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Axon Guidance at the Midline: From Mutants to Mechanisms

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How axons in the developing nervous system successfully navigate to their correct targets is a fundamental problem in neurobiology. Understanding the mechanisms that mediate axon guidance will give important insight into how the nervous system is correctly wired during development and may have implications for therapeutic approaches to developmental brain disorders and nerve regeneration. Achieving this understanding will require unraveling the molecular logic that ensures the proper expression and localization of axon guidance cues and receptors, and elucidating the signaling events that regulate the growth cone cytoskeleton in response to guidance receptor activation. Studies of axon guidance at the midline of many experimental systems, from the ventral midline of *Drosophila* to the vertebrate spinal cord, have led to important mechanistic insights into the complex problem of wiring the nervous system. Here we review recent advances in understanding the regulation of midline axon guidance, with a particular emphasis on the contributions made from molecular genetic studies of invertebrate model systems.

Keywords growth cone, attraction, repulsion, slit, netrin, robo, DCC

INTRODUCTION

During development neuronal growth cones, the specialized structures at the tips of extending axons, follow specific pathways and navigate a series of intermediate choice points to find their correct targets. At each decision point, growth cones encounter a number of guidance cues in their extracellular environment (Dickson, 2002; Yu *et al*., 2002). Several phylogenetically conserved families of guidance cues and receptors have been discovered including: 1) Semaphorins (Semas) and their Plexin (Plex) and Neuropilin receptors (Pasterkamp & Kolodkin, 2003); 2) Netrins and their Deleted in colorectal carcinoma (DCC) and UNC5 receptors (Kennedy, 2000); 3) Slits and their Roundabout (Robo) receptors. (Brose & Tessier-Lavigne, 2000); and 4) Ephrins and their Eph receptors (Kullander & Klein, 2002) (Figure 1). More recently additional protein families previously recognized for other developmental functions have been implicated in growth cone guidance including Sonic Hedgehog (Shh) (Charron *et al*., 2003), Bone Morphogenetic Proteins (BMPs) (Augsburger *et al*., 1999; Butler & Dodd, 2003) and Wingless-type (Wnt) proteins (Lyuksyutova *et al*., 2003; Yoshikawa *et al*., 2003). Guidance cues can act at short or long range to elicit either attractive or repulsive responses (Tessier-Lavigne & Goodman, 1996). An emergent theme from many studies is that it is the type of receptor or receptor complex expressed on the surface of the growth cone, rather than a given guidance cue, that determines the direction of axon growth (Yu & Bargmann, 2001).

In order to ensure correct and efficient wiring of the nervous system, an intricately choreographed sequence of events must take place. First neurons and their surrounding target tissues must be specified to express the correct complement of receptors and guidance cues, respectively. Second, receptors must be assembled into the appropriate complexes and localized to the axonal or dendritic growth cones, while guidance cues must be correctly trafficked and localized in the extra-cellular environment. Third, signaling mechanisms must be in place to integrate and transmit signals from the surface receptors into changes in the growth cone actin cytoskeleton which result in stereotyped steering decisions. Each of these steps provides many potential levels for the regulation of axon guidance decisions, and although recent work has enriched our understanding of the complexities of guidance regulation, many questions remain.

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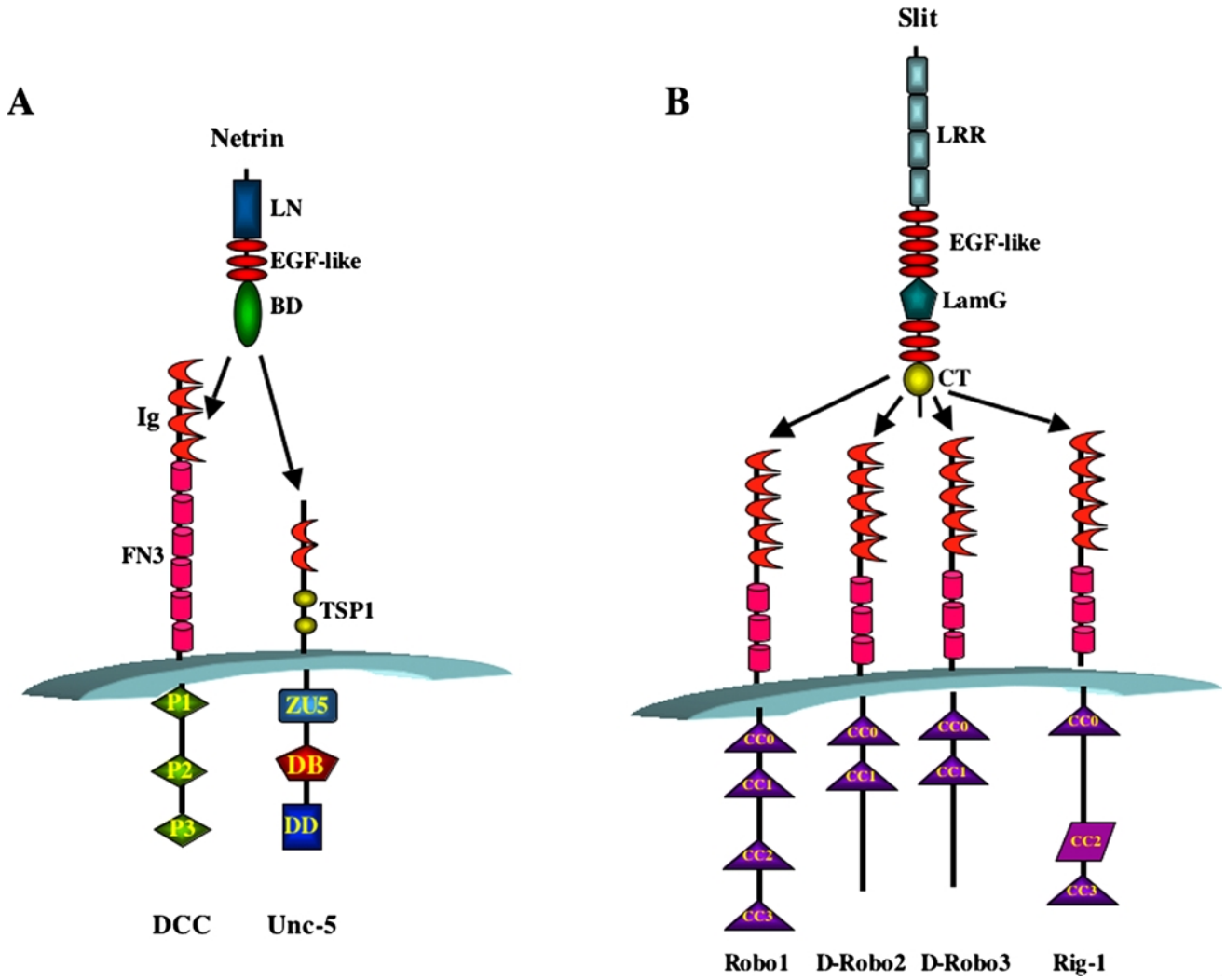

FIG. 1. Slits, Netrins and their receptors. A) The Netrin ligand and its receptors DCC/UNC-40/Frazzled (indicated as DCC in the schematic for simplicity) and UNC-5. Netrin binds DCC and UNC-5 and elicits an attractive or repulsive response, respectively. Netrin is a secreted molecule containing laminin EGF-like repeats, a Laminin VI Homology Domain (LN) and a Basic Domain (BD; also called Domain C) at its C-terminus. DCC/UNC-40 is a transmembrane receptor containing four Immunoglobulin (Ig) domains and six Fibronectin Type III domains (FN3) in its ectodomain and three conserved sequence motifs, P1, P2, and P3 in its cytoplasmic domain. UNC-5 consists of Immunoglobulin and Thrombospondin Type I (TSP1) domains in its extracellular domain, while intracellularly, it contains a ZU5 domain (present in the Zonula-Occludens 1 protein), a DCC binding domain (DB) and a Death Domain (DD). B) Slit and its complement of Robo receptors. Drosophila Robos 1, 2 and 3 are diagrammed. Slit, a secreted guidance cue containing EGF-like and Leucine Rich repeats (LRR), a Laminin G homology domain (LamG) and a cysteine knot (CT) binds to Robo family receptors and mediates repulsion. The Robo1 receptor consists of an extracellular domain with five Ig domains and three FNIII repeats, a single transmembrane domain and a long cytoplasmic tail containing four conserved sequence motifs, CC0-CC3. D-Robo 2 and D-Robo-3 are structurally similar to Robo1, but are missing the CC2 and CC3 motifs. Vertebrate Robos most closely resemble Robo 1. There are no known vertebrate homologues of D-Robo2 and D-Robo3. Vertebrate Rig-1 is also similar to Robo 1 but it lacks CC1 and has additional cytoplasmic differences.

THE MIDLINE CHOICE POINT

In diverse organisms, the midline is an important intermediate target for many classes of navigating axons, which must decide whether or not to cross (Figure 2) (Kaprielian *et al.*, 2001). Most axons in both invertebrate and vertebrate nervous systems cross the midline once and then project on the contralateral side of the CNS, never to cross again, while a smaller percentage of axons remain on their

