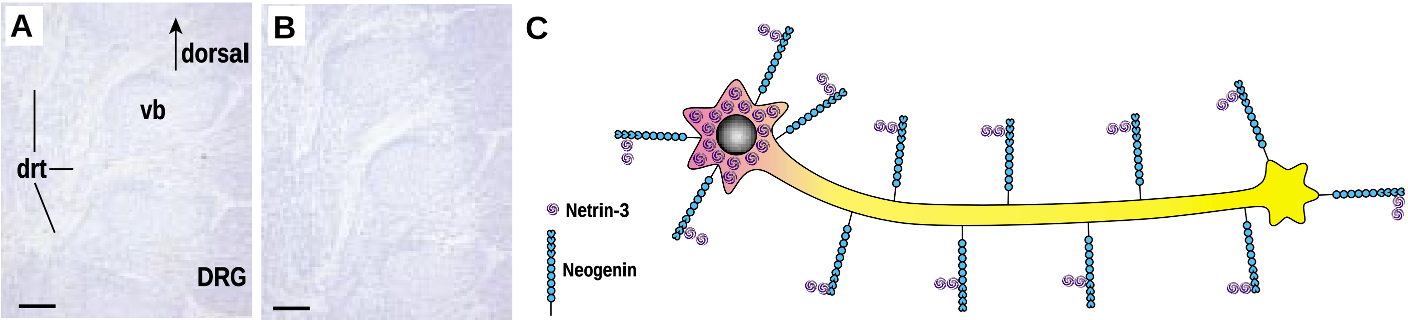

Fig. 3. The Netrin-3 protein is tightly associated with axons projecting from dorsal root ganglia. Netrin-3 may be transported down the axon by its receptor, Neogenin. (A) *Immunohistochemical analysis of Netrin-3 localization in the E14.5 mouse embryo using an anti-peptide antiserum raised against a unique N-terminal peptide of mouse Netrin-3. Netrin-3 protein is present on axons projecting within the dorsal roots of DRGs throughout the rostro-caudal axis of the neural tube.* **(B)** *Pre-incubation of the antiserum with the immunizing peptide results in the complete loss of immunoreactivity.* **(C)** *Our observations suggest that in the mouse PNS, Netrin-3 may be actively transported along the length of the axon by its receptor Neogenin. (A) and (B) are sagittal sections at the level of the caudal neural tube. DRG, dorsal root ganglion; drt, dorsal root; vb, vertebral body. Scale bars, 125 μm.*

the axonal membrane (Kidd *et al.*, 1998b). Taken together, these observations lead to the hypothesis that Comm locally down-regulates Robo protein levels on the growth gone as it contacts the midline, thereby silencing the chemorepulsive Robo-Slit interaction and allowing the axon to move into the midline and subsequently cross to the contralateral side (Fig. 1C). The absence of Comm on the contralateral side would then allow the Robo receptors to accumulate such that the growth cone again becomes responsive to the Slit.

Removal of Netrin Receptors by Ubiquitin-Dependent Degradation

Evidence that guidance receptors can be restricted to discrete regions along the length of the axon in mammals comes from our analysis of DCC protein distribution in the developing mouse CNS (Gad *et al.*, 2000). Our studies show that DCC protein expression is high along the entire length of retinal ganglion cell (RGC) axons as they navigate through the optic disc at embryonic day 15.5 (E15.5) and E16.5. However, we observed that by E18.5, no DCC protein was detectable on the distal regions of the axons within the optic nerve while DCC was still present on the proximal regions of the RGC axons that lay within the nerve fibre layer. These observations suggest that high levels of DCC protein are present on RGC axons only when they are actively navigating through the optic disc. A similar phenomenon was observed in a variety of projecting axons within the developing mouse forebrain (Shu *et al.*, 2000). High levels of DCC protein are present on cortical axons as they actively project through the internal capsule. Again, expression is greatly reduced on the distal segments of these axons after the targeting phase has been completed whereas significant DCC expression is still apparent on the proximal axonal membranes of these neurons. Localized down-regulation of DCC protein on projecting axons appears to coincide with the arrival of the axon at choice points expressing significant levels of Netrin-1. This suggests that the DCC-Netrin-1 interaction may trigger removal of DCC from the membrane permitting the growth cone to escape domains of high Netrin concentration.

