

Dynamic regulation of axon guidance

Timothy W. Yu and Cornelia I. Bargmann

Howard Hughes Medical Institute, Program in Neuroscience, Departments of Anatomy and of Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA

Correspondence should be addressed to C.I.B. (cori@itsa.ucsf.edu)

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To reach their proper targets, axons rely upon the actions of highly conserved families of attractive and repulsive guidance molecules, including the netrins, Slits, semaphorins and ephrins. These guidance systems are used to generate an astonishingly varied set of neuronal circuits. Here we consider the mechanisms by which a few guidance systems can be used to generate diverse outcomes. Recent studies have revealed extensive transcriptional and post-transcriptional regulation of guidance cues and their receptors, as well as combinatorial mechanisms that integrate information from different families of guidance cues.

Neurons respond to a variety of attractive and repulsive guidance cues to navigate to their targets¹. Genetic, biochemical and molecular approaches have identified four conserved families of guidance cues with prominent developmental effects: the netrins, Slits, semaphorins and ephrins (Fig. 1). Netrins, Slits and some semaphorins are secreted molecules that associate with cells or extracellular matrix, whereas ephrins and other semaphorins are expressed at the cell surface. Netrins can act as attractants or repellents; Slits, semaphorins, and ephrins act primarily as repellents but can be attractive or adhesive in some contexts. For each of these cues, one or more transmembrane receptors have been identified: UNC-40 (also known as DCC, the 'deleted in colorectal cancer' protein) and UNC-5 receptors for netrins², Roundabout (Robo) receptors for Slit proteins^{3,4}, neuropilin and plexin receptors for semaphorins^{5,6} and Eph receptors for ephrins^{7,8}. In the case of netrins and Slits, a small number of ligands interact with a small number of receptors; in the case of semaphorins and ephrins, large families of related ligands interact with corresponding families of receptor proteins. These are not the only guidance factors: neurotrophins, hepatocyte growth factor (HGF)/scatter factor and transforming growth factor β (TGF- β) family members can guide axons, and additional candidate receptors include the protocadherin family, odorant receptors, immunoglobulin family cell adhesion molecules (Ig-CAMs) and neuroligins. Nonetheless, the number of guidance cues and receptors seems small relative to the immense complexity of the nervous system.

The conserved netrin, Slit, semaphorin and ephrin pathways are remarkably versatile. Each guidance cue has been implicated in numerous axon guidance or targeting events. Moreover, the functions of the guidance molecules are not restricted to axon migrations. Netrins and Slits affect neuronal and mesodermal cell migrations⁹⁻¹²; semaphorins mediate bone and heart morphogenesis¹³; ephrins direct neural crest migration and angiogenesis⁸. How are a few guidance molecules used to generate a variety of structures in the nervous system (and elsewhere)? Recent work has revealed extensive regulation of guidance cues and their receptors, and uncovered mechanisms that integrate

information from different families of guidance cues. Here we consider three general mechanisms that regulate guidance molecules to yield diverse outcomes. First, the expression of guidance cues and their receptors can be regulated in specific patterns. Second, the signal transduction pathways downstream of receptors can be intrinsically different between cells, or extrinsically regulated by other pathways. Third, the activity of receptors can be regulated by the assembly of receptor complexes with new signaling properties.

Guidance molecules are developmentally regulated

Any particular guidance cue and its receptor are expressed at many times in development, and often in the adult brain¹⁴⁻¹⁸. Transcriptional regulation of guidance cues and receptors confers both spatial and temporal controls for axon guidance.

The expression of guidance cues is often dynamic and precisely tuned to the decisions that occur at a particular time and place. For example, the developing *Xenopus* tadpole has laterally placed eyes, and axon guidance at the optic chiasm initially drives all axons to the contralateral brain (Fig. 2a). Later in development, both eyes migrate medially so that left and right visual fields overlap. Corresponding ipsilateral axon projections from the eye to the brain form to accommodate the overlapping visual fields. The appearance of ipsilateral axons during frog development correlates with the appearance of repulsive ephrin-B cues at the midline of the optic chiasm¹⁹. The late appearance of ephrin-B directs the axons of later-born retinal neurons expressing the ephrin receptor EphB to the ipsilateral brain, creating visual projections that represent the modified eye location in the frog.

Both gene activation and gene repression by transcriptional regulators are involved in shaping guidance decisions. The UNC-130 forkhead transcription factor is required in *Caenorhabditis elegans* to repress expression of a secreted UNC-129 axon guidance cue by ventral muscles²⁰ (Fig. 2b). The ventral-only expression of the transcription factor UNC-130, followed by dorsal-only expression of the UNC-129 guidance cue, are essential for a normal dorsoventral guidance axis.