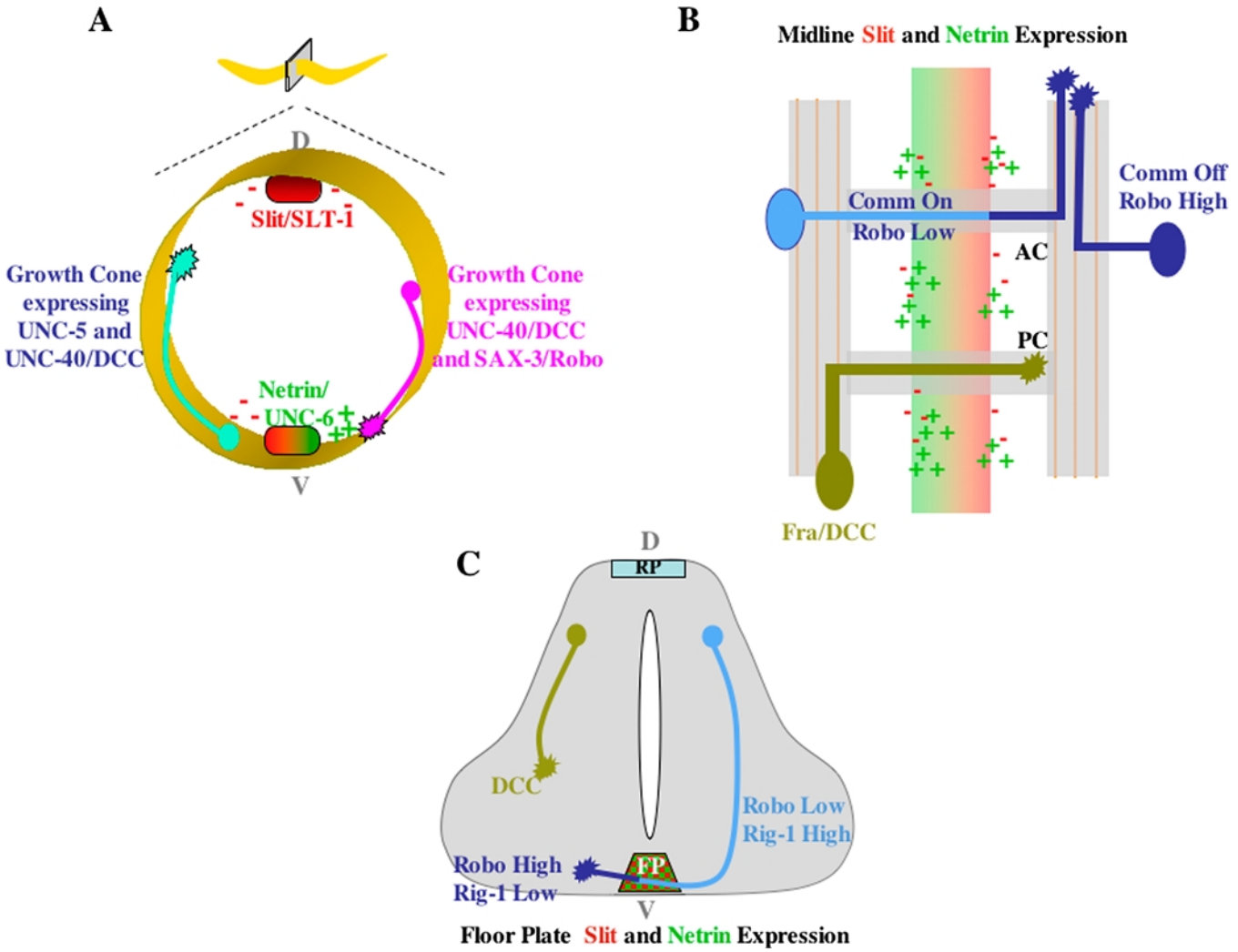

FIG. 2. Selected paradigms of midline guidance. A) Circumferential axon guidance in the nematode *C. elegans*. A cross section is shown. Growth cones that express UNC-40/DCC and SAX-3/Robo (purple) are repelled away from dorsal cells expressing SLT-1/Slit and are attracted toward the ventral source of Netrin. On the other hand, growth cones that express UNC-5 and UNC-40/DCC (teal) are repelled dorsally, away from Netrin. D = Dorsal, V = Ventral. B) Axon guidance at the midline of *Drosophila*. Slit (red minus symbols), which is produced by cells at the ventral midline, repels neurons that express high levels of Robo (dark blue) and keeps them ipsilateral. Commissural axons, which express DCC/Frazzled (tan), are attracted toward the midline by Netrin (green plus symbols) and are allowed to cross via the precise down-regulation of Robo by Comm (light blue). For clarity, two commissural axons are shown. AC = anterior commissure. PC = posterior commissure. C) Guidance of commissural axons in the developing vertebrate spinal cord. For clarity two commissural axons are shown. As in *Drosophila*, commissural axons expressing DCC (tan) are attracted ventrally toward Netrin. As these axons migrate, they express low levels of Robo1 and Robo2 but remain insensitive to Slit due to Rig-1 expression (light blue). Upon crossing the ventral floor plate (FP), Robo expression is increased and Rig-1 is turned down (dark blue). As Robo levels and thus repulsion from the midline increase, attraction back toward the midline is "silenced" by a Slit-gated interaction between Robo and DCC (see Figure 3C). RP = Roof Plate.

own side of the midline. Once across, axons of CNS interneurons turn at a stereotyped lateral position relative to the midline and continue towards their synaptic targets. The midline of many model organisms, including the ventral midline of the invertebrates *C. elegans* and *Drosophila*, and the spinal cord and optic chiasm in various vertebrate species, has proven to be a very powerful system for the study of guidance mechanisms because of the relative ease of interpreting midline guidance defects and the clearly defined source of guidance cues emanating from

well-characterized classes of midline cells (Tear *et al*., 1993). In addition, there is a striking structural and functional conservation among many of the molecules that control midline axon guidance (Figure 2). For example, Netrin is secreted by cells of the ventral midline in fly and worm and by cells of the floor plate of the spinal cord in vertebrates, where it acts through its DCC receptor to attract commissural axons towards and across the midline (Chan *et al*., 1996; Harris *et al*., 1996; Hedgecock *et al*., 1990; Keino-Masu *et al*., 1996; Kolodziej *et al*., 1996; Mitchell *et al*., 1996; Serafini *et al*., 1996). In contrast, Slit is secreted by the floor plate of the spinal cord, the midline glia in *Drosophila* or dorsal cells in *C. elegans*, and in all cases acts to repel axons that express Robo receptors-pushing them ventrally in the worm, while preventing inappropriate midline crossing in the vertebrate spinal cord and embryonic CNS of the fly (Battye *et al*., 1999; Brose *et al*., 1999; Hao *et al*., 2001; Kidd *et al*., 1999; Kidd *et al*., 1998a; Long *et al*., 2004; Zallen *et al*., 1998).

A number of large-scale genetic screens for mutations affecting midline guidance in *C. elegans* and *Drosophila* have been instrumental in the identification and functional characterization of two of the major conserved guidance systems that control axon guidance at the midline—the Netrins and their UNC-5 and UNC-40/DCC receptors and the Slits and their Robo receptors (Hedgecock *et al*., 1985; Hummel *et al*., 1999a; Hummel *et al*., 1999b; Seeger *et al*., 1993; Zallen *et al*., 1999). For example, screens for mutants affecting dorsal and ventral circumferential axon guidance in *C. elegans* and subsequent molecular genetic characterization identified mutations in the Netrin/UNC-6 ligand, and in the UNC-5 and UNC-40 receptors (Hedgecock *et al*., 1990). *unc-6* mutants have both dorsal and ventral guidance defects, while *unc-5* and *unc-40* mutants disrupt dorsal and ventral projections respectively, leading to the suggestion (later borne out by extensive genetic and biochemical analysis) that *unc-5* is a repulsive Netrin receptor and UNC-40 is an attractive Netrin receptor (Hamelin *et al*., 1993; Hedgecock *et al*., 1990; Hong *et al*., 1999). On the other hand, screens in *Drosophila* played a pivotal role in the discovery of the Slit/Robo repulsive guidance system, and a potent negative regulator of Robo repulsion, the *commissureless (comm)* gene (Seeger *et al*., 1993). Mutations in *slit* or *robo* both result in inappropriate midline crossing; in *slit* mutants all CNS axons collapse onto the midline, while in *robo* mutants axons cross back and forth across the midline (Kidd *et al*., 1999; Kidd *et al*., 1998a). The paradoxical difference in the mutant phenotypes between ligand and receptor was clarified when it was found that there are three Robo receptors in *Drosophila*: Robo, Robo 2 and Robo 3. Robo and Robo 2 function together to prevent inappropriate midline crossing, while Robo2 and Robo3 have a distinct function in specifying the lateral position of longitudinal axons with respect to the CNS midline (Rajagopalan *et al*., 2000a, b; Simpson *et al*., 2000a, b). Over the past several years, it has become abundantly clear that these conserved families of molecules also control many other axon guidance events in the developing nervous system, and in addition play important roles in both neuronal and non-neuronal cell migration.

In this review, we will discuss recent advances from studies of midline guidance that illuminate: 1) how axon guidance cues and receptors are deployed during development, 2) how the assembly of heteromeric receptor complexes and different combinations of guidance receptors influence guidance decisions; and 3) how guidance receptors signal to the downstream actin regulatory machinery to steer the growth cone. To limit the scope of the review we will focus primarily on the midline guidance functions of the Slits and Netrins and their respective receptors and signaling pathways, although studies of other molecules and systems that provide special insight into particular aspects of axon guidance will also be highlighted. Finally, special emphasis will be given to the important contributions that genetic approaches in fly and worm have made, and will continue to make, as the study of axon guidance moves forward.

DEPLOYMENT OF AXON GUIDANCE CUES AND RECEPTORS

The expression of guidance cues and receptors is exquisitely tailored to allow appropriate path-finding decisions at specific times and places throughout development. There are a wide variety of mechanisms in place to ensure the correct presentation and receipt of guidance signals, ranging from spatially and temporally restricted transcriptional regulation of cues and receptors to their specific post-translational trafficking. There are doubtless additional regulatory mechanisms awaiting discovery.

Transcriptional Regulation

Considerable evidence indicates that combinatorial codes of transcription factor expression function to specify motor neuron subtype identity and targeting in the vertebrate spinal cord, and in the *Drosophila* and *C. elegans* ventral nerve cords (Hobert *et al*., 1998; Jessell, 2000; Shirasaki & Pfaff, 2002; Thor & Thomas, 2002). Lim homeobox genes have been shown to play an instructive role in the targeting of lateral motor column neurons (LMC) to the correct dorsal and ventral domains in the limb (Kania *et al*., 2000). Genetic studies of motor neuron targeting in the *Drosophila* embryo and additional studies in *C. elegans* support an evolutionary conserved role for the Lim domain genes in target specification (Hobert *et al*., 1998; Thor *et al*., 1999). Much less is known about how these transcriptional codes

are read out at the level of specific axon guidance receptors to control pathfinding and target selection; however, recent studies are forging direct links between neuronal identity and the transcriptional regulation of specific guidance receptors. For example, in the vertebrate spinal cord LIM homeodomain proteins have been shown to regulate the expression of EphA receptors to control targeting of subsets of LMC neurons to appropriate domains in the limb (Kania & Jessell, 2003).

In contrast to target selection in the motor system, transcriptional control of guidance at intermediate targets, including the CNS midline is less well defined. Nevertheless, progress has been made, and several studies illustrate that transcriptional control can be used to dynamically modulate axon responses ensuring that appropriate guidance cues and receptors are available at precisely the time when they are needed to instruct guidance decisions. For example, temporal up-regulation of Ephrin-b expression at the optic chiasm in the developing *Xenopus* tadpole is correlated with formation of the ipsilateral (non-crossing) axon projections (Nakagawa *et al*., 2000). Responsiveness to Ephrin-b in this instance is conferred by temporally regulated axonal expression of the ephB repulsive Ephrin-b receptor in the later-born ipsilateral retinal neurons (see the following). Temporal changes in UNC-6/Netrin expression in *C. elegans* also contribute to distinct guidance events at discrete developmental times (Wadsworth, 2002).

Dynamic control of guidance responses can also be regulated at the level of guidance cue expression. For example, in *C. elegans*, at a certain stage in gonadal development the distal tip cells migrate away from ventral sources of Netrin and the timing of this guidance event is controlled by the regulation of UNC-5 transcription; premature ectopic UNC-5 expression leads to premature repulsive migration (Su *et al*., 2000). At the optic chiasm in the vertebrate visual system, a large percentage of the axons project contralaterally to the opposite side of the brain, while a smaller percentage (the exact percentage depending on the degree of binocularity of a given species) remain on their own side of the midline (Williams *et al*., 2004). Recent findings indicate that Zic2, a zinc finger transcription factor, plays a pivotal role in specifying the uncrossed projection. Zic2 expression is spatially and temporally restricted to the cell bodies of ipsilateral projecting retinal ganglion cell neurons as they project their axons towards the optic chiasm (Herrera *et al*., 2003). Though the direct transcriptional targets of Zic2 have yet to be identified, EphB2 receptors, known to respond to and be repelled by Ephrin B cues expressed at the chiasm, are excellent candidates (Williams *et al*., 2003b).