Regulation of DCC protein expression at the post-translational level can occur via the ubiquitin-proteasome degradation pathway (Hu and Fearon, 1999) which has now emerged as a rapid and efficient mechanism for the regulation of cellular protein levels (Hershko and Ciechanover, 1998). Addition of ubiquitin moieties to the ε-amino group of lysine residues targets proteins to the large proteolytic proteasome complex where they are degraded. Ubiquitin ligases (E3s) covalently link ubiquitin to the target protein in a multi-step process. The cytoplasmic domain of DCC has been shown to bind an E3-like protein, Siah-2, resulting in the ubiquitination of DCC and its subsequent degradation *in vitro* (Hu and Fearon, 1999). These experiments indicate that DCC can be removed from the membrane in a ubiquitin-dependent manner and that this process is regulated by the ability of the Siah proteins to bind to the cytoplasmic domain of the receptor. We have recently discovered that a second Netrin receptor, Neogenin, also interacts with a member of the Siah family, Siah1b (Cooper and Tebbutt, unpublished observations). Thus, the cell surface density of both Netrin receptors may be regulated by a Siah-dependent degradation pathway.

The ability of Siahs to specifically target Netrin receptors for ubiquitin-dependent degradation offers an explanation for the observation that DCC is down-regulated on the distal domain of the projecting axon. One hypothesis would be that engagement of the Netrin receptor by its ligand stimulates Siah-dependent ubiquitination of the receptor's cytoplasmic domain. Thus, in low Netrin concentrations, the degree of receptor ubiquitination is minimal. However, as the growth cone moves towards the Netrin source the local concentration of Netrin increases amplifying the rate of ubiquitin addition and triggering transport of the receptor to the proteasome complex for degradation (Fig. 4). The threshold concentration of Netrin required for the activation of DCC-dependent chemoattraction would be lower than that required for Siah-dependent ubiquitination of the receptors such that the growth cone would continue to migrate towards the Netrin source. In high concentrations of Netrin, however, the density of DCC receptors on the axonal membrane would be minimal due to targeted degradation allowing the growth cone to become unresponsive to Netrin and escape from the region of high Netrin concentration.

Proteolytic Cleavage controls Guidance Receptor Signalling

A second mechanism for controlling the local density of guidance receptors on the exploring growth cone is proteolytic cleav-