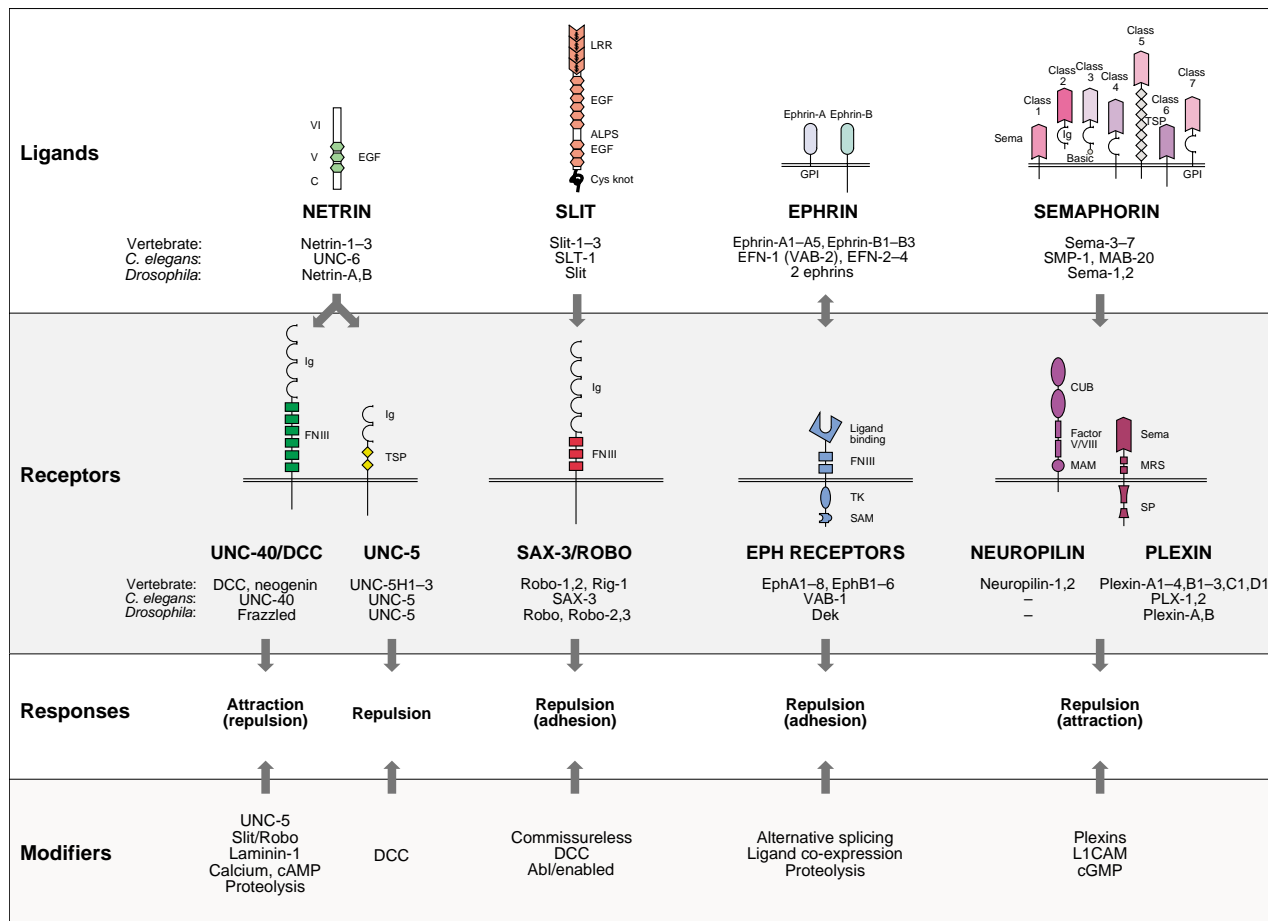


Fig. 1. Summary of the four families of instructive guidance cues and receptors discussed in this review. Major families are indicated by column headings, with species-specific names underneath. Typical guidance responses, alternate responses (in parentheses) and known modifiers of each pathway are shown. ALPS, agrin–laminin–perlecan–Slit domain; C, netrin C terminus; CUB, CI/Uegf/BMP-1 domain; DCC, deleted in colorectal cancer; EGF, epidermal growth factor; FNIII, fibronectin type III domain; GPI, glycosylphosphatidyl–inositol anchor; Ig, immunoglobulin domain; LRR, leucine-rich repeat; MAM, mephrin/A5 antigen motif; MRS, Met tyrosine kinase–related sequence; RK, arginine/lysine-rich basic domain; SAM, sterile alpha motif; SP, ‘sex and plexins’ domain; TK, tyrosine kinase domain; TSP, thrombospondin domain; VI and V, homology to laminin domains VI and V, respectively.