Of course, gene transcription is also employed to make sure the right cells make the right cues and receptors, but in contrast to the examples described above, transcriptional control is often not the key regulated step for guidance decisions. At the ventral midline of the *Drosophila* CNS, a special set of glial cells express both Slit and the two fly Netrins, Net A and Net B. A wealth of elegant cellular and molecular genetic studies have described the specification, differentiation and migration of these midline glia, which play critical roles in establishing the normal axon scaffold with its distinct anterior and posterior commissures in each embryonic segment (Jacobs, 2000; Klambt *et al*., 1997). Single-minded (Sim), a PAS domain containing transcription factor, controls many aspects of midline development, including the direct transcriptional regulation of Slit (Hummel *et al*., 1997; Ma *et al*., 2000). In *sim* mutants the midline glia fail to develop, and fail to express Slit and many other genes required for the normal program of glial development, resulting in the collapse of the all CNS axons onto the midline. Transcription of the Robo receptors for Slit is in part controlled by the Longitudinals-lacking (Lola) family of BTB domain containing zinc finger transcription factors, although it is not yet known if Robos are direct Lola targets (Crowner *et al*., 2002). Mutations in *lola* were originally identified in the same screen that identified *robo* and result in misrouting of axons across the midline (Seeger *et al*., 1993). Dose-dependent genetic interactions between *slit, robo* and *lola* suggest function in a common pathway, and in addition there is a marked reduction in expression of both Robo and Slit in *lola* mutants, suggesting that the precise levels of both ligand and receptor may be tuned by the same transcription factor (Seeger *et al*., 1993). However, the fact that many neurons that do not respond to midline Slit express Robo receptors, indicates that transcriptional regulation is not the only strategy for the control of Slit repulsion.

Post-Transcriptional and Post-Translational Regulation

In addition to transcriptional regulation, many post-transcriptional mechanisms also regulate the availability and spatiotemporal distribution of guidance cues and receptors with profound consequences for midline guidance and neuronal connectivity. These mechanisms include local translation, control of ligand and receptor trafficking, regulated proteolysis and control of ligand distribution. Here we highlight recent advances in understanding some of these regulatory mechanisms.

Local translation provides a neuron with the means to rapidly change the protein composition in discrete locations such as synaptic sites and axonal growth cones; indeed poly- ribosomes and other translational machinery have been observed in both axons and dendrites (Martin, 2004). Such a mechanism could allow a navigating axon to quickly alter its responsiveness to cues in the extracellular environment as it negotiates an intermediate target. Perhaps the clearest example of a role for local translation

during axon guidance comes from studies of EphA2 receptor expression during commissural axon guidance in the chick spinal cord (Brittis *et al*., 2002). EphA2 translation is restricted to post-crossing axonal segments via a specific element in its 3′ UTR (Brittis *et al*., 2002). The restriction of Eph-A2 expression to post-crossing portions of axons is strikingly similar to the distribution of the Robo receptors in the *Drosophila* CNS, although as we shall see it is likely to be achieved by a different mechanism. Several studies of *Xenopus* retinal and spinal axons also support a role for local translation during axon guidance (Campbell & Holt, 2001; Ming *et al*., 2002). Although to date there are only a few examples, it seems likely that regulation of local protein synthesis during axon guidance will prove to be a wide-spread mechanism for controlling axon responses.

Several post-translational strategies for controlling the extracellular distribution and levels of a number of guidance cues have recently come to light. In *Drosophila*, as in *C. elegans* and the vertebrate spinal cord, Netrins attract commissural axons towards the midline. However, in contrast to the mouse knockouts of Netrin and DCC, where very few commissural axons are observed to cross the floor plate, mutations in their *Drosophila* homologues have relatively mild phenotypes, with many axons still projecting normally across the midline. Axons of the posterior commissure are preferentially disrupted in both the *frazzled (fra)*(i.e. *Drosophila* DCC) and *netrin AB* double mutants (Harris *et al*., 1996; Kolodziej *et al*., 1996; Mitchell *et al*., 1996). This suggests that additional mechanisms are in place to allow midline crossing in the absence of Netrin/DCC attraction. Indeed, other mutants have been identified that result in too few axons crossing the midline, including a mutant called *schizo*, which in contrast to *fra/netrin* preferentially disrupts formation of the anterior commissure (Hummel *et al*., 1999a). The recent cloning and characterization of *schizo* revealed that it encodes an ARF-GEF, a group of proteins known to regulate endocytosis. Cell-specific rescue experiments, mis-expression studies, and genetic manipulations to block endocytosis in midline glia led to the unexpected finding that rather than encoding a component of a second midline attraction system, as its mutant phenotype suggested, Schizo is a negative regulator of Slit endocytosis (Onel *et al*., 2004). Thus, the identity of any existing additional attractive functions at the *Drosophila* midline remains obscure.

In contrast to Schizo's function to negatively regulate the amount of Slit secreted by midline glia, two additional studies have implicated the axonally-expressed heparin sulfate proteoglycan Syndecan (Sdc) in the positive control of the extracellular distribution and signaling efficacy of Slit (Johnson *et al*., 2004; Steigemann *et al*., 2004). Consistent with this idea, Sdc interacts genetically and biochemically with Slit and Robo and the extra-cellular distribution of Slit is altered in *sdc* mutants. Another mechanism to control ligand distribution, akin to the proposed role for Sdc in controlling Slit distribution, but with an interesting twist was revealed by studies of the role of Netrin and Fra during the projection of longitudinal axons within the embryonic CNS. Here, surprisingly, Fra was shown to function cell non-autonomously to "capture" Netrin and present it to other receptors on neighboring cells. How widespread this "ligand presentation" mechanism will prove to be in other paradigms of axon guidance remains to be determined (Hiramoto *et al*., 2000). Interestingly, in addition to ligand receptor interactions, biochemical interactions between Netrin and Slit ligands, as well as between Slit and the extracellular matrix protein Laminin have also been observed, suggesting the possibility that ligand-ligand interactions could also function to refine the distribution and availability of guidance cues (Brose *et al*., 1999).

In addition, recent studies of Slit-Robo repulsion in *Drosophila* have established selective protein trafficking as a novel and potent mechanism for the post-translational control of the surface expression of guidance receptors. Again, molecular genetic approaches in *Drosophila* opened the door to discovery. Of all the mutants recovered in the forward genetic screens at the *Drosophila* midline, the one with arguably the most striking phenotype is *comm*, where mutation results in the complete absence of axon commissures (Seeger *et al*., 1993). That *comm, robo* double mutants resemble *robo* indicated that *comm* acts upstream of *robo* and suggested that *comm* might function to negatively regulate *robo*. Initial molecular characterization of Comm provided surprisingly little clue to its function; indeed, comm encodes a protein with a single transmembrane domain, no conserved domains and no significant homology to either *C. elegans* or vertebrate proteins (Tear *et al*., 1996). This is in stark contrast to other guidance cues and receptors, which have proven, in general, to be quite highly conserved. In addition, initial expression analysis led to the puzzling observation that although Comm protein was detected on commissural axons, its mRNA was not detected in neurons, but rather it appeared to be enriched in midline glia, leading to the proposal that Comm is synthesized in midline glia and then transferred to commissural axons as they cross the midline (Tear *et al*., 1996).

Despite these early enigmatic findings, further Comm over-expression experiments and dose-dependent genetic interactions established that Comm does in fact act to down-regulate Robo expression (Kidd *et al*., 1998b). Comm gain-of-function studies were also critical to the discovery of ligand-receptor relationship between Slit and Robo and correctly suggested that additional Robo receptors must be encoded in the fly genome (Kidd *et al*., 1999). More recently, several studies have resolved the ambiguities of the earlier work and have found that Comm is not transferred from glia to neuron, but rather it is

expressed and functions in commissural neurons to down-regulate surface expression of Robo (Georgiou & Tear, 2002; Keleman *et al*., 2002; Myat *et al*., 2002). While cell transplantation experiments have been interpreted to suggest that Comm functions predominantly cell autonomously in neurons, other genetic data including mosaic rescue experiments also support an additional role for Comm in midline glia (Georgiou & Tear, 2002; Keleman *et al*., 2002). High-resolution mRNA expression analysis revealed that *comm* message is detected in commissural neurons precisely at the time when the decision to cross is being made, and is then rapidly extinguished in post-crossing axons; non-crossing ipsilateral neurons do not detectably express *comm* mRNA (Figure 2) (Keleman *et al*., 2002).

How does Comm function to regulate Robo? Several lines of evidence including sub-cellular localization experiments and transgenic expression of mutant forms of *comm* indicate that Comm can recruit Robo receptors directly to endosomes for degradation before they ever reach the cell surface (Keleman *et al*., 2002). In addition, an independent study has shown that Comm's ability to regulate surface levels of Robo depends on its interaction with the Nedd 4 ubiquitin ligase; mutation in either the dNedd4 binding site or the ubiquitin acceptor sites in Comm disrupts its ability to regulate Robo (Myat *et al*., 2002). At first glance this regulatory strategy seems wasteful to the neuron- why bother going to the trouble of synthesizing a large protein like Robo if you are just going to turn around and degrade it before it is even used? While, there is no definitive answer to this question, one possibility is that this mechanism allows for the rapid deployment of Robo in post-crossing commissural neurons, ensuring that they do not re-cross the midline.

Many questions remain about Comm and the regulation of Robos, not the least of which is whether or not there are vertebrate Comm homologues that serve similar functions during commissural axon guidance in the spinal cord, or instead are there other molecules that play this role? So far, no clear vertebrate Comm proteins have been found, however Comm is very poorly conserved at the level of primary sequence—even other insect Comms are difficult to recognize; therefore, it remains possible that homologues may still be found. On the other hand, there is new and compelling genetic evidence that other non-Comm molecules may have an analogous function in the spinal cord. Rig-1/Robo3, a divergent Robo family member, is required in pre-crossing commissural neurons to down-regulate the sensitivity to midline Slit proteins (Figure 2), though it is likely to achieve this regulation by a distinct mechanism (see the following) (Sabatier *et al*., 2004). Another set of intriguing questions is how *comm* mRNA expression is so dynamically regulated during midline crossing to ensure a pulse of expression just as axons cross. What is the signal that activates Comm expression as the growth cone approaches the midline? How is Comm repression of Robo relieved in post-crossing neurons? Dissecting the *comm* promoter and upstream regulatory sequences should begin to shed light on these questions.

Although it remains to be determined how widespread direct sorting of guidance receptors to endosomes for subsequent degradation will prove to be, additional examples of controlling guidance by controlling the surface expression of receptors are accumulating; these include two recent studies of the regulation of surface levels of the vertebrate Netrin receptors UNC5H1 and DCC respectively. In the first example, activation of Protein Kinase C (PKC) triggers the formation of a complex between the cytoplasmic domain of UNC5H1, Protein Interacting with C-Kinase 1 (Pick1) and PKC and leads to the specific removal of UNC5H1 (but not DCC) from the growth cone surface; reducing surface levels of UNC5H1 correlates with the inhibition of the Netrin dependent collapse of cultured hippocampal growth cones (Williams *et al*., 2003a). In the second example, Protein Kinase A (PKA) activation has been shown to selectively increase surface levels of DCC and concomitantly increase axon outgrowth in response to Netrin (Bouchard *et al*., 2004). This is an intriguing observation because it may help to explain how increasing cyclic AMP (cAMP) and PKA activity promotes Netrin-mediated chemoattraction.