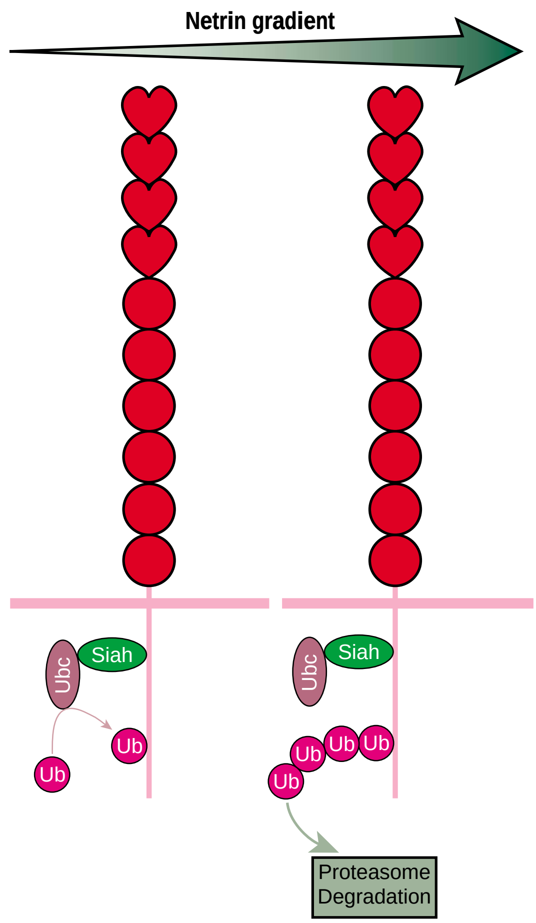

Fig. 4. Proposed model for the regulation of the density of Netrin receptors on the axonal membrane by the Siah-dependent protein degradation pathway. *Engagement of the Netrin receptor by its ligand stimulates Siah-dependent ubiquitination of the receptor's cytoplasmic domain. In low Netrin concentrations, the degree of receptor ubiqutination is minimal. As the growth cone moves towards the Netrin source, the local concentration of Netrin increases amplifying the rate of ubiquitin addition and triggering transport of the receptor to the proteasome complex for degradation.*

age of the receptor's extracellular domain at the membrane surface. Galko and Tessier-Lavigne (2000) have presented evidence that the activity of DCC can be modulated by proteolytic degradation of the receptor by an unidentified metalloprotease. In the presence of metalloprotease inhibitors the levels of DCC protein on axons projecting from dorsal spinal cord explants is significantly enhanced and their response to Netrin-1 potentiated. These observations indicate that metalloproteases may modulate the growth cone's response to Netrin by removing the DCC extracellular domain. This mechanism would again allow growth cones to escape regions of high Netrin concentration.

Guidance receptor activity can also be curtailed by proteolytic cleavage of the guidance cue. The interaction between the Eph receptors on the projecting growth cone and their membrane-bound ligands, the Ephrins, is a high affinity, multivalent interaction which leads to a chemorepulsive response. Since the chemorepulsive response requires that the Eph-Ephrin coupling be transitory, the question arises as to how the high affinity Eph-Ephrin interaction is efficiently terminated. One mechanism that triggers the localized dissociation of the Eph-Ephrin pair and therefore the termination of Eph signalling is proteolytic cleavage of the Ephrin by ADAM metalloproteases. Hattori *et al.* have demonstrated that Ephrin A2 forms a complex with the metalloprotease, Kuzbanian, which is then specifically activated upon ligation of EphA3 with EphrinA2 (Hattori *et al.*, 2000). Thus receptor-dependent cleavage of the membrane-bound guidance cue provides a mechanism by which proteolysis is restricted to the membrane interface where the ligand-receptor pair resides allowing the growth cone to detach from the Ephrin once the chemorepulsive signal has been initiated.

Guidance Receptor Cross-Talk determines the Biological Response to the Local Environment

In order for the growth cone to respond in a coherent manner when it encounters the many guidance cues in its local environment there must be a hierarchical system which allows the directional signals transduced by the appropriate guidance receptor to predominate. To date the hierarchical nature of growth cone responses to guidance cues is best demonstrated in the Netrin-DCC guidance system within the vertebrate.

A Hierarchy in Guidance Receptor Responses determines Growth Cone Directionality

In the developing *Xenopus* embryo, axons arising in the presumptive telencephalon project longitudinally along the ventral aspect of the forebrain before turning into the ventral commissure to cross to the contralateral side. Disruption of these axon projections were observed as they crossed the ventral commissure when cRNA encoding a truncated form of DCC (comprising the entire extracellular domain and the transmembrane domain) was expressed in living *Xenopus* embryos. In this case, the cRNA was injected into the blastomeres of early 2- or 4- cells stage embryos (Anderson *et al.*, 2000). When the truncated DCC receptor was present on the commissural axons not only did these axons fail to cross the midline but they abruptly turned away from the midline and actively grew back towards the longitudinal tract. These aberrant axon projections revealed that an underlying chemorepulsive activity was operating in the absence of a functional DCC receptor. This chemorepulsive activity may be provided by the Semaphorin receptor, Np-1, which also resides on the same population of commissural axons (Anderson *et al.*, 2000). These studies demonstrate the existence of a hierarchy in guidance receptor activity which ultimately determines the directionality of growth cone migration. In this instance, it is the chemoattractive activity of the DCC receptor that overrides chemorepulsive guidance cues encountered by this population of commissural axons. In the wildtype embryo this chemorepulsive mechanism is likely to come into play only after the growth cones have crossed the ventral midline where it is required to drive the growth cones away from the midline.

