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REVIEW

# Molecular Mechanisms of Axon Guidance

**Barry J. Dickson**

**Axons are guided along specific pathways by attractive and repulsive cues in the extracellular environment. Genetic and biochemical studies have led to the identification of highly conserved families of guidance molecules, including netrins, Slits, semaphorins, and ephrins. Guidance cues steer axons by regulating cytoskeletal dynamics in the growth cone through signaling pathways that are still only poorly understood. Elaborate regulatory mechanisms ensure that a given cue elicits the right response from the right axons at the right time but is otherwise ignored. With such regulatory mechanisms in place, a relatively small number of guidance factors can be used to generate intricate patterns of neuronal wiring.**

The correct wiring of the nervous system relies on the uncanny ability of axons and dendrites to locate and recognize their appropriate synaptic partners. To help them find their way in the developing embryo, axons and dendrites are tipped with a highly motile and exquisitely sensitive structure, the growth cone. Extracellular guidance cues can either attract or repel growth cones, and can operate either at close range or over a distance (*1*). By responding to the appropriate set of cues, growth cones are able to select the correct path toward their target.

Ten years ago (*2*), very few of the molecules that guide axons in vivo were known. But the 1970s and '80s had seen the introduction of several powerful in vitro assays to detect guidance activities in the developing vertebrate nervous system, and the growing interest of invertebrate geneticists in the problem of axon guidance. So by the early 1990s, the stage had been set for a burst of activity that led to the discovery of several conserved families of axon guidance molecules. Prominent among these are the netrins, Slits, semaphorins, and ephrins (Fig. 1). These are not the only known guidance molecules, but they are by far the best understood. With these molecules in hand, we can now begin to ask how growth cones sense and respond to guidance cues, and how a relatively small number of cues can be used to assemble complex neuronal networks.

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## Guidance Cues and Their Receptors

*Netrins.* The discovery of netrins came as the remarkable convergence of the search for a chemoattractant for vertebrate commissural axons (*3*, *4*), and the analysis of genes required for circumferential axon guidance in *Caenorhabditis elegans* (*5*, *6*). Across more than 600 million years of evolution, netrins have retained the function of attracting axons ventrally toward the midline (*7*). Netrins can also repel some axons, and this function too has been conserved. This was initially inferred from defects in dorsal as well as ventral guidance in *unc-6/netrin* mutant worms (*5*), and subsequently confirmed by the direct demonstration of netrin's repulsive activity in vertebrates (*8*) and in flies (*9*, *10*).

Identification of the netrin receptors followed from the characterization of two other worm mutants with defects in circumferential guidance: *unc-40*, which primarily disrupts ventral guidance; and *unc-5*, which affects only dorsal guidance (*5*). Both *unc-40* and *unc-5* encode conserved transmembrane proteins (*7*), with UNC-40 belonging to the DCC (deleted in colorectal carcinoma) family. Biochemical and genetic studies have confirmed their functions as netrin receptors in several different species (*7*, *10*). DCC receptors mediate attraction to netrins but can also participate in repulsion. UNC-5 receptors appear to function exclusively in repulsion, either alone or in combination with DCC receptors. UNC-5 receptors may require a DCC coreceptor for repulsion farther away from the netrin source, where ligand concentration is likely to be lower (*5*, *10*). This may involve a direct interaction between the cytoplasmic domains of the two receptors (*11*).

Netrins guide many different axons in vivo. In some cases, netrin can exert its effects from distances of up to a few millimeters (*12*), but in others it appears to act only at short range (*9*). Netrins have high affinity for cell membranes (*3*, *4*), and it is unclear how far they can diffuse in vivo and how their diffusion is regulated. Indeed, a netrin gradient has not yet been visualized directly in any system, and formal proof that netrin must diffuse away from its source to exert its long-range effects is lacking.