RECEPTOR COMPLEXES AND COMBINATIONS

As detailed above, both transcriptional and post-transcriptional control of the expression and localization of guidance cues and receptors play critical roles in the wiring of the nervous system. Yet once guidance receptors are trafficked and localized to their proper intracellular destinations, often receptor self-association and the formation of receptor complexes are essential for modifying, switching and/or inhibiting a particular response. Additional receptor-receptor interactions and combinations provide yet another level of control that contributes to proper growth cone guidance during development.

Receptor Self Association

Members of the DCC family of Netrin receptors, including UNC-40 in *C. elegans*, Frazzled in *Drosophila*, and DCC in vertebrates contain extracellular domains consisting of six immunoglobulin (Ig) repeats and four fibronectin type III (FNIII) repeats and cytoplasmic domains consisting of three conserved sequence motifs, P1, P2, P3 (Figure 1) (Kolodziej, 1997). Previous studies have shown that DCC family members bind to Netrin and mediate growth cone attraction (Chan *et al*., 1996; de la Torre *et al*., 1997; Keino-Masu *et al*., 1996; Kolodziej *et al*., 1996; Stein *et al*., 2001). For example, in *C. elegans*, during circumferential axon guidance, the UNC-40/DCC receptor is required in

ventrally migrating cells that respond to Netrin; *unc-40* mutants display ventral guidance defects that can be rescued by transgene expression of UNC-40 in these cells (Chan *et al*., 1996). In addition, using the single cell *Xenopus* spinal axon turning assay developed by Poo and colleagues, it was shown that axons grown in a collagen matrix are attracted to an exogenous source of Netrin, and this response can be suppressed by adding function blocking DCC antibodies (de la Torre *et al*., 1997). Taken together, these data establish that the DCC/Frazzled/UNC-40 family of receptors responds to Netrin to mediate axon outgrowth and attraction.

Further analysis of the DCC receptor demonstrated that ligand-gated self-association is required for proper function (Stein *et al*., 2001) (Figure 3A). Similar self-association has also been shown for the Eph tyrosine kinase receptors; in this case, the SAM (sterile alpha) motif appears to be involved in the assembly of receptor multimers (Bruckner & Klein, 1998; Thanos *et al*., 1999). In the case of DCC, it has been established that the conserved cytoplasmic P3 sequence motif is necessary for receptor multimerization and Netrin induced attractive turning of stage 22 *Xenopus* neurons (Stein *et al*., 2001). Versions of DCC lacking the P3 motif cannot self-associate and neurons expressing this form of DCC are no longer able to transduce Netrin turning and outgrowth responses. Replacing the P3 motif with the SAM multimerization domain from Eph receptors can restore an appropriate DCC response, suggesting that one of the major roles of the P3 domain is to mediate self-association (Stein *et al*., 2001). In the future, similar structure/function studies of the UNC-40 and Frazzled receptors in both *C. elegans* and *Drosophila* respectively, will determine if the P3 domain (or another domain) is responsible throughout evolution for functional self-association.

Many lines of evidence, including the results described above demonstrate that specific motifs within guidance receptor cytoplasmic domains confer specific functions. In the case of DCC, the P3 domain is required for self-association, which in turn is required for guidance in cultured spinal neurons. Likewise, in vertebrates, the P1 and DB domains of DCC and UNC-5 respectively, are required for receptor/receptor interactions (see the following) (Hong *et al*., 1999). It has also been shown that conserved sequence motifs of the Robo1 receptor are required for interaction with other known guidance receptors and with specific downstream effectors. For example, the CC1 domain of vertebrate Robo is required for association with DCC (Stein & Tessier-Lavigne, 2001), while in *Drosophila* the CC2 and CC3 domains of Robo are required for interaction with downstream signaling components such as Dock, Ena, and Abl (Bashaw *et al*., 2000; Fan *et al*., 2003). Additionally, Lim and Wadsworth have identified domains of Netrin/UNC-6 that mediate attractive or repulsive guidance responses of ventrally and dorsally migrating neurons in *C. elegans* via the UNC-40 and UNC-5 receptors, respectively (Lim & Wadsworth, 2002). Further identification of functional domains will help determine how guidance cues and their precise interactions (for example: ligand/receptor and/or receptor/receptor) are integrated to elicit proper downstream responses.

Bifunctional Guidance Cues

Through numerous elegant genetic, molecular and biochemical experiments, it is now clear that many guidance cues are bifunctional, producing an attractive response for some growth cones while producing a repulsive response for others. Several possible mechanisms could exist to underlie the bifunctionality of these cues. For example, bifunctionality could result from the formation of heteromultimeric receptor complexes containing distinct downstream signaling cascades from each individual receptor. In support of this type of mechanism, Netrin, when binding to only DCC/Frazzled/UNC-40 mediates attraction (Chan *et al*., 1996; Keino-Masu *et al*., 1996; Kolodziej *et al*., 1996). However, Netrin can also stimulate repulsion when it binds to either UNC-5 alone or the DCC and UNC-5 receptor complex (Figure 3B) (Hamelin *et al*., 1993; Hong *et al*., 1999).

Additional mechanisms also exist to elicit a bifunctional response. For example, the activation of *one* particular guidance receptor could be linked to different downstream effectors within the growth cone, (the presence of these effectors might depend on various environmental and intracellular cues) thereby modifying the response to a particular cue. For instance, in *Xenopus* spinal axons grown in culture, a guidance response can be switched from attraction to repulsion (or vise versa) by changing the concentration of intracellular cyclic nucleotides (Nishiyama *et al*., 2003; Song *et al*., 1998; Song *et al*., 1997). Cyclic nucleotide signaling, which will be discussed in more detail later in this review, appears be an effective way to modulate and even reverse guidance responses. Receptor complex formation and downstream signaling modulation may represent efficient mechanisms by which a growth cone, given the limited number of guidance molecules that have been uncovered, is able to display precise responses within a complicated environment.

Heteromeric Assembly of Receptors

Like DCC, UNC-5 receptor family members, including UNC-5 in *C. elegans*, dUNC-5 in *Drosophila*, and three vertebrate homologues, are also Ig super family members and also bind to Netrin (Ackerman *et al*., 1997; Keleman and Dickson, 2001; Leonardo *et al*., 1997; Leung-Hagesteijn *et al*., 1992). However, UNC-5 family

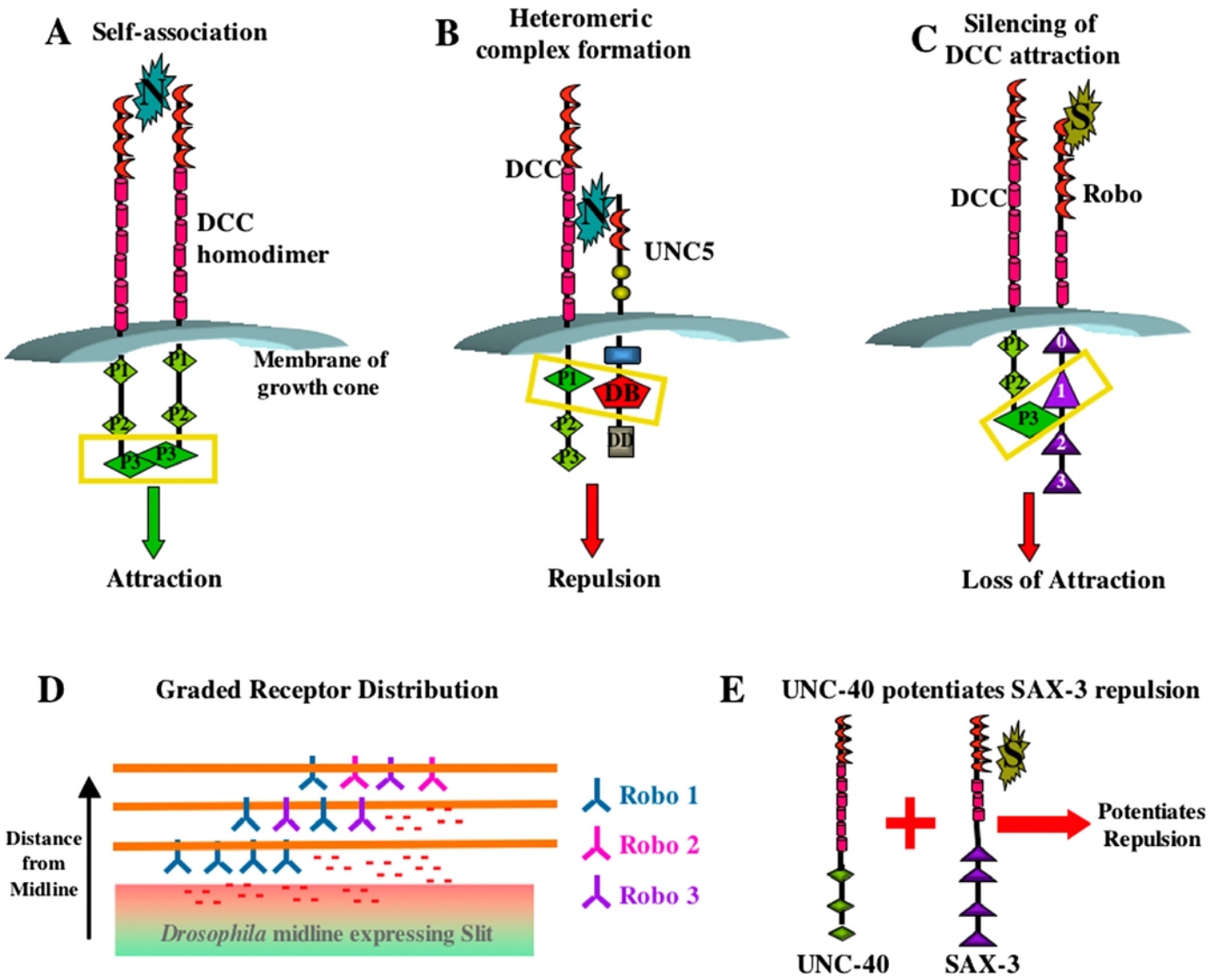

FIG. 3. Receptor complexes and combinatorial control of guidance responses. A) Ligand-gated self-association of DCC, mediated by the P3 sequence motif, is needed for a proper attractive signaling response. N = Netrin. B) Ligand-gated heteromeric association between DCC/UNC-40 and UNC-5 via the P1 and DB domains respectively, produces a repulsive signaling response. N = Netrin. C) Slit-stimulated interaction between the P3 domain of DCC and the CC1 domain of Robo silences midline attraction in post-crossing neurons. S = Slit. D) Graded receptor distribution of the Robo receptor family members. Robo1 (blue) is expressed on most axons, including three prominent longitudinal FasII positive axon bundles that can be observed in a single focal plane, whereas Robo3 (dark purple) is expressed more intermediate-to-lateral and Robo2 (light purple) is confined most laterally. Robo receptor distribution, and their individual responses to Slit (red), creates a code that specifies lateral position relative to the midline. E) In *C. elegans*, *unc-40* mutations can suppress an ectopic Slit gain-of-function phenotype suggesting that in this case, UNC-40/DCC potentiates SAX-3/Robo repulsion. S = Slit.

members differ from DCC receptors in both their structure and their response to Netrin. While DCC/UNC-40 mediates an attractive response in most neurons, UNC-5 mediates repulsion (Hamelin *et al*., 1993; Hedgecock *et al*., 1990; Leung-Hagesteijn *et al*., 1992). Early work in *C. elegans* demonstrated that overexpression of UNC-5 in touch receptor neurons steers their axons dorsally, away from ventral cells expressing Netrin (Hamelin *et al*., 1993). UNC-5 overexpression can repel not only "neutral" axons (axons that do not show polarity toward Netrin) away from the ligand source but can also repel cells that were initially attracted by Netrin. Interestingly, this ectopic repulsion was found to partially require *unc-40* (Colavita and Culotti, 1998), and together with the observations that many neurons co-express UNC-5 and UNC-40, and *unc-40* mutants exhibit partial defects in dorsal axon projections (Chan *et al*., 1996), these findings led to the suggestion that both UNC-40 and UNC-5 are required in the same cell for robust Netrin-mediated repulsion (Figure 3B).