The hierarchy in responses to the array of guidance cues in the local environment of the growth cone, at least in some instances, can now be understood in molecular terms. Tessier-Lavigne and colleagues have elegantly demonstrated that the final outcome of ligand engagement by a given guidance receptor can be governed by direct physical interactions between different guidance receptors at the growth cone membrane. This receptor cross-talk determines the biological outcome of receptor-ligand interactions.

Robo silences the DCC Chemoattractive Response to Netrin-1

In the developing mammalian neural tube, DCC protein is present on the surface of commissural axons as they migrate toward the floorplate, the source of the Netrin gradient. Once these axons have crossed the floorplate they no longer respond to Netrin despite the fact they the still retain expression of DCC on the axonal membrane (Shirasaki *et al.*, 1998). Instead, they become responsive to the chemorepellents Slit2 and class 3 Semaphorins which are produced by the floorplate and the ventral neural tube, respectively (Zou *et al.*, 2000). This switch in responsiveness to chemorepulsive cues once having crossed the midline is believed to propel the axons away from the midline and explains why axons are never seen to recross the midline after reaching the contralateral side (Fig. 1 B,C).

The key to the silencing of the chemoattractive response of the Netrin-1-DCC interaction in this context lies in the absence or presence of the Slit receptor, Robo. Axons projecting towards the midline express DCC but not Robo on their surface. When on the ipsilateral side, Netrin engagement by DCC homodimers triggers a chemoattractive response. It is proposed that upon crossing the midline, Robo is up-regulated on the growth cone membrane leading to a direct interaction between the cytoplasmic domains of DCC and Robo (Stein and Tessier-Lavigne, 2001). This cytoplasmic interaction is mediated by the CC1 subdomain of Robo and the P3 domain of DCC (Fig. 5B) and may confer a conformational change on the tertiary structure of the DCC cytoplasmic domain. This model proposes that once on the contralateral side, DCC still binds Netrin-1 but can no longer interact appropriately with signalling molecules that potentiate the chemoattractive response (Stein and Tessier-Lavigne, 2001). In contrast, the Slit-Robo signalling cascade does not appear to be affected by formation of the DCC-Robo heterodimer. Thus, the direct coupling of the DCC and Robo receptors provides a precise temporal and spatial mechanism that accurately controls growth cone responses at a given choice point comprising conflicting directional information. In this system, Slit-Robo chemorepulsion overrides Netrin-DCC chemoattraction thus becoming the driving force for that growth cone.

UNC5 reverses the Polarity of the DCC Response to Netrin-1

It has also been demonstrated that DCC is subservient to another chemorepulsive guidance receptor, UNC5. Recent studies using *Xenopus* spinal cord neurons have demonstrated that the chemoattractive response of DCC-Netrin interactions is converted to chemorepulsion by the direct interaction between the cytoplasmic domains of DCC and UNC5 in the presence of Netrin-1 (Hong *et al.*, 1999). In this case it is the P1 cytoplasmic subdomain of DCC that interacts with the DB subdomain of the UNC5 cytoplasmic region (Fig. 5C). Taken together these studies suggested that in the presence of UNC5, DCC is forced to change its polarity and act as chemorepulsive Netrin receptor (or co-receptor in conjunction with UNC5).

Therefore, it seems that the dimerization state of DCC governs the polarity of the DCC response to Netrin-1 (Fig. 5). DCC homodimerization promotes chemoattraction upon Netrin-1 binding. However, homodimerization is disrupted in the presence Robo or UNC5 allowing Robo-DCC or UNC5-DCC heterodimers to predominate. This indicates that in the presence of Netrin-1 the affinity of DCC is higher for Robo and UNC5 than it is for another DCC receptor. Thus, the hierarchy in the DCC response to Netrin-1 may be governed by the relative affinities of receptor pairs. It will be of interest to determine which heterodimers form when all three receptors are present on a single growth cone. Since UNC5 binds the P1 subdomain of the DCC cytoplasmic region and Robo interacts with the P3 subdomain it is possible that a trimer of receptors may form. Which receptor will predominate in such a situation would need to be determined experimentally.