The transcription of guidance receptors as well as guidance cues can be regulated in development. In *C. elegans*, certain cells that migrate away from netrin sources are exposed to netrin throughout their development, but they turn on the expression of the repulsive netrin receptor UNC-5 at the exact time that they make the guidance decision. For these cells, the timing of *unc-5* transcription is the central regulatory event: precocious UNC-5 expression induces precocious repulsion from netrin²¹.

Complex guidance decisions may be directed by complex expression patterns, as seen in the retinotopic map of the visual system in the vertebrate brain. The expression of ephrin-A ligands in the tectum occurs in an anterior-to-posterior gradient, and the expression of EphA receptors in retinal ganglion cells follows a complementary nasal-to-temporal gradient⁷ (Fig. 2c). Axons expressing high levels of EphA receptor are repelled from low levels of ephrin-A ligand, whereas axons with lower EphA receptor levels can tolerate higher ephrin-A levels. A competition between axons, rather than the absolute level of receptor, drives their relative positions in the tectum²². The opposing ligand and receptor gradients are largely specified by transcriptional mechanisms^{23–25} and are essential for orderly targeting and creation of the visual map.

Many axon pathfinding mutants identified in fly, worm or mouse genetic screens correspond to mutations in specific transcription factors^{26–34}. Transcription factors often have tissue-specific effects on guidance, but in most cases the genes they regulate are unknown. We suggest that these transcription factors are the tissue-specific regulators that define local expression of general guidance molecules.

Post-transcriptional regulation of cues and receptors

The activity of guidance pathways is regulated not only by transcriptional mechanisms but also by post-transcriptional mechanisms that regulate the availability of receptors and ligands. These include receptor downregulation, ligand inactivation of receptor, alternative splicing, regulated proteolysis and ligand presentation.

Regulation of the guidance receptor Robo is important in the patterning of the *Drosophila* nervous system. Most axons in the fly CNS cross the midline once, but do not recross because of Robo-mediated repulsion from the midline repellent Slit. *Robo* RNA is present in neurons when they initially cross the midline, but the Robo protein is transiently inactivated by the *commis sureless* (*comm*) gene until after midline crossing. When *comm* is absent,

Robo function is de-repressed and axons never cross the midline^{35,36}. The *comm* gene encodes a transmembrane protein that decreases the surface expression of Robo in commissural axons, rendering them insensitive to Slit until after midline crossing^{36,37}. In wild-type animals, Robo protein is sequestered in internal vesicles in axons that cross the midline³⁸. This result suggests that Robo stability, delivery to the cell surface or internalization is regulated during axon guidance.

Guidance receptors can also be regulated by their own ligands. Temporal retinal axons express high levels of EphA3 receptors and are repelled by target tissues expressing high levels of ephrin-A ligands (Fig. 2c). Interestingly, however, overexpression of ephrin-A2 or ephrin-A5 in temporal axons abolishes their repulsive response to ephrin-A proteins in other tissues^{39,40}. This result suggests that the expression of ephrin-A can desensitize or inactivate Eph receptors on the same cell. Nasal axons express ephrin-A2 and ephrin-A5 as well as a moderate level of Eph receptors, and they are not repelled by tissues expressing ephrin-A. Enzymatic removal of ephrin-A ligands from nasal retinal axons, or genetic inactivation of ephrin-A2 and ephrin-A5, increases the sensitivity of nasal axons to repulsive target tissues^{39,41}. These results indicate that endogenous ephrin-A proteins reduce Eph function on nasal axons. Ephrin-A2 and ephrin-A5 are expressed in high-nasal-to-low-temporal gradients in retinal neurons, suggesting that inhibition of Eph receptor function by retinal ephrins contributes to the proper formation of the topographic projection.

Eph receptor signaling can also be regulated by alternative splicing of the Eph receptor. Ephrin-A5 and EphA7 mediate closing of the neural folds and formation of the neural tube by an adhesive interaction, unlike the repulsive interactions characteristic of Eph signaling⁴². The adhesive response is generated by an alternatively spliced form of EphA7 that lacks the tyrosine kinase domain and suppresses tyrosine phosphorylation of full-length EphA7. Loss of EphA7 catalytic activity shifts the cellular response from repulsion to adhesion. It will be interesting to see whether this mechanism contributes to attractive functions of ephrins in axon guidance⁴³.