*Slits.* Slits are large secreted proteins that signal through Roundabout (Robo) family receptors. Robo was first identified in a genetic screen for midline guidance defects in *Drosophila* (*13*, *14*). Genetic studies suggested that Robo is the receptor for a midline repellent (*14*), subsequently identified as Slit (*15*, *16*). This repulsive action of Slit was found to be conserved in vertebrates (*17*, *18*). However, in a parallel approach, Slit was also purified as a factor that stimulates sensory axon branching

and elongation (*19*). Thus, Slits, like netrins, are multifunctional. The importance of Slit-Robo signaling in axon guidance and cell migration is underscored by the recovery of *robo* mutations in genetic screens for guidance defects in at least three different species (*13*, *20*, *21*) and the purification of Slit in at least three independent biochemical assays (*19*, *22*, *23*).

The best-understood functions of Slit proteins are in midline guidance in *Drosophila* and in the formation of the optic chiasm in vertebrates. In *Drosophila*, Slit is expressed at the ventral midline, where it acts as a short-range repellent signaling through Robo to prevent ipsilateral axons from crossing the midline and commissural axons from recrossing (*15*, *16*). Two other Slit receptors, Robo2 and Robo3, specify the lateral positions of axons that run parallel to the midline, presumably in response to a long-range gradient of Slit activity diffusing away from the midline (*24*, *25*).

Vertebrate Slit proteins are also expressed by ventral midline cells (*17*), and commissural axons are repelled by Slit after they have crossed the midline (*26*). Mice deficient for both Slit1 and Slit2 lack any obvious defects in midline guidance in the spinal cord (*27*), but Slit3 is still expressed at the midline in these mice.

Slit1/2-deficient mice do have striking defects in the formation of the optic chiasm, where Slit3 is not expressed (*27*). These defects are strikingly reminiscent of those seen in *astray/robo2* mutant fish, in which retinal axons make multiple guidance errors before, during, and after crossing the midline (*21*). Similar errors also occur in wild-type fish but are always corrected (*28*). In fish, all retinal axons project contralaterally, but in mice, which have binocular vision, some axons project contralaterally and others ipsilaterally. By analogy to the role of *Drosophila* Slit in midline guidance, it was anticipated that the vertebrate Slit proteins might be expressed at the chiasm and control the choice of an ipsilateral or contralateral projection. This is not the case (*29*). Instead, Slit1 and Slit2 are expressed by cells surrounding the chiasm and repel ipsilateral and contralateral axons alike (*23*, *27*, *30*, *31*). This has led to the idea that Slits form a repulsive corridor to guide all retinal axons through the chiasm.