Indeed, in cultured *Xenopus* spinal axons, overexpression of *C. elegans* UNC-5 and vertebrate UNC-5H1 and UNC-5H2 can convert Netrin-mediated attraction to repulsion (Hong *et al*., 1999). Function blocking antibodies against the extracellular domain of DCC inhibited this repulsion suggesting that the response conversion requires DCC. DCC and UNC-5 can form a receptor complex upon ligand stimulation and the P1 and DB domains of DCC and UNC-5 respectively mediate this interaction (Hong *et al*., 1999). Receptor complex formation is an essential requirement for UNC-5-mediated repulsion, since Netrin can no longer repel *Xenopus* axons expressing either UNC-5 missing the DB domain, or DCC missing the P1 domain(Hong *et al*., 1999). In vertebrates, it is interesting that the extracellular domain of UNC-5 is dispensable for DCC/UNC-5 mediated repulsion; a membrane bound form of UNC-5 can still dictate a repulsive output provided the interaction with full length DCC is intact (Hong *et al*., 1999). Data from *Drosophila* differs somewhat from the results seen in vertebrates (Keleman & Dickson, 2001) in that a constitutive membrane bound form of dUNC-5, when expressed in all neurons, does not result in ectopic Netrin repulsion, as the vertebrate data would predict (Hong *et al*., 1999; Keleman and Dickson, 2001). Likewise, the Death Domain (DD) of *Drosophila* UNC-5 appears to be required for repulsion; however, it is not necessary in vertebrates (Figure 1), suggesting significant mechanistic differences between the repulsion of *Drosophila* commissural axons and *Xenopus* spinal axons (Hong *et al*., 1999; Keleman & Dickson, 2001). Short-range Netrin repulsion of commissural axons at the *Drosophila* midline, mediated by ectopic UNC-5, does not require Frazzled/DCC; however, long-range ectopic UNC-5 repulsion of the apterous subset of interneurons partially does (Keleman & Dickson, 2001). These results support the idea that when levels of Netrin are limited, Frazzled/DCC acts in concert with dUNC-5 to mediate repulsion, but that when Netrin is in excess, UNC-5 alone is sufficient.

Additional receptors for Netrin also exist although their role in receptor complex formation and axon guidance is still unclear. Initially, in vertebrates, the adenosine A2b receptor was shown to bind both Netrin and DCC, and pharmacological studies suggested that inhibition of A2b leads to suppression of a cAMP production and Netrin/DCC-induced outgrowth of dorsal spinal cord neurons (Corset *et al*., 2000). However, these results were directly challenged by other pharmacological experiments demonstrating that A2b activation is not required for Netrin-induced attraction (Stein *et al*., 2001). Additional experiments from the same report also show that the A2b receptor is not expressed on commissural axons, suggesting that A2b is not required for Netrin signaling, at least not in commissural neurons. More recently, *in vivo* support for a role of the A2b receptor in contributing to Netrin responses has come from studies of the guidance of retinal axons in *Xenopus* (Shewan *et al*., 2002). Retinal axons are initially attracted to Netrin as they exit the eye, but later in their trajectory the response to Netrin changes from attraction to repulsion. The change in response to Netrin is correlated with decreased expression of the A2b receptor and can be blocked by specific A2b agonists, supporting a role for A2b in mediating Netrin responses (Shewan *et al*., 2002). It will be of great interest to determine whether in the context of retinal axon guidance, A2b functions independently or as a DCC co-receptor in modifying Netrin responses.

Receptor Interactions and the Modification of Guidance Responses

The DCC/UNC-5 results demonstrate the importance of bifunctional receptor complex formation and response conversion during axon guidance. This theme is revealed yet again by examining the interactions between the DCC and Robo receptors. Early experiments using rat spinal cord explants demonstrated that commissural neurons lose responsiveness to Netrin upon crossing the midline/floor plate (Figure 3C) (Shirasaki *et al*., 1998). This observation helped to answer, but did not solve, the perplexing question of how a neuron that was once attracted to a particular destination (i.e. the floor plate) could now leave this location even though it still expresses the original attractive guidance receptor. Recently, however, using cultured *Xenopus* spinal axons, it was shown that the attractive output of the DCC receptor can be blocked by a Slit2 stimulated interaction between Robo and DCC (Figure 3C) (Stein and Tessier-Lavigne, 2001). The P3 domain of DCC and the CC1 domain of Robo are important for this

receptor-receptor association, as Robo no longer silences DCC receptors lacking the P3 domain. Appropriate receptor interactions and output (i.e. silencing) could be achieved by replacing the P3 and CC1 domains of DCC and Robo, respectively, with the SAM multimerization domain of the Eph receptor (Stein & Tessier-Lavigne, 2001). The silencing mechanism is a very attractive model that helps to explain how precise guidance responses can be modulated during development; however, the significance of these observations for midline axon guidance in the organism await further investigation. Future experiments in other model systems, such as *C. elegans* and *Drosophila*, should help to establish if this regulatory mechanism is evolutionarily conserved or specific for vertebrate axon guidance.

In *C. elegans*, an UNC-40/DCC-Sax-3/Robo interaction has also been described (Yu *et al*., 2002). In this case, UNC-40 has been proposed to potentiate SAX-3/SLT-1 signaling independent of its ligand Netrin (Figure 3E). Ubiquitous overexpression of SLT-1 in muscle cells causes both dorsal-ventral and anterior-posterior defects in the guidance of a subset of neurons called AVM, and these defects are indicative of hyperactive SAX-3/SLT-1 signaling (Yu *et al*., 2002). Unexpectedly, a mutation in *unc-40*/DCC can suppress the gain of function phenotype caused by excess SAX-3/SLT-1 signaling suggesting that *unc-40* positively contributes to SAX-3 mediated guidance. However, *unc-6*/Netrin mutations do not suppress the SLT-1 gain of function phenotype leading to the conclusion that UNC-40's role, as a component of the SAX-3 signaling pathway, is Netrin independent (Yu *et al*., 2002).

Given the fact that UNC-40 and SAX-3 can interact biochemically *in vitro* (Yu *et al*., 2002), the most reasonable explanation is that an UNC-40/SAX-3 receptor complex potentiates SLT-1 signaling. Perhaps SAX-3 is able to mediate guidance without UNC-40, but in the absence of UNC-40, SAX-3 signaling may be less efficient or more sensitive to perturbation. This type of regulation might be similar to the short-range/long-range requirements of Frazzled/DCC for UNC-5 mediated repulsion explained above. Additionally, UNC-40/DCC appears to potentiate SAX-3/Robo signaling, whereas in the case of *Xenopus* spinal axons, Robo diminishes DCC signaling. These differences could be explained by differences in temporal requirements and/or the presence of other guidance molecules. Other examples of Netrin-independent functions of UNC-40/DCC have also been documented (Hedgecock *et al*., 1990; Kim *et al*., 1999), implying that DCC may have other unknown roles during axon guidance.

Additional Receptors and Potential Complexes Mediating Axon Guidance

It is now appreciated (as noted above) that in *Drosophila*, the Commissureless protein (Comm) down-regulates the Robo receptor ultimately allowing axons to cross the midline. Perplexingly, a Comm homologue has not yet been identified in vertebrates. However, recent work has identified Rig-1/Robo3, a guidance molecule proposed to be functionally analogous to Comm (Marillat *et al*., 2004; Sabatier *et al*., 2004). Rig-1, a divergent member of the Robo family of receptors, is mutated in patients with horizontal gaze palsy with progressive scoliosis (HGPPS) (Jen *et al*., 2004), binds Slit and is expressed on pre-crossing neurons as they migrate toward the floor plate (Sabatier *et al*., 2004). Rig-1/Robo-3, in contrast to the known function of other Robo receptors, is proposed to function by preventing pre-crossing axons from being prematurely responsive to the Slit ligand, thus allowing them to cross the midline (Sabatier *et al*., 2004). Similar to *comm* mutants in *Drosophila,* in the absence of Rig-1, all commissural axons fail to cross the floor plate, and this effect is partially suppressed by the simultaneous removal of Robo 1. Although exact mechanistic details remain to be determined, it is clear that Rig-1 does not regulate the Robo 1 receptor via the same mechanism as Comm; Rig-1 does not interact with Robo 1 and it does not appear to regulate the surface level of Robo 1. Sequence analysis shows that unlike other Robo receptors, Rig-1 does not contain the conserved CC1 sequence motif (Sabatier *et al*., 2004) (Figure 1). Perhaps this sequence divergence may help explain the unexpected functional divergence. The absence of the CC1 motif is particularly satisfying, since it implies that Rig-1 should not be able to silence DCC attraction in pre-crossing axons. Given the fact that Rig-1 does not appear to interact with Robo 1 *in vitro*, one can propose many models by which Rig-1 regulates Slit sensitivity. For example, since Rig-1 can directly bind to Slit, perhaps it can function as an endogenous dominant negative protein by sequestering Slit and preventing its interaction with Robo 1. Alternatively, by analogy to the Slit-dependent silencing of DCC attraction, perhaps Slit binding to Rig-1 gates an interaction between Rig-1 and Robo 1, thereby preventing Robo repulsion.

The interpretation of a guidance signal as a repellent or an attractant also appears to depend on another class of receptors, the Receptor Protein Tyrosine Phosphatases (RPTPs) (Chang *et al*., 2004; Desai *et al*., 1996). Although the ligands that activate RPTPs are not known, RPTPs are presumed to affect axon guidance by antagonizing tyrosine kinases. Indeed, previous work demonstrated that RPTPs are required for motor axon guidance in the *Drosophila* embryo (Desai *et al*., 1996; Krueger *et al*., 1996). Recently, *clr-1*, a *C. elegans* gene encoding a RPTP, was identified in a screen for suppressors of the AVM guidance defect of *slt-1* mutants (Chang *et al*., 2004). Mutations in *clr-1* can suppress guidance defects caused by null mutations in SLT-1/SAX-3 signaling pathway but do not suppress similar defects caused by mutations in the

UNC-6/UNC-40 pathway, suggesting that *clr-1* inhibits Netrin-mediated axon attraction. On the other hand, *clr-1* mutants can potentiate UNC-5-dependent repulsion from UNC-6/Netrin, demonstrating that CLR-1 also has a positive role in Netrin-mediated repulsion (Chang *et al*., 2004). These results further implicate RPTPs (and perhaps their cognate kinases) in axon guidance and this is the first example that integrates these receptor phosphatases with other guidance receptors. It will be interesting to know whether this modulation reflects direct guidance receptor/RPTP interactions.