Regulated proteolytic cleavage of guidance receptors and their ligands can either inhibit or facilitate signaling. Many cell surface proteins, including the netrin receptor DCC, undergo metalloprotease-mediated ectodomain shedding. Proteolytic activity seems to downregulate the netrin response: inhibition of metalloprotease function stabilizes full-length DCC and potentiates netrin-stimulated axon outgrowth⁴⁴.

Proteases can also facilitate guidance responses. Guidance cues elicit rapid neurite retraction after interactions with repulsive ligands such as semaphorins or ephrins. However, the signaling between guidance receptors and ligands involves multivalent, high affinity receptor-ligand interactions, generating adhesive forces that must be overcome for axon repulsion or retraction to occur. Proteolytic cleavage of ephrin is critical in this disadhesion step⁴⁵. Mouse hippocampal growth cones contacting the repulsive cue ephrin-A2 respond by collapsing and rapidly retracting. Ephrin-A2 forms a stable complex with the metalloprotease kuzbanian, which triggers cleavage of ephrin-A2 upon Eph receptor binding. When cleavage is blocked by mutations in ephrin-A2, disadhesion does not occur and axon withdrawal is delayed.

Finally, for secreted guidance ligands, the final pattern of expression can be regulated by interactions with other cell-associated proteins or extracellular matrix proteins. In *Drosophila*, netrin can be concentrated at particular choice points through

binding to cell-associated DCC⁴⁶. In this context, DCC acts to present netrin to other cells. Slit binds to netrin and laminin, suggesting that binding interactions between guidance ligands might affect their localization⁴⁷.

Cytoskeletal pathways mediate responses to cues

Axon guidance receptors can act both by conferring selective adhesion to substrates and by transducing signals to the growth cone. Simple adhesion of the growth cone to substrate may be sufficient for some forms of axon guidance, as the growth of neurites *in vitro* can be controlled by gradients or sharp boundaries of fibronectin or laminin⁴⁸. Although navigation through corridors of extracellular matrix may require only adhesion and traction, the guidance cues listed in Fig. 1 function by

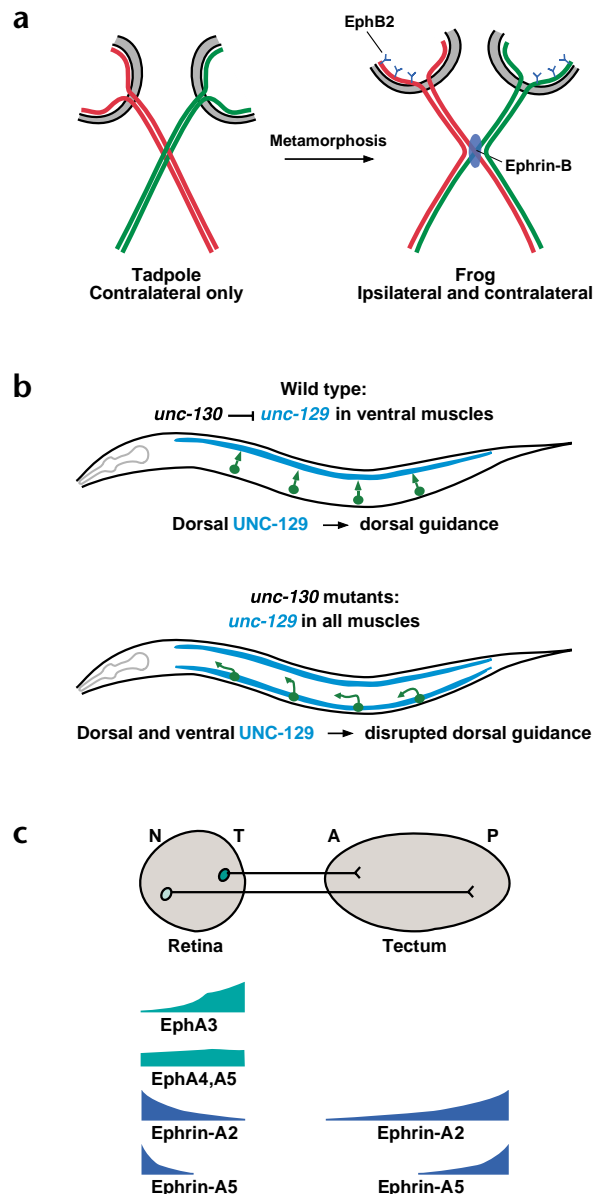


Fig. 2. Transcriptional regulation of axon guidance cues and their receptors. (a) Induction of ephrin-B at the *Xenopus* optic chiasm induces ipsilateral projections. (b) Transcriptional repression of the *C. elegans* guidance cue *unc-129* controls dorsal axon guidance. (c) Ephrin-A and EphA gradients specify topography of chick retinotectal projections.