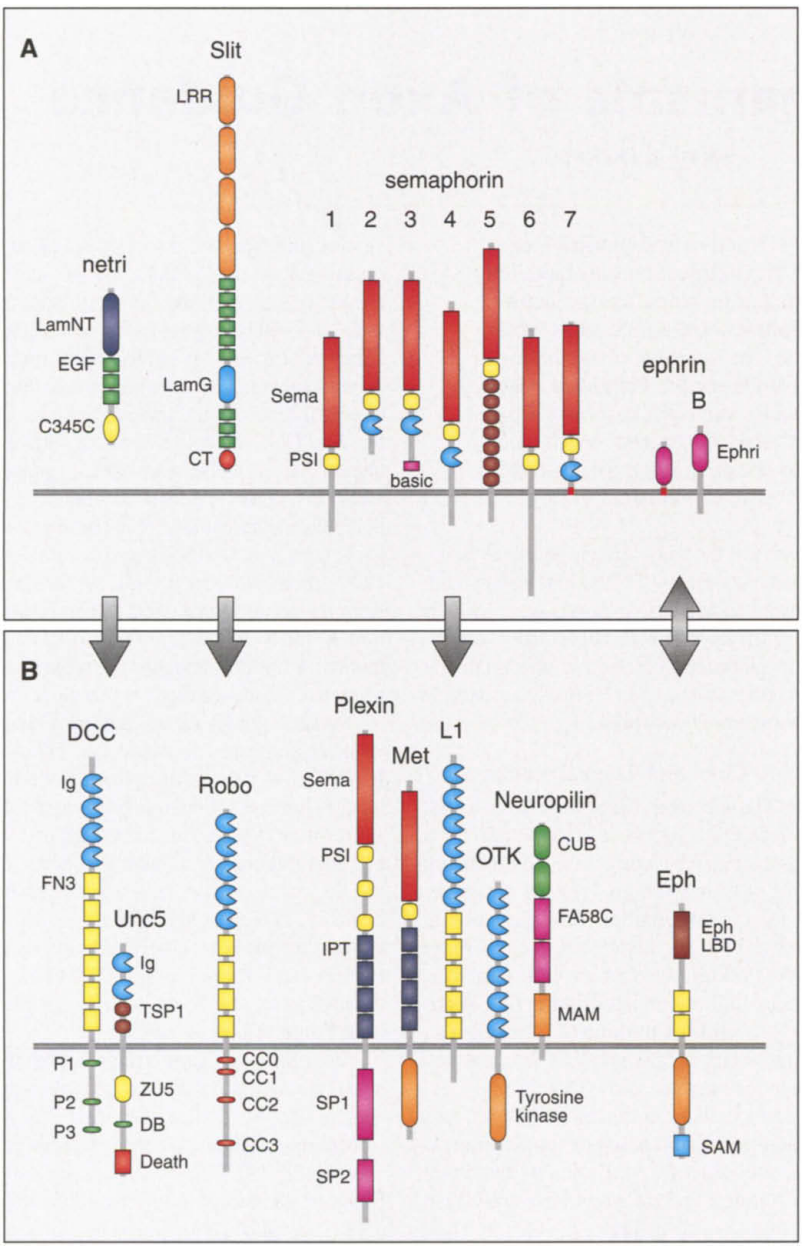


**Fig. 1.** Conserved families of guidance molecules (**A**) and their receptors (**B**). Domain names are from SMART (http://smart.embl-heidelberg.de). P1 to P3, DB (DCC-binding), CC0 to CC3, and SP1 and SP2 indicate conserved regions in the cytoplasmic domains of DCC, UNC-5, Robo, and Plexin receptors, respectively.

*Semaphorins.* Semaphorins are a large family of cell surface and secreted guidance molecules, defined by the presence of a conserved ~420–amino acid Sema domain at their $NH_2$-termini. The first semaphorins were identified by searching for molecules expressed on specific axon fascicles in the grasshopper central nervous system (CNS) (*32*) and by purifying a potent inducer of vertebrate sensory growth cone collapse in vitro (*33*). Semaphorins are divided into eight classes, on the basis of their structure. Classes 1 and 2 are found in invertebrates, classes 3 to 7 are found in vertebrates, and class V semaphorins are encoded by viruses (*34*).

Semaphorins signal through multimeric receptor complexes. The composition of these receptor complexes is not fully known. Many, and perhaps all, semaphorin receptor complexes include a plexin protein. Plexins comprise a large family of transmembrane proteins divided into four groups (A to D), on the basis of sequence similarity (*35*). *Drosophila* PlexinA is a functional receptor for the transmembrane Sema1a (*36*), vertebrate plexin-As are functional receptors for secreted class 3 semaphorins (*35*, *37*), and other plexins bind directly to semaphorins of different classes (*35*, *38*, *39*). Receptor complexes for the vertebrate class 3 semaphorins also include neuropilins, which bind directly to both semaphorins and plexins (*34*). Neuropilins do not appear to have a signaling function, but rather contribute to ligand specificity. Other essential components of semaphorin receptor complexes include the neural cell adhesion molecule L1 (for Sema3A) (*40*), the receptor tyrosine kinase Met (for Sema4D) (*41*), and the catalytically inactive receptor tyrosine kinase OTK (for *Drosophila* Sema1a) (*42*).

Genetic analysis of semaphorin function in flies and in mice suggests that they primarily act as short-range inhibitory cues that deflect axons away from inappropriate re-

gions, or guide them through repulsive corridors (*34*, *37*). Evidence suggests that semaphorins may also act as attractive cues for certain axons (*34*, *43*), although this remains to be verified by genetic analysis. Interestingly, semaphorins do not seem to function in axon guidance in *C. elegans*, but instead have an analogous role in discouraging inappropriate cell contacts. Worms have three semaphorin and two plexin genes, all of which have been mutated (*44*–*46*). In these mutants, epidermal cells that should only transiently contact one another instead make more perdurant contacts.