Graded Receptor Distribution and the Assembly of a Code

Several recent studies of the diverse roles *Drosophila* Robo receptors during midline guidance suggest that in addition to the potent effects of receptor complex formation on guidance responses described above, graded and combinatorial expression of receptors provides yet another mechanism to influence neuronal connectivity.

In *Drosophila* there are three Robo receptors—Robo, Robo 2, and Robo 3 (note that Robo-3 in *Drosophila* is different than Robo3/Rig-1 in vertebrates). The Robo receptors consist of an extra-cellular domain with five immunoglobulin (Ig) domains and three fibronectin (FN) type III repeats, a single transmembrane domain, and a long cytoplasmic tail (Brose & Tessier-Lavigne, 2000) (Figure 1). All three Robos have identical extra-cellular domain organizations and bind to their only known ligand, Slit, with similar affinity (Robo 2 and Robo 3 bind Slit with about two fold greater affinity than Robo, but it is not known whether this relatively small difference is of biological significance). In contrast to the similarity of their extracellular domains, the cytoplasmic domains of the three Robos are quite divergent; Robo 2 and Robo 3 lack the two conserved proline rich motifs, CC2 and CC3, suggesting that they may signal differently than Robo 1 (Figure 1).

All three receptors appear to mediate repulsive guidance responses, and these responses are thought to be dependent on activation of the receptors by Slit. Robo and Robo 2 function together to prevent inappropriate midline crossing, while Robo2 and Robo3 have a distinct function in specifying the lateral position of longitudinal axons with respect to the CNS midline (Rajagopalan *et al*., 2000a, 2000b; Simpson *et al*., 2000a, 2000b). For example, the *robo1*, *robo2* double mutant phenocopies that of the ligand Slit, suggesting that Robo2 plays a role in repulsive guidance by preventing axons from lingering at the midline (Rajagopalan *et al*., 2000b; Simpson *et al*., 2000b). Furthermore, several lines of evidence suggest that the combination of Robo receptors a given neuron expresses are important to determine its lateral position with respect to the midline (Figure 3D). Robo1 is distributed over the entire width of the longitudinal axon bundles. On the other hand, Robo3 is expressed more laterally, in a domain spanning the outer two thirds of the longitudinal axons, while expression of Robo2 is confined most laterally (Rajagopalan *et al*., 2000b; Simpson *et al*., 2000a) (Figure 3D). Disruption of Robo2 and Robo3 results in the inward shifting of the longitudinal axon bundles towards the midline, whereas mis-expression of Robo2 and Robo3 in medial axon bundles shifts these axons laterally (Rajagopalan *et al*., 2000b; Simpson *et al*., 2000a). Robo does not appear to share this property. It is generally assumed that the ability of Robo 2 and Robo 3 receptors to influence lateral position is dependent on the midline expression of Slit; however, direct genetic evidence for Slit in mediating lateral position is lacking. Thus it remains a formal, though unlikely, possibility that lateral position specification is Slit independent.

These data demonstrate that the distribution of the various Robo receptors creates a graded response within the CNS, perhaps creating a "code" which determines the approximate positioning of particular axons. Yet how does the cell interpret this code? How does the differential expression of Robo proteins dictate distinct zones or locations within the CNS? One idea is that the slightly increased affinity of Robo 2 and Robo 3 for the Slit ligand makes neurons that express them more sensitive to Slit. Therefore, at the midline, where Slit is assumed to form a gradient emanating from glial cells, the code is determined not only by the expression pattern of individual Robo receptors, but also by how well a particular receptor is able to bind and respond to Slit. Another hypothesis is that the overlapping expression pattern of the Robo family members determines the formation of heteromeric receptor complexes, each with distinct downstream signaling capabilities. Alternatively, since Robo 2 and Robo 3 have quite divergent cytoplasmic domains from Robo 1 (i.e., absence of CC2 and CC3 and presence of other motifs), different signaling interactions may underlie the distinct roles of Robo2 and 3 in specifying lateral position. It will be interesting to determine the effects that receptor domain swapping, between Robo1, Robo2 and Robo3, has on cellular positioning and repulsive guidance. It has also been suggested, based on the discrete step-wise axon shifting that is observed in both Robo 2, 3 loss of function and mis-expression experiments, that the Robo proteins may determine the "general" location where specific neurons project, while other local guidance cues, such as cell adhesion molecules, determine the precise pathway selection. Further study of the Robo family receptors will determine how distribution of receptors, affinity for ligand and/or distinct signaling outputs leads to proper guidance.

SIGNALING MECHANISMS

Once guidance cues and receptors are correctly deployed and assembled into the appropriate combinations and complexes they must activate signaling pathways to steer the growth cone. While guidance receptor signaling mechanisms are incompletely understood, they are likely to act locally to modulate actin cytoskeletal dynamics in the axon and growth cone, rather than through signaling to the cell body. Activation of specific signaling pathways can promote attraction, repulsion, result in growth cone collapse or affect the rate of axon extension. How a given guidance signal is interpreted also depends on the activities of a number of second messenger pathways within the cell, and as we shall see, these pathways are potent modulators of axon responses. In addition, several proteins implicated in the regulation of actin cytoskeletal dynamics, such as the SH3-SH2 adaptor protein Dreadlocks (Dock)/Nck, the Abelson (Abl) tyrosine kinase and its substrate Enabled (Ena) also contribute to guidance receptor signaling pathways. Finally, considerable evidence supports important roles for the Rho family of small GTPases, their upstream regulators and their effectors in transducing a number of specific guidance receptor signals, including those of Slit and Netrin receptors. Here we provide a progress report on the current understanding of guidance receptor signaling and modulation by second messengers, with a particular emphasis on Slit and Netrin receptor signaling (Figure 4) (Guan & Rao, 2003).

Second Messengers and the Modulation of Guidance Responses

Nearly ten years ago, Mu Ming Poo and colleagues made the startling discovery that reducing the levels of the cyclic nucleotide cAMP or inhibiting protein kinase A (PKA) in the growth cones of cultured *Xenopus* spinal neurons could convert attraction towards sources of Brain Derived Neurotrophic Factor and Acetylcholine into repulsion (Song *et al*., 1997). Additional studies in the *Xenopus* culture system demonstrated that cyclic nucleotide (cAMP or cGMP)-dependent response conversion could also be observed for other attractive guidance cues such as Netrin (Ming *et al*., 1997), as well as a number of repulsive cues, including Semaphorins (Song *et al*., 1998). The general picture that emerged from these studies is that high cyclic nucleotide levels favor attraction, while low levels favor repulsion. Moreover, response conversion for some guidance cues is dependent on cAMP levels and extracellular calcium, while response conversion for a non-over lapping set of guidance cues is dependent on cGMP levels and is calcium independent. There were a number of early hints that the story was not as simple as this; for example, cGMP dependent switching of Semaphorin repulsion into attraction was blocked by cAMP antagonists (Song *et al*., 1998), suggesting that there is an interplay between the different cyclic nucleotide signaling pathways. Indeed, recent findings support the model that the ratio of cAMP and cGMP is critical for determining the polarity of the turning response to Netrin, with high cAMP to cGMP ratios favoring attraction and vice versa (Nishiyama *et al*., 2003).

Despite significant progress in understanding some of the signals that lead to changes in cAMP and cGMP levels, and how cyclic nucleotide signaling influences cytoskeletal regulation in growth cones, a direct molecular link between these pathways and axon guidance receptors had remained elusive until recently. Furthermore, the significance of effects of cyclic nucleotides on receptor responses for *in vivo* midline axon guidance is still lacking. However, a recent study of motor axon guidance in *Drosophila* has shown that the *Drosophila* A-kinase anchoring protein (AKAP), Nervy, links the Plexin receptor to PKA to modulate Semaphorin repulsion (Terman & Kolodkin, 2004), providing *in vivo* support for the importance of cyclic nucleotide signaling and forging a direct molecular link between cyclic nucleotides and guidance receptors. Distinct AKAPs localize type II PKAs to distinct sub-cellular regions, facilitating the spatially specific phosphorylation of target proteins in response to local elevations of cAMP, and thus are uniquely poised to coordinate multiple PKA inputs and outputs (Diviani & Scott, 2001; Feliciello *et al*., 2001). An elegant series of biochemical and dose dependent genetic interactions, together with the finding that a single amino acid substitution ($Nervy^{V523P}$) that prevents Nervy and PKA RII association generates a dominant negative Nervy, beautifully support the model that by linking PKA to Plexin, Nervy negatively regulates the repulsive response to Sema1a (Terman & Kolodkin, 2004). The simplest interpretation of the genetic analysis of *nervy* and*sema/plexin* interactions is that rather than effecting a conversion of Sema repulsion into attraction, Nervy and PKA weaken the strength of the repulsive response. Together with a number of recent studies in vertebrate neuronal culture that suggest a similar inhibitory effect of cAMP/PKA on the strength of Sema repulsion, the role of *nervy* in Sema signaling suggests that in addition to a whole-sale conversion of guidance responses, cyclic nucleotide signaling can also play a more refined modulatory role on the strength of guidance outputs (Chalasani *et al*., 2003; Dontchev & Letourneau, 2002; Terman & Kolodkin, 2004). Understanding the signals that lead to activation of Plexin-tethered PKA and the substrates of PKA that allow regulation of Plexin output are major challenges for future study. It will also be of great interest to determine whether there are specific AKAPs for other guidance receptors, such as the Slit and Netrin receptors, and if so how they function to regulate *in vivo* axon guidance.