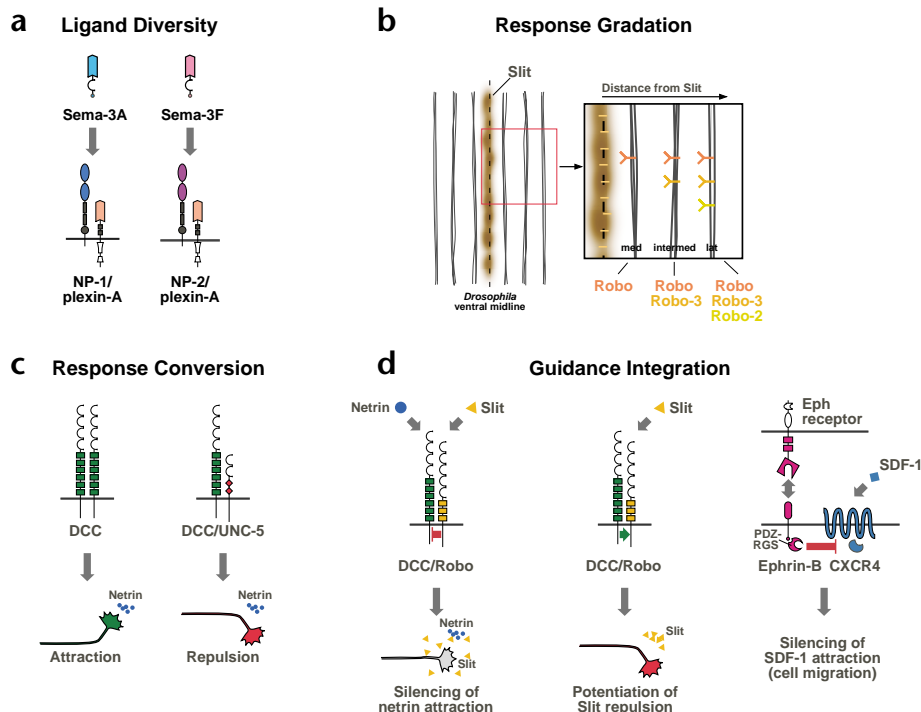


Fig. 3. Combinatorial regulation of axon guidance receptor signaling. **(a)** Combinatorial assembly of semaphorin receptors increases the diversity of ligands to which a family of guidance receptors can respond. **(b)** Combinatorial codes of Robo receptors confer different, graded responses to a single Slit guidance cue. **(c)** Addition of UNC-5 subunits to a DCC receptor complex can convert the guidance response from attractive to repulsive. **(d)** Interactions between receptors allow combinatorial integration of a variety of different guidance pathways. DCC, deleted in colorectal cancer (netrin receptor); SDF-1, stromal cell-derived factor (chemokine); CXCR4, chemokine receptor; PDZ-RGS, PDZ domain-containing regulator of G protein signaling; SDF-1, stromal cell-derived factor (chemokine).

activating specific signaling pathways and not purely by adhesion and disadhesion.

Guidance receptors can initiate signaling cascades that instruct the growth cone to turn toward or away from a source of ligand, change speed, expand or collapse. The signaling pathways in axon guidance are not fully understood, but it is likely that they act locally on the growth cone and not by long-range signaling to the cell body. Signaling is converted into local changes in the actin cytoskeleton of the growth cone, which modulate the stability of the growth cone and its conversion to a microtubule-rich axon shaft⁴⁹. The Rho family of GTPases, which direct the formation of a wide range of cytoskeletal structures⁵⁰, are strongly implicated in axon guidance^{51–54}. Local activation of Rho-family GTPases is likely to be a central component of growth cone turning responses, although the pathways that link specific GTPases to specific guidance receptors are not fully understood⁵⁵. Cytoskeletal effectors are discussed in more detail elsewhere^{56,57}.

In this discussion of regulation of guidance pathways, three general aspects of intracellular signal transduction are particularly interesting. First, the intrinsic properties of a cell's cytoskeleton dictate its response to guidance information. Netrin or Slit can direct either cell migration or axon migration depending upon the intrinsic properties of the cell that senses netrin or Slit (am I migrating, or is my axon migrating?)^{9–12}. The intracellular mechanisms that direct this decision are unknown.