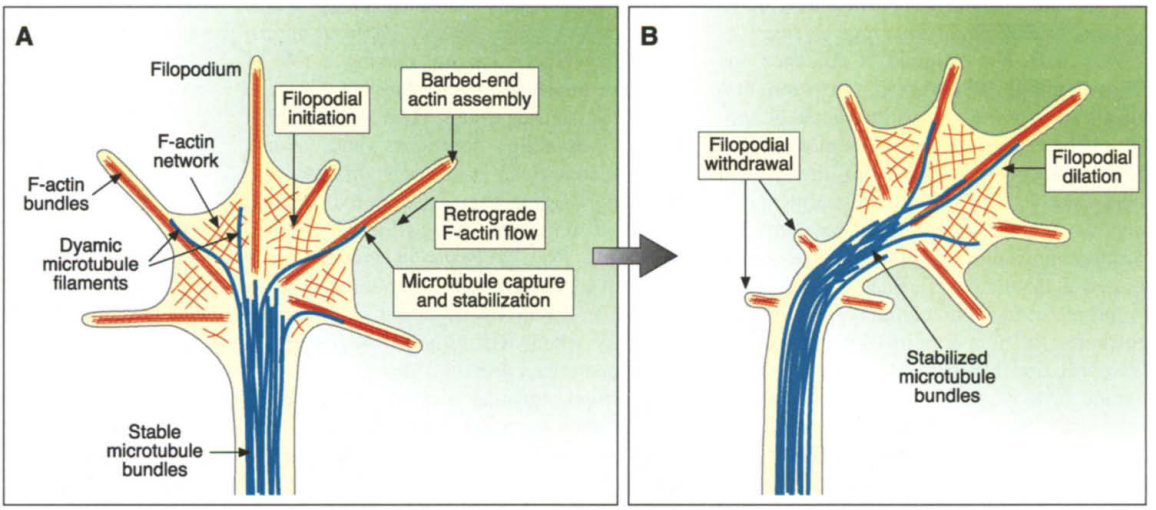


**Fig. 2. (A and B)** A model showing one way in which a growth cone might turn toward an attractant (green).

*Ephrins.* In a classic paper (*47*), Sperry postulated that vertebrate retinal axons are guided to their appropriate topographic locations in the optic tectum by an orthogonal system of molecular gradients in the retina and the tectum. The search for these graded cues led to the identification of the ephrins, membrane-bound ligands for the Eph family of receptor tyrosine kinases (*48*, *49*). Ephrins and Eph receptors fall into two classes: ephrin-As, which are anchored to the membrane by a glycosylphosphatidylinositol (GPI) linkage and bind EphA receptors; and ephrin-Bs, which have a transmembrane domain and bind EphB receptors (*50*).

In the visual system, topographic mapping of retinal axons along the anterior-posterior axis depends on repulsion mediated by ephrin-A ligands and their EphA receptors (*50*). Ephrin-A ligands are expressed in a gradient in the tectum [or its mammalian equivalent, the superior colliculus (SC)], and EphA receptors are expressed in a complementary gradient in the retina. Retinal axons with successively higher EphA levels map to successively lower points along the ephrin-A gradient. If the ephrin-A gradient is eliminated in the mouse SC, then retinal axons do not all shift to one end of the SC, as would be expected if each retinal axon simply mapped to a specific threshold value on the ephrin-A gradient. Instead, retinal axons still fill the entire SC, but their topographic order is disrupted—some axons shift posteriorly and others anteriorly (*51*). This suggests that the ephrin-A gradient establishes the topographic order of retinal axons, but not their precise termination sites. Further support for this model comes from a clever genetic experiment in which half the retinal axons were forced to express higher levels of an EphA receptor (*52*). Those axons with extra EphA receptors shifted down the ephrin-A gradient, whereas those with only their endogenous levels shifted up the gradient. The result was two smooth maps, one in each half of the SC. The conclusion is that the mapping of retinal axons depends on their relative EphA levels, not their absolute levels.