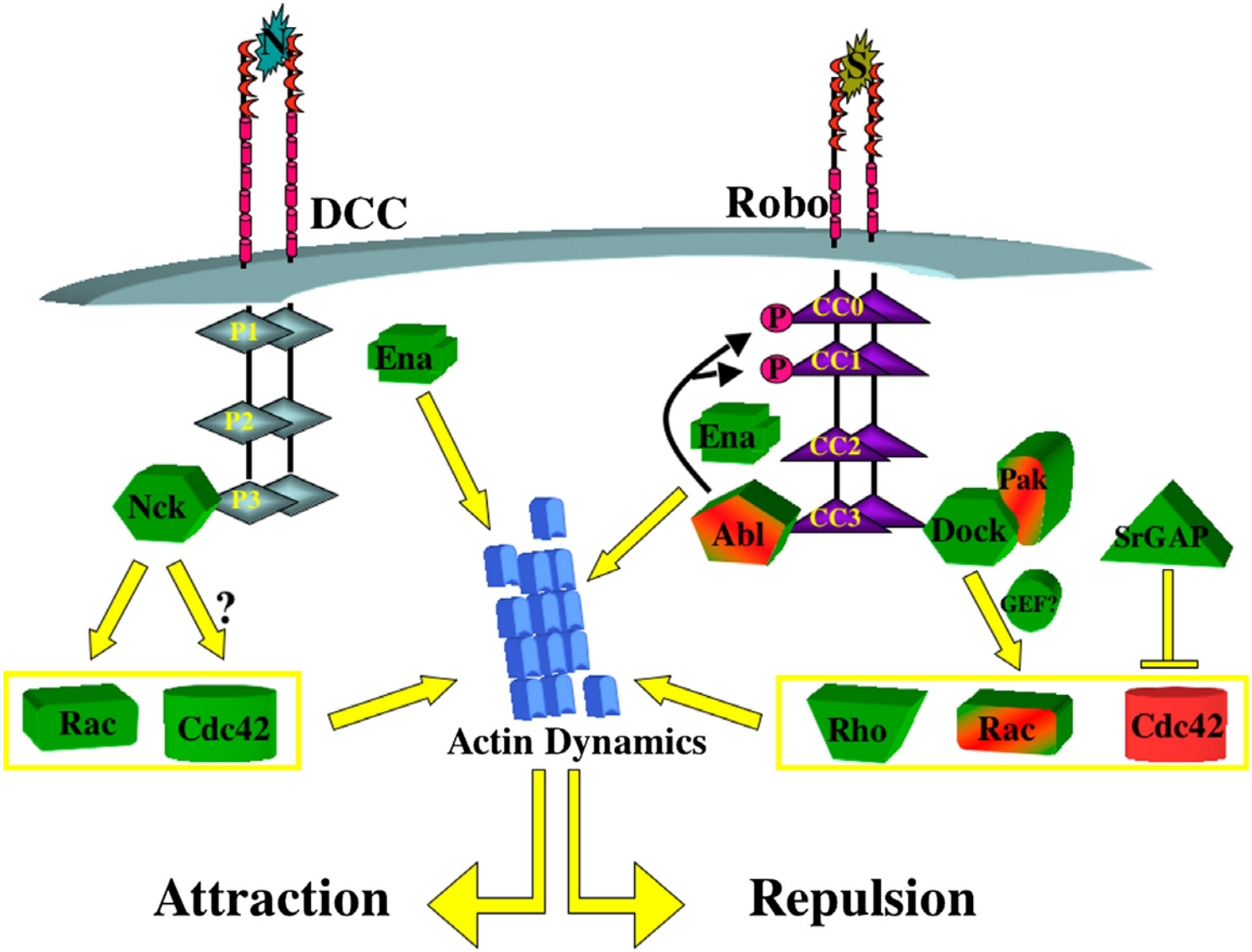

FIG. 4. Robo and DCC signaling components. Selected molecules implicated in DCC attraction and/or Robo repulsion. Robo and DCC receptors are drawn as dimers, although dimerization has been shown empirically only for DCC. Proteins that function as positive factors in a given signaling pathway are shaded green, proteins that function as negative factors are shaded red, and proteins that have been implicated as both positive and negative factors are shaded red/green. For example, Cdc42 is up-regulated in response to DCC activation and is colored green downstream of DCC, while Cdc42 is down-regulated in response to Robo activation and is colored red downstream of Robo. Molecules that bind to specific receptor motifs are drawn adjacent to the motifs with which they are known to interact. Ena does not directly interact with DCC and is shown at a greater distance from the P1 motif, since genetic evidence implicates Ena in a P1-dependent pathway. See text for further details.

Abelson, Enabled and Dock/NCK in Robo and DCC Signaling

Although the cytoplasmic domains of Robo and DCC do not contain any obvious catalytic signaling motifs, they do have proline rich regions, tyrosine phosphorylation sites, and other short stretches of evolutionarily conserved sequences (Figure 1) (Kidd *et al*., 1998a; Kolodziej *et al*., 1996). As detailed below, these motifs are likely to be important to link cytoplasmic domains to regulators of the cytoskeleton either directly or through adaptor proteins. In Robo there are four of these small cytoplasmic sequence motifs—CC0, CC1, CC2 and CC3. CC0 and CC1 are tyrosine-containing motifs that can be phosphorylated *in vitro* by the Abl tyrosine kinase, CC2 is a proline rich sequence (LPPPP) that matches the consensus binding site for the EVH1 domain of the *Drosophila* Enabled protein and CC3 is a poly-proline stretch. In DCC there are three conserved motifs- termed P1, P2 and P3, and as we have seen these motifs play important roles in mediating various receptor-receptor interactions and are likely important for downstream signaling events as well. Several studies using chimeric receptors have established that the specificity of Slit and Netrin signaling outputs is controlled by the cytoplasmic domains of their respective receptors (Bashaw & Goodman, 1999; Hong *et al*., 1999; Keleman & Dickson, 2001).

In *Drosophila*, the cytoplasmic tyrosine kinase Abl and its substrate, the actin binding protein Ena, have been proposed to play direct and opposing roles during Robo repulsion (Bashaw *et al*., 2000). Abl and Ena are both potent regulators of actin dynamics that have been shown to function in various aspects of cell motility and axon guidance (Krause *et al*., 2003; Lanier & Gertler, 2000). Initial genetic and biochemical data were consistent with a model in which Ena functions to transduce part of Robo's repulsive signal by binding to Robo's CC2 motif. Genetic and biochemical evidence from studies of the *C. elegans* Robo, Sax3, support a similar role for UNC34/Ena in Sax3 dependent guidance events, suggesting evolutionary conservation of this interaction (Yu *et al*., 2002), yet as in *Drosophila*, the cell biological mechanism by which Ena contributes to repulsion remains unclear.

In contrast to Ena's positive role in Robo repulsion, initial observations supported the idea that Abl functions to antagonize Robo signaling, likely through a mechanism involving direct phosphorylation of the Robo receptor on the CC0 and CC1 motifs (Bashaw *et al*., 2000), but also suggested that Abl could bind via its SH3 domain to the CC3 motif in Robo. The importance of the SH3/CC3 interaction is a bit unclear, since deletion of the Robo CC3 motif does not result in major disruption in Robo function. Genetic evidence for Abl antagonism of Robo signaling is derived from the observation that increasing Abl expression results in ectopic midline axon crossing, particularly in sensitized genetic backgrounds. More recently, other studies have suggested a positive role for Abl (in addition to its antagonistic function) during Robo signaling, as loss of *abl* function also appears to partially disrupt midline axon repulsion (Hsouna *et al*., 2003; Wills *et al*., 2002). In this context Abl appears to cooperate with two distinct cytoskeletal interacting proteins: 1) Capulet, a homolog of the adenylyl cyclase protein that regulates actin dynamics in yeast and 2) Orbit/MAST/Clasp, a microtubule binding protein proposed to coordinate actin and microtubule dynamics during growth cone repulsion (Lee *et al*., 2004; Wills *et al*., 2002). Future studies to address the consequences of Robo activation on Abl recruitment and kinase activity will provide important insight into the dual role of Abl during midline repulsion.

In addition to a conserved role in contributing to Robo repulsion, Ena has also been implicated in both attractive and repulsive responses to Netrin. In *C. elegans*, *unc-34/ena* was identified in a genetic screen for suppressors of an UNC-5 gain of function phenotype (Colavita & Culotti, 1998), suggesting a role for Ena proteins in diverse repulsive responses. Somewhat surprisingly two independent reports have shown that Ena also plays important roles in DCC/UNC-40 attraction (Gitai *et al*., 2003; Lebrand *et al*., 2004), indicating that Ena is unlikely to be the key determinant of whether a particular guidance response is attractive or repulsive. In the first report, *unc-34/ena* mutants were found to act as suppressors of *unc-40* gain of function and additional genetic analysis supports a role for *unc-34/ena* in endogenous *unc-40* signaling, specifically through the UNC-40 conserved P1 cytoplasmic domain (Gitai *et al*., 2003). In contrast to Ena function during Robo signaling, where Ena has been shown to directly bind to Robo's cytoplasmic domain (Bashaw *et al*., 2000; Yu *et al*., 2002), Ena does not appear to bind directly to UNC-40/DCC, suggesting that additional unidentified factors must link Ena and DCC/UNC-40; genetic analysis argues against a bridging role for the Sax-3/Robo receptor in this context (Gitai *et al*., 2003).

In the second study using cultured hippocampal neurons, where Ena/VASP has been previously shown to localize to the distal tips of filopodia (Lanier *et al*., 1999), loss of Ena/VASP function (generated by a clever method to deplete these proteins from the membrane) revealed an important role for Ena/VASP in the formation and elongation of filopodia (Lebrand *et al*., 2004). Furthermore, DCC-mediated filopodia formation was also dependent on Ena/VASP and correlated with the phosphorylation of Ena/VASP at a specific PKA regulatory site (Lebrand *et al*., 2004); this is particularly satisfying in light of the known role of cAMP and PKA in the promotion of Netrin attraction. Together these findings suggest that Ena/VASP functions in neurons primarily to regulate filopodial dynamics,

and it can be involved in mediating either attractive or repulsive responses. Given the lack of conservation of the PKA phosphorylation site in *Drosophila* Ena, it will be interesting to see if Ena also has a role in Frazzled signaling at the fly midline.

In addition to Ena and Abl, the SH3-SH2 adaptor protein Dreadlocks (Dock), the *Drosophila* homologue of vertebrate Nck (Garrity *et al*., 1996), has also been implicated in both DCC and Robo signaling pathways. Dock/Nck has previously been shown to function downstream of a number of axon guidance receptors. For example, *Drosophila* Dock has been suggested to act downstream of the Down Syndrome Cell Adhesion Molecule (Dscam) axon guidance receptor during pathfinding of Bolwig's nerve (Schmucker *et al*., 2000). Since the ligand for Dscam has not been identified, it is not known whether Dscam is functioning in this context as an attractive or a repulsive receptor. Vertebrate Nck has also been linked to several guidance receptors *in vitro*, including Eph receptors and c-Met receptors (Kochhar & Iyer, 1996; Stein *et al*., 1998). Dock is also known to interact with key regulators of the actin cytoskeleton, including members of the SCAR/WAVE family of actin regulatory proteins (Rohatgi *et al*., 2001), and the p21 activated protein kinase (Pak) (Hing *et al*., 1999), which in turn can interact with members of the Rho family of small GTPases, such as Rac 1 and Cdc42 (Burbelo *et al*., 1995; Eby *et al*., 1998; Manser *et al*., 1994).

More recently, Dock/Nck has been shown to directly interact with the cytoplasmic domains of both the vertebrate DCC receptor (Li *et al*., 2002a) and the *Drosophila* Robo receptor (Fan *et al*., 2003). In both cases the interaction is mediated through the Dock/Nck SH3 domains, and in the case of Robo, ligand stimulation has been shown to enhance Dock/Robo interaction and lead to the formation of a ternary complex including Pak (Fan *et al*., 2003; Li *et al*., 2002a). Genetic evidence suggests that the Robo/Dock interaction is important for Robo repulsion, while the significance of Pak recruitment remains unclear, since genetic disruption of Pak does not lead to dramatic embryonic guidance defects. The Nck and DCC interaction is important for DCC's function to stimulate axon extension *in vitro* (Li *et al*., 2002a), however, the *in vivo* significance of these observations remains to be established.

Rho GTPases and Their Upstream Regulators

The key role of the Rho family of small GTPases in regulating the actin cytoskeleton in response to extracellular signals is well established (Hall, 1998). The Rho GTPases cycle between a GDP-bound inactive state and a GTP-bound active state. In their active GTP bound form, they can bind to and turn on downstream effector proteins to elicit a range of cellular responses (Van Aelst & D'Souza-Schorey, 1997). The canonical members of the Rho family of small GTPases—Rac, Rho and CDC42—have differential effects on the cytoskeletal morphology of many cell types. Their functions are perhaps best characterized in fibroblasts where activation of Rho tends to promote cell rounding and actin stress fiber formation, while activation of Rac and Cdc42 promote lamellopodial and filopodial extension respectively (Hall, 1998). In neuronal cells activation of Rac and Cdc42 have been found to promote neurite extension, while Rho activation leads to neurite retraction (Jalink *et al*., 1994; Luo *et al*., 1994).