Second, the regulated expression of specific intracellular signaling components can be a source of guidance information. The

cytoplasmic VAB-8 protein is required mostly for posterior axon guidance in normal *C. elegans* development⁵⁸. Ectopic expression of VAB-8 is sufficient to specify a posterior direction of axon outgrowth to the *C. elegans* ALM neuron, which normally sends its axon anteriorly⁵⁹. At a more subtle level, the ratios of activity of a cytoplasmic tyrosine kinase, ABL, and several other regulators (the ENA protein, which is phosphorylated by ABL, and the tyrosine phosphatases, which antagonize its activity) can determine the outcome of Robo-mediated guidance decisions, particularly in sensitized genetic backgrounds^{60,61}.

Third, there is evidence from *in vitro* studies that the activity of second-messenger pathways within the cell can specify the outcome of guidance decisions. Netrin is normally attractive to *Xenopus* spinal cord neurons, but is repulsive when cAMP levels are low. Similar effects have been reported with manipulations of calcium^{62,63}. Conversely, semaphorin is normally repulsive to spinal neurons but becomes attractive in high cGMP levels^{64,65}. Cortical neurons in slice preparations exhibit paradoxical opposite responses to semaphorin:

their axons are repelled from semaphorin but their dendrites are attracted to it^{66,67}. Attraction of cortical dendrites to semaphorin is associated with guanylyl cyclase activity that is localized to dendrites, suggesting that cGMP levels act as an endogenous regulator of semaphorin signaling that distinguishes axons from dendrites. Although their mechanisms of action remain mysterious, current results suggest that calcium and cyclic nucleotides modulate the cytoskeletal components underlying attraction and repulsion.

Receptors function as multimeric complexes

Dimerization or multimerization is an essential step in the activation of many classes of receptors, and this principle seems to apply in axon guidance as well. The mechanisms of multimerization are best defined for the Eph transmembrane tyrosine kinase receptors^{68,69}. The exact valency of the physiological Eph signaling complex is not clear: it has at least two subunits, and possibly more. The cytoplasmic SAM (sterile-alpha) motif, a conserved motif within Eph receptors, is involved in the formation of tetrameric and possibly higher order oligomers^{70–72}, but mice bearing EphA4 receptors that lack the SAM domain have no detectable defects in EphA4 function⁷³. In all likelihood, the multimerization function provided by the SAM domain is redundant with other clustering mechanisms used by Eph receptors. The ligand itself may have this function, as activation of Eph receptors by glycosylphosphatidylinositol (GPI)-anchored and transmembrane ephrin ligands requires that the ligand be presented in multimeric form⁶⁸. Ligand clustering may be a

common motif that contributes to complex formation and signaling. Crystal structures of receptors bound to their ligands demonstrate that in addition to receptor–receptor interactions, dimerization can be facilitated by ligand–ligand, ligand–extracellular matrix or ligand–receptor contacts⁷⁴.

Netrin also induces multimerization of its receptor DCC via the P3 domain, a short motif within the DCC cytoplasmic region⁷⁵. The essential function of the P3 domain in netrin signaling can be supplied by replacing P3 with the SAM multimerization domain from Eph receptors. This result suggests that axon guidance receptors have a modular organization, with discrete domains that confer functions such as dimerization.

Combinatorial assembly of receptors

Eph and DCC signaling can create homomultimeric receptor complexes; further complexity can arise from the formation of heteromultimer complexes between different receptor molecules. Heteromeric interactions between receptors create the possibility for specificity by a combinatorial logic, in which individual subunits can act in several complexes with distinct properties. Combinatorial action is involved in the establishment of ligand specificity, formation of graded responses, response conversion and signal integration.

Combinatorial assembly of receptor complexes can expand the repertoire of ligands to which growth cones respond, as exemplified by the semaphorin receptors. Semaphorins are a large family of secreted, GPI-linked or transmembrane proteins that share an extracellular Sema domain. In three examples, their receptors have been shown to be members of the transmembrane plexin receptor family: Sema-3 proteins signal through plexin-A1 or plexin-A2 (plexin-A), transmembrane Sema-4D binds to plexin-B1 and GPI-anchored Sema-7A binds to plexin-C1 (refs. 76, 77). Interestingly, plexins also contain a Sema domain with an autoinhibitory function; relief of plexin autoinhibition by Sema domain displacement seems to be a component of receptor activation⁷⁸.

The Sema-3 family of semaphorins contains different members with distinct biological activities. Specificity is generated because their functional receptors are not plexin-A alone but heteromultimers containing both a neuropilin and a plexin-A molecule (Fig. 3a). Neuropilins are transmembrane molecules that bind semaphorins and plexins; they seem to modulate Sema–plexin binding and are probably not directly involved in signaling^{77,79}. The combination of neuropilin-1 and plexin-A confers sensitivity to the ligand Sema-3A^{77,80–82}, whereas the combination of neuropilin-2 and plexin-A confers sensitivity to Sema-3F⁸³ (Fig. 3a). The functions of neuropilins are not limited to semaphorin signaling. When co-expressed with the receptor tyrosine kinase VEGF-R2, neuropilin-1 confers enhanced sensitivity to an entirely different peptide, the angiogenic VEGF-165 protein^{84,85}.