That the polarity of Robo-dependent guidance may also be modulated in a similar fashion to that of DCC comes from *Robo* loss-of-function mutants in *Drosophila* (Kramer *et al.*, 2001). Migrating mesodermal cells initially move away from Slit at the midline, however, a few hours later these same cells change their behavior to migrate toward Slit expressing muscle attachment sites. Thus, the chemorepulsive signalling pathway triggered by Slit-Robo interactions at the mesodermal cell membrane is superseded by a Robo-dependent chemoattractive response initiated by the same ligand-receptor pair at a later point in time. It seems logical to propose that such a switch in signal polarity may be

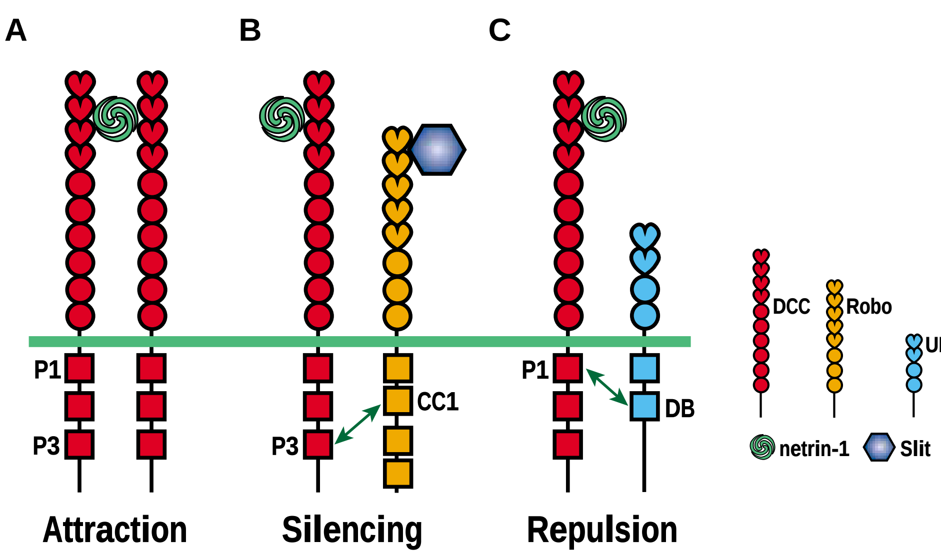

Fig. 5. Receptor cross-talk determines the biological outcome of DCC receptor-ligand interactions. (A) *DCC homodimerization promotes chemoattraction upon Netrin-1 binding.* **(B)** *Silencing of the chemoattractive response of the growth cone to Netrin-1-DCC interactions results from a direct coupling of the cytoplasmic domains of DCC and Robo. This cytoplasmic interaction is mediated by the CC1 subdomain of Robo and the P3 domain of DCC.* **(C)** *The chemoattractive response to DCC-Netrin interactions is converted to chemorepulsion by the direct coupling of the cytoplasmic domains of DCC and UNC5 in the presence of Netrin-1. In this case it is the P1 cytoplasmic subdomain of DCC that interacts with the DB subdomain of the UNC5 cytoplasmic region.*

induced by the interaction of Robo with another as yet unidentified co-receptor.