In addition to their role in regulating neurite extension, overwhelming evidence argues that the Rho GTPases and their upstream positive and negative regulators—guanine nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs)—also play important roles in the control of growth cone guidance in the developing nervous system (Dickson, 2001; Luo, 2000; Yuan *et al*., 2003). Genetic disruption of various Rho family GTPases, either through mutation or expression of dominant negative versions of the GTPases, leads to defects in axon guidance in many different systems, including worms, flies and frogs. For example, mutations in *C. elegans* Rac genes, or expression of dominant negative Rac in the developing nervous system of *Drosophila*, results in guidance errors at specific choice points (Kaufmann *et al*., 1998; Lundquist *et al*., 2001; Zipkin *et al*., 1997). More recently, loss of function mutations in the three *Drosophila* Rac genes- Rac1, Rac2 and Mtl- have been characterized and found to have defects in both axon growth and guidance, as well as dendrite morphogenesis and branching, further supporting an *in vivo* role of the GTPases in axon guidance (Hakeda-Suzuki *et al*., 2002; Ng *et al*., 2002). Finally, a steadily growing number of mutations in upstream regulators, including the GEF Trio, and down stream effectors of the small GTPases, such as the Pak serine-threonine kinase, have also been shown to cause axon guidance defects (Bateman *et al*., 2000; Billuart *et al*., 2001; Hing *et al*., 1999; Liebl *et al*., 2000; Newsome *et al*., 2000).

The opposing roles of the Rho family of GTPases during neurite outgrowth have led to the suggestion that repulsive axon guidance cues could exert their effects by causing local activation of Rho, while attractive guidance cues could work through the activation of Rac and/or Cdc42 (Dickson, 2001; Patel & Van Vactor, 2002). Studies aimed at elucidating the potential role of the Rho family of small GTPases in signaling downstream from these receptors is for the most part consistent with the idea that Rho is involved in growth cone collapse and/or repulsion (Luo, 2000). For example, recent studies of Eph receptor signaling have shown that the RhoGEF ephexin couples Ephrin stimulation to the activation of RhoA, and is required *in vitro* for Ephrin induced growth cone collapse (Shamah

et al., 2001). Genetic analysis of Semaphorin/Plexin induced axon repulsion in *Drosophila* suggests that RhoA activation is required to mediate Plexin's repulsive effects (Hu *et al*., 2001). In addition, the PDZ-RhoGEF LARG has been shown to couple Semaphorin-Plexin signaling to the activation of RhoA; a "dominant negative" form of this RhoGEF that binds to Plexin, but cannot activate Rho prevents Semaphorin mediated collapse of hippocampal neurons in culture (Swiercz *et al*., 2002).

Several genetic studies of the regulation of midline crossing in *Drosophila* embryos also support an important role for Rho function in Robo repulsion (Fan *et al*., 2003; Fritz & VanBerkum, 2002), although it is unclear how direct these effects are, since biochemical assays show that Slit stimulation does not lead to a marked increase in Rho activity (Fan *et al*., 2003). In contrast to these findings, several reports have also suggested a role for Rac in mediating the repulsive effects of Sema and Slit, suggesting perhaps that the role of the GTPases in mediating specific repulsive responses is more complex (Fan *et al*., 2003; Hakeda-Suzuki *et al*., 2002; Jin & Strittmatter, 1997; Vastrik *et al*., 1999; Wong *et al*., 2001). For example, various genetic manipulations to reduce *rac* activity exhibit dose-dependent genetic interactions with *slit* and *robo* (Fan *et al*., 2003; Matsuura *et al*., 2004). Paradoxically, similar perturbations of midline crossing can sometimes be observed by expressing a constitutively active form of Rac (Fritz & VanBerkum, 2002), suggesting that disrupting cycling between inactive and active states can disrupt function. Indeed, similar phenotypic effects of constitutive active and dominant negative versions of the GTPases have frequently been observed (Luo, 2000).

How is Robo receptor activation coupled to the regulation of RhoGTPase activity? Studies of Slit-mediated neuronal cell migration in mammals, together with studies of Slit/Robo repulsion at the *Drosophila* midline are consistent with important roles for the small GTPases in Robo repulsive signaling and have shed light on the biochemical mechanisms of GTPase regulation (Fan *et al*., 2003; Fritz & VanBerkum, 2002; Matsuura *et al*., 2004; Wong *et al*., 2001). For example, the GTPase activating proteins (GAPs), srGAP1 and srGAP2, interact with Robo's cytoplasmic domain and are required for Slit's repulsive effect on the migration of cultured precursor cells of the anterior subventricular zone (SVZa). The srGAPs interact with Robo predominantly through the proline rich CC3 motif of the receptor's cytoplasmic domain, and activation of Robo leads to the srGAP dependent down-regulation of the small GTPase Cdc42. In addition, Slit stimulation was observed to result in a significant increase in Rac activity (independent of srGAP function), a finding consistent with biochemical evidence from studies of Slit and Robo in *Drosophila* (Fan *et al*., 2003). Various manipulations of Cdc42 function support the idea that it is the down-regulation of Cdc42 that is important to generate the repulsive effect of Slit on SVZa cell migration (Wong *et al*., 2001). Consistent with this idea, expression of constitutively active Cdc42 in a small subset of ipsilaterally-projecting neurons, in embryos that are heterozygous for *robo,* results in significant levels of ectopic midline crossing (Fritz & VanBerkum, 2002; Matsuura *et al*., 2004), suggesting that navigating axons and migrating cells both down-regulate Cdc42 in response to Robo activation, and moreover that this signaling mechanism is conserved between invertebrates and vertebrates.

One of the hallmarks of Cdc42 activation is the induction of filopodia extension. Blocking Cdc42 function de-stabilizes existing filopodia and inhibits the extension of new filopodia (Hall, 1998). Interestingly, in time-lapse studies of filopodia dynamics in wild-type and *robo* mutant embryos, the striking observation was made that non-crossing axons in both wild-type and *robo* mutants often would extend filopodia that crossed the midline (Murray & Whitington, 1999). In wild-type animals all of these crossing filopodia were rapidly retracted, while in *robo* mutants, crossed filopodia were often stabilized on the contra-lateral side of the midline resulting in axon bundles that inappropriately crossed the midline (Murray & Whitington, 1999). A clear analogy to the alterations in filopodia dynamics in *robo* mutants in the fly can be made to defective pathfinding error correction in *astray (robo)* mutants in zebra fish (Hutson & Chien, 2002). The ability of Robo to down-regulate Cdc42 activity may provide a molecular explanation for both of these phenomena.

In contrast to Slit/Robo negative regulation of Cdc42 activity, where the srGAPs link the Robo cytoplasmic domain directly to Cdc42, how Slit stimulation leads to Rac activation in both vertebrates and *Drosophila* is less clear. Since the srGAPs appear to function specifically in the negative regulation of Cdc42 activity, they are not good candidates to explain the up-regulation of Rac activity (Wong *et al*., 2001). Indeed, because Slit stimulation results in an increase in Rac activity, one might predict that Rac-GAP activity would be negatively regulated in response to Slit stimulation of Robo. Intriguingly, in the course of a genome-wide analysis of all RhoGEFs and RhoGAPs in *Drosophila*, one Rac-specific GAP has been identified that when over-expressed, results in phenotypes reminiscent of *robo* loss of function (Hu *et al*., submitted). There are a number of candidate GEFs that could explain how Rac activity is up-regulated by Slit activation of Robo, most notably the dual Ras/Rho GEF Son-of-Sevenless (Sos), rtGEF (pix) and Trio (Fritz & VanBerkum, 2000; Newsome *et al*., 2000; Parnas *et al*., 2001). Indeed, *sos* has been shown to genetically interact with *slit* and *robo* during midline guidance (Fritz & VanBerkum, 2000, 2002), and studies of vertebrate Sos indicate it can regulate Rac; interestingly this function is dependent on

the tyrosine phosphorylation of Sos by Abl (Sini *et al*., 2004). It will be interesting to determine which if any of these molecules could play such a role in Robo signaling. It is also possible that the observed increase in Rac activity could be explained by the Slit-dependent down-regulation of a constitutive Rac-GAP.

The Rho family small GTPases have also been implicated in DCC-mediated Netrin attraction and findings are consistent with positive roles for both Rac and Cdc42, but not Rho in transmitting DCC signals to the regulation of the cytoskeleton (Li *et al*., 2002b; Shekarabi & Kennedy, 2002). Recent evidence from neuronal cell culture has suggested that stimulation of the attractive Netrin receptor DCC leads to an increase in Rac activity through the Nck-1 adaptor protein, since a dominant negative form of Nck prevents Rac activation in response to Netrin (Li *et al*., 2002a, 2002b). In addition, disruption of Rac or Cdc42 prevents Netrin/DCC-induced cell spreading and filopodia formation respectively. Finally, genetic evidence in *C. elegans* has also implicated *rac* in signaling downstream of UNC-40/DCC in a parallel genetic pathway to *enabled* primarily through the P2 motif of UNC-40/DCC (Gitai *et al*., 2003).

How are Attraction and Repulsion Specified?

The finding that Dock/Nck, Ena and Rac 1 can all function both in DCC-mediated attractive axon guidance and Robo-mediated repulsive axon guidance raises the obvious question of how the specificity of attraction and repulsion is controlled, and argues against a committed role for any of these signaling molecules to either one or the other type of responses. This is perhaps not too surprising, given the fact that Robo and DCC receptors themselves are intimately connected through their ability to form a heteromeric receptor complex with potentially unique signaling properties (Stein & Tessier-Lavigne, 2001). One possible explanation for how the specificity of the axon response is controlled is that there are additional signaling molecules that are unique to either repulsive or attractive responses. For example Cdc42 appears to be activated in the context of Netrin/DCC attraction and inhibited in the context of Slit/Robo repulsion. It remains possible that additional signaling molecules or adaptors will be identified that can further account for the specificity of responses. An alternative possibility is that it is the coordinate regulation, relative activity levels and combinatorial action of a core group of common signaling molecules that makes the difference in attraction versus repulsion.

FUTURE DIRECTIONS

The last several years have seen remarkable progress in elucidating the mechanisms by which axon guidance is coordinated during development, but much remains to be done. Though many conserved families of cues and receptors have been identified over the past decade, others undoubtedly await discovery. Studies in diverse systems continue to catalog novel transcriptional and post-transcriptional regulatory strategies that ensure the high-fidelity of guidance decisions, but as we have seen, many outstanding questions remain to be answered about how these events are coordinated during development. Although details of signaling pathways are beginning to emerge, our understanding of the key ligand-regulated events that control receptor activation and signaling is fragmentary; this is particularly true for guidance receptors such as DCC and Robo, which lack catalytic activity in their cytoplasmic domains. Progress in this area will rely on the development of biochemical and optical strategies to reveal the dynamic changes in multi-protein signaling complexes that are set in motion by guidance receptor activation. It is also clear that many signaling and additional regulatory components await discovery and molecular and genetic approaches, including sensitized genetic screens in *Drosophila* and *C. elegans* will continue to identify these missing components. Finally, understanding how signaling pathways are integrated to promote appropriate responses in the context of axon guidance in the developing organism is another major challenge in the field that will require continued investigation using diverse approaches in diverse experimental systems.

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