In addition to the core receptor complex that contains neuropilin and plexin, studies of mice lacking L1-CAM activity suggest that a third molecule, the transmembrane cell adhesion protein L1-CAM, has a modulatory effect⁸⁶. Axons from cortical explants or DRG neurons from wild-type mice are efficiently repelled by COS cells expressing Sema-3A, but explants from L1-deficient animals are not. These results suggest that L1 contributes to the Sema-3A repulsive response. Bath application of an L1 fragment to cortical slices blocked Sema-3A–induced collapse and had the surprising effect of converting Sema-3A–induced repulsion into attraction. The mechanism of L1 action is unknown, and it may be an indirect effect mediated through

cGMP levels or another process like axon fasciculation (bundling). Nonetheless, its activity is of interest as a potential modifier of semaphorin receptors.

Combinatorial receptor function has implications for signaling as well as ligand binding, and can underlie the generation of graded responses to a single ligand, as exemplified by the action of *Drosophila* Robo proteins. *Drosophila* Robo was first characterized according to its ability to prevent inappropriate midline crossing by axons, but Robo proteins also act in positioning of longitudinal tracts around the midline. This was first discovered through characterization of Slit, the Robo ligand⁸⁷. In addition to disruption of midline crossing, *slit* mutants exhibit a striking defect in which all of the longitudinal tracts coalesce into a single midline bundle. Conversely, overexpression of Slit leads to lateral displacement of longitudinal tracts away from the midline. Thus Slit can define the distance between axons and the midline. Different axon bundles occupy medial, intermediate or lateral positions with respect to the midline. The position of a given tract is determined largely by its particular complement of three different Robo receptors, Robo, Robo2 and Robo3 (Fig. 3b)^{88–91}. The three receptors are expressed in partially overlapping subsets of medial, intermediate and lateral tracts (Fig. 3b). Robo is expressed on all axons, and it acts primarily to keep the medial axons from crossing the midline. Disruption of intermediate and laterally expressed Robo2 and Robo3 shifts the corresponding axon tracts to a medial position. Ectopic expression of laterally expressed Robo2 or Robo3 in medial axons drives them to a lateral position. Thus the different Robos have different biological activities, which may reflect different affinities for Slit or the formation of heteromeric complexes with different signaling potencies. These findings demonstrate that the expression of particular Robos can confer graded responsiveness to a single Slit guidance cue.

Drosophila midline guidance is modulated by another set of transmembrane proteins, the receptor protein tyrosine phosphatases (PTPs)⁶¹. It is possible that these act as part of the combinatorial Robo receptor complex. A conserved tyrosine residue in Robo has significant effects on Robo signaling and has been proposed as a site of PTP regulation⁶⁰. However, the PTPs act in many guidance decisions, so—as mentioned above for L1-CAM and semaphorin signaling—their action at the midline could be an indirect effect mediated through axon fasciculation or other mechanisms, not only through Robo.

Interactions between different receptors in a signaling complex can lead to the dramatic conversion of a guidance response from attraction to repulsion. Netrin is a bifunctional axon guidance signal that can mediate either attraction or repulsion. The transmembrane receptor DCC is the major conserved receptor involved in attraction to netrin⁹. DCC binds netrin directly⁷⁵, although DCC expression is not always sufficient for a response to netrin⁹². A second proposed netrin receptor, the A2B adenosine receptor, seems to bind netrin but is not essential for the netrin response^{75,93}.

Repulsion from netrin primarily depends on the action of the UNC-5 transmembrane receptor, which is structurally unrelated to DCC^{9,94}. UNC-5 also binds directly to netrin⁹⁵. Ectopic expression of UNC-5 can drive repulsion not only in cells that were formerly netrin-insensitive⁹⁶ but also in cells that were formerly attracted to netrin via DCC^{96,97} (Fig. 3c). This conversion to repulsion is the result of direct interactions between DCC and UNC-5 cytoplasmic domains that are induced by binding to netrin. In the new UNC-5/DCC receptor complex, the attractive function of DCC is silenced. Instead, the DCC protein potentiates

the UNC-5 repulsive response^{9,97,98}, although DCC is not as important as UNC-5 for repulsion⁹. These results demonstrate that inclusion of different receptor subunits in a complex can modulate not only the strength of the guidance response but also its polarity.