L1-Neuropilin-1 Cross-Talk converts Semaphorin Repulsion to Attraction

The above demonstration that molecular cross-talk between guidance receptors from different receptor families modulate the individual receptor's response to its specific ligand has implications beyond the DCC/UNC5/Robo system. It is now clear that other guidance receptors also form multi-receptor complexes at the axonal membrane. Recent studies now suggest that activation of the Semaphorin receptor complex can lead to chemoattractive or chemorepulsive responses depending on the molecular compostion of the receptor complex. In mice lacking the L1 cell adhesion molecule (CAM) the axons of the corticospinal tract (CST) fail to decussate in the caudal hindbrain at the point were the CST normally projects dorsally to form the pyramidal decussation in the wild type. L1 is a member of the immunoglobulin superfamily of CAMs and is known to promote neurite outgrowth by acting as either a homophilic or a heterophilic CAM. Since L1 is present on CST axons, an L1 ligand was likely to be present in the pyramidal region at the level of the decussation. Unexpectedly, Castellini and colleagues identified this ligand as Sema3A and further demonstrated that L1 physically interacts with Np-1 (but not Np-2) via its extracellular domain (Castellani *et al.*, 2000). Moreover, the addition of a soluble form of the L1 molecule was found to convert the response of wildtype axons to Sema3A from one of chemorepulsion to one of attraction, probably due to homophilic interaction between soluble L1 and L1 bound to the axonal membrane. These findings suggest that the polarity of the growth cone response to Sema3A may be governed by the presence or absence of L1 binding partners in the local environment. Both the Sema3A driven chemorepulsive and chemoattractive activity are dependent on the presence of L1, since CST axons from the *L1* knock-out mouse cannot respond to Sema3A (Castellani *et al.*, 2000). Thus the growth cone response to Semaphorins can be modulated by the direct physical interaction between Neuropilins and members of other receptor families, in this case L1, again highlighting the importance of receptor cross-talk in determining growth cone responses to guidance cues. The role of the Plexins has not yet been determined for this system, however, since they are likely to be one of the signalling components of the Semaphorin receptor it will be of great interest to determine the molecular relationship between L1 and the Plexins.

Components of Eph Receptor Complexes determine Biological Outcome of Eph-Ephrin Interactions

How is the physiological outcome from the activation of the Eph receptors determined? Several independent studies have now demonstrated that Eph receptors have the potential to directly associate with other receptors at the membrane surface. Thus, the repertoire of molecular interactions in these multi-molecular complexes is likely to determine the physiological response of Eph receptor activation upon Ephrin binding. A possible candidate for Eph receptor cross-talk in the context of Eph-driven chemorepulsion is the Ryk receptor, a kinase-dead member of the receptor tyrosine kinase family. Ryk has been shown to directly associate with, and, is phosphorylated by EphB2 and EphB3 (Halford *et al.*, 2000). Loss of the Ryk orthologue (Derailed) in *Drosophila* embryos results in aberrant axon pathfinding (Callahan *et al.*, 1995). In addition, Bonkowsky *et al.* (1999) have demonstrated that Derailed behaves as a chemorepellent guidance receptor. Taken together these observations suggest that Ryk is a component of a multi-molecular complex that promotes the chemorepulsive activity of EphB receptors when in the context of the axon growth cone.

Holmberg and colleagues have demonstrated that an alternatively spliced form of EphA7, lacking the kinase domain, is responsible for the silencing of the chemorepulsive activity normally observed with the full length receptor (Holmberg *et al.*, 2000). When co-expressed, the truncated form of the receptor suppresses tyrosine phosphorylation of the full length receptor thereby switching the cellular response from one of repulsion to one of adhesion. These observations argue that the phosphorylation state of the Eph receptors can determine the biological outcome of Ephrin engagement. Thus Eph receptors are likely to be part of a multi-molecular complex which comprises protein moieties that regulate the phosphorylation state of these receptors.