Mapping along the dorsal-ventral axis, in contrast, involves attractive signaling mediated by ephrin-B ligands and EphB receptors (*53*, *54*). Correct mapping of retinal axons along this axis evidently requires both "forward" signaling, in which ephrin-B ligands activate EphB receptors, and "reverse" signaling, in which EphBs serve as ligands to signal back through the transmembrane ephrin-Bs.

Ephrins control axon guidance in many other places too, and the ability to signal in either direction is a common theme, as is the ability to mediate either attraction or repulsion (*50*). For example, ephrin-B reverse signaling repels forebrain commissural axons away from regions of EphB expression (*55*) while attracting them to regions of EphA4 expression (*56*). The GPI-anchored ephrin-As are also able to signal in the reverse direction (*57*) and may act in this mode to mediate attraction or adhesion during mapping of vomeronasal axons to the accessory olfactory bulb (*58*).

Mammals have 13 Eph receptors and 8 ephrins. Worms and flies both have just a single Eph receptor, with four and one ephrin ligands, respectively. Somewhat surprisingly, the invertebrate ephrin and Eph mutants do not have dramatic axon guidance defects (*59*–*63*). The *C. elegans* ephrins and the Eph receptor do, however, have critical functions in multiple aspects of epithelial morphogenesis, as do their vertebrate counterparts (*50*). It seems that ephrins and Eph receptors are an ancient but versatile system for cell-cell communication that has diversified and acquired its axon guidance functions primarily during vertebrate evolution.

## Steering the Growth Cone

*Cytoskeleton.* Growth cone turning is a complex process in which actin-based motility is harnessed to produce persistent and directed microtubule advance (Fig. 2). Actin filaments are organized into two distinct populations: dense, parallel filaments that radiate outward and into filopodia; and intervening networks of loosely interwoven filaments (*64*). Filopodial filaments are oriented with their fast-growing barbed ends toward the filopodium tip. The extension and retraction of a filopodium reflect the balance between the polymerization of actin at barbed ends and the retrograde flow of entire filaments (*65*–*67*). Filopodia often extend asymmetrically before the entire growth cone turns (*68*–*70*), and without filopodia, growth cones become disoriented (*69*, *71*, *72*). The precise role of filopodia in growth cone turning remains unclear, but they have been postulated to steer the growth cone by differential adhesion (*73*), generating mechanical force (*74*), or transducing distal signals (*75*).

Microtubules form stable, cross-linked bundles in the axon shaft. Single microtubule filaments also emerge into the growth cone. These filaments display the classic properties of dynamic instability, extending and retracting as they explore the peripheral region of the growth cone (*76*). These dynamic microtubules grow preferentially along the filopodial actin filaments (*76*, *77*), and the capture or stabilization of microtubule bundles in a specific filopodium may be a critical event in growth cone turning. Consistent with this view, stabilization and dilation of a single filopodium appear to be a common feature of growth cone turning in vivo (*78*–*80*).

There are many different ways in which a guidance signal might intervene to steer the growth cone. For example, a guidance cue might promote the initiation, extension, stabilization, or retraction of individual filopodia, or the capture or stabilization of microtubules in specific regions of the growth cone. Likely targets for the signaling pathways downstream of guidance receptors are therefore molecules such as Arp2/3 (to nucleate new actin filaments), Ena/VASP proteins (to promote filament elongation), adhesion molecules (to couple actin filaments to the substrate), and myosins (to regulate the retrograde flow of actin filaments). Molecules that capture microtubule ends (e.g., IQGAP1) or suppress microtubule instability (e.g., MAP1B) are also potential targets for guidance signals. We still need to determine which aspect(s) of actin or microtubule dynamics are the primary targets for regulation by each of the known guidance cues. It is difficult to trace the signaling pathways downstream of a guidance receptor without knowing what lies at the "business end."

*Signaling.* With their well-known roles in regulating cytoskeletal