Conversion from attraction to repulsion is observed in several other netrin signaling systems. Lowering calcium or cAMP levels or adding laminin-1 fragments to a bath can switch *Xenopus* neurons from attraction to repulsion by netrin^{62,64,99}. However, the molecular targets of calcium, cAMP and laminin are unknown. The UNC-5/DCC example is the first to demonstrate a direct interaction between guidance receptors that changes their signaling properties.

Multiple guidance cues can be arranged in opposing or complementary patterns, and a growth cone must prioritize its responses to these cues. Heteromeric receptor complexes also provide the possibility of integration, where information from several guidance cues can be sorted out in a single decision. Several recent observations suggest that interactions between receptors can dictate the response to guidance cues. Experiments in *Xenopus* neurons provide evidence of hierarchical integration of guidance information. Stage 22 *Xenopus* spinal neurons grow toward netrin-1, but when Slit is applied, the growth cones lose their ability to turn towards netrin¹⁰⁰. This silencing of netrin responses occurs through direct binding of Robo to DCC that is induced by Slit (Fig. 3d). Interestingly, the Robo-DCC complex has properties that are not predictable from the individual responses of DCC and Robo. First of all, stage 22 *Xenopus* neurons are not repelled by Slit, so for these neurons Slit-Robo function is manifested only as a modification of the netrin response. Second, Slit silences only the turning response to netrin: netrin stimulates outgrowth of stage 22 neurons whether Slit is present or not. The signaling complex has a set of properties that are related to its components, but are more than just the sum of the parts.

Later in development, stage 28 *Xenopus* neurons respond to Slit, but not netrin. Unlike stage 22 neurons, they are repelled by Slit. This observation suggests that the interactions between guidance receptors are themselves regulated by other factors. A third situation has been observed in *C. elegans*, where genetic analysis suggests that DCC binds to Robo and potentiates Robo signaling in a netrin-independent fashion (Fig. 3d) (T. W. Y. *et al.*, unpublished data). Receptor interactions can thus lead to cooperative guidance functions that depend on either of the normal ligands of the receptors.

A different kind of silencing has been described in the cerebellum, where the G protein-coupled receptor CXCR4 mediates attractive guidance towards the chemokine SDF-1 during granule cell migration¹⁰¹. The activity of the chemokine receptor is inhibited by 'reverse' ephrin signaling, in which EphB receptors signal back to the transmembrane ephrin-B protein. Ephrin-B binds to a regulator of G protein signaling (RGS) protein and silences chemokine receptor signaling, presumably by inactivating G proteins through the RGS protein (Fig. 3d). In this case, silencing involves an interaction between two downstream signaling molecules, not a direct interaction between receptors.

Mechanistic implications

Guidance receptors signal as multimeric complexes, and the composition of guidance complexes can define ligand interactions and the nature of guidance responses. Some receptors seem to be relatively specific for a particular function: for example, UNC-5

is only known to act in netrin repulsion. Other receptors seem to be more promiscuous, interacting with multiple partners in a variety of signaling events: DCC is involved in netrin attraction, netrin repulsion, netrin presentation and Robo signaling, and neuropilins act in semaphorin signaling and vascular endothelial growth factor (VEGF) signaling.

The existence of combinatorial signaling mechanisms is a fascinating aspect of axon guidance, but it should not be taken to mean that everything signals through everything. Both genetic and biochemical evidence strongly implicate DCC in attractive netrin signaling, but its involvement in other guidance processes is usually more subtle—suggestions rather than demands. Communication between guidance molecules is valuable because it modulates their function in a context, not because it supplants their specificity.

Why should neurons use multifunctional receptor proteins such as DCC and neuropilin during axon guidance? First, as mentioned above, they provide the opportunity to link responses to different ligands in a combinatorial fashion. Second, they could provide biochemical activities that potentiate the functions of other proteins. One receptor subunit might stabilize the expression, subcellular localization or ligand-binding properties of other receptor subunits. Neuropilins may be a candidate for this sort of activity. Alternatively, one receptor might provide specific intracellular signaling motifs that are useful in many guidance decisions. For example, DCC stimulates neurite outgrowth, and might add an outgrowth-stimulatory activity to several different complexes by activating a signaling module.

Conclusion

The regulation of guidance receptors and ligands allows a single guidance system to generate a variety of different responses. Some regulatory mechanisms, such as transcriptional regulation, are characteristic of all guidance systems. Others, such as regulated ligand cleavage, have only been described in a single case but may be more widespread. The identification of these regulatory pathways will facilitate the next step in understanding axon guidance—determining how guidance molecules function together in a biological context.

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