The ability of Eph receptors to phosphorylate other components within a multi-molecular complex may also have significant repercussions for the type of response evoked. Recent experiments have demonstrated that activation of EphB receptors is required for dendritic spine morphogenesis and also for excitatory synapse formation in cultured neurons (Dalva *et al.*, 2000). The transmembrane HSPG, Syndecan-2, has been shown to cluster in dendritic spines and induces spine formation upon phosphorylation in cultured hippocampal neurons (Ethell *et al.*, 2001). EphB receptors have now been identified as the tyrosine kinases responsible for activating Syndecan-2-dependent dendritic spine formation leading to the hypothesis that activation of Eph receptors (probably by clustered Ephrins located in the presynaptic terminal) is the trigger for dendritic spine formation (Ethell *et al.*, 2001). At mature excitatory synapses, clustered EphrinB binding to EphB receptors promotes a direct physical interaction between the extracellular domains of EphB receptors and the NR1 subunit of the NMDA receptor (Dalva *et al.*, 2000). It is proposed that this interaction is responsible for recruiting and clustering of NMDA receptors at postsynaptic densities. The interaction between the extracellular domains of the EphB and NR1 proteins was shown to be independent of Eph receptor tyrosine kinase activity. However, it was further demonstrated that Eph kinase activity enhanced the number of both pre- and post-synaptic specializations that formed in cultured neurons. In summary, the Eph receptors appear to play a strategic role in the formation and stabilization of postsynaptic specializations. Moreover, the phosphorylation of key synaptic transmembrane components by Eph receptor tyrosine kinases appears to be a pivotal molecular event promoting these processes. Here Eph receptor activation results in the stabilization of the molecular complexes underpinning synaptic structure rather than destabilization of the cytoskeleton components leading to growth cone collapse when Eph receptors act as chemorepulsive axon guidance receptors. Thus, it seems likely that it is the cross-talk within multi-molecular complexes, and the nature of the receptor components, that determine the physiological outcome of Eph receptor activation.

Where to From Here?

The formation of multi-molecular complexes at the axonal membrane comprising guidance receptors, other key transmembrane components, and intracellular signalling molecules (constitutively associated or recruited upon receptor activation) promotes cross-talk between these components which subsequently determines the biological consequences of receptor-ligand interactions. Since many different receptor activation events impinge on the growth cone at any given point in time and space, the integration of ensuing signal transduction cascades is necessary to achieve a synchronous response to the extracellular environment. It must also be kept in mind that these guidance receptor complexes do not operate in isolation but in the same spatiotemporal environment as other receptor-ligand interactions. Thus the incoming signals from activated guidance receptors must be interpreted in the context of other relevant environmental signals. For example, Laminin directly influences the nature of the biological response to Netrin-1 by lowering the intracellular levels of cAMP resulting in a switch in the polarity of the Netrin response from one of chemoattraction to one of chemorepulsion (Höpker *et al.*, 1999). Here the parallel signalling cascade activated by the Laminin receptors must at some point intersect with the Netrin-triggered signal transduction pathway to switch the polarity of the signal.

At present, our understanding of the intricacies of the signal transduction cascades responsible for relaying and integrating the incoming information about receptor state is limited (for a comprehensive review of this topic see Song and Poo, 2001). However, several key integration points for the numerous signal transduction cascades have now been identified. The levels of cAMP, cGMP and Ca^{2+} as well as Protein Kinase A or G (PKA, PKG) activity appear to be key determinants in the growth cone response to guidance cues. Low intracellular cAMP levels convert the chemoattractive response to Netrin-1 into one of chemorepulsion, while Sema3A driven chemorepulsion is converted to attraction in low intracellular cGMP concentrations. An elevation in intracellular Ca^{2+} levels is required for the induction of the growth cone response to Netrin-1 (see Song and Poo, 2001). Inhibition of PKA activity results in the conversion of Netrin-dependent attraction to repulsion whereas activation of PKA converts the chemorepulsive response to MAG (an immunoglobulin superfamily CAM) to one of attraction (see Song and Poo, 2001). In the context of axon navigation, the ultimate outcome of these signal transduction cascades must be to trigger dynamic rearrangement of the actin cytoskeleton within the growth cone, promoting cycles of extension and retraction of filopodia at the growth cone tip. One point of convergence for the myriad of signal transduction pathways is the Rho family of GTPases which governs the directionality of cell motility by directly linking many actin-binding proteins to upstream signalling molecules (Schmidt and Hall, 1998). It is anticipated that these small GTPases will act as major integration points for the network of signal transduction cascades triggered by receptor occupation.

Understanding how the individual molecular guidance systems work at the level of a single axon and how the different signalling cascades work in concert to initiate and steer axonal migration will be the future goal of laboratories focussing on the molecular mechanisms underlying axon guidance. These studies will also provide insights into mechanisms driving cell migration, tissue patterning and boundary formation during embryogenesis since many of the axon guidance receptor families and their cognate ligands are also essential for these developmental processes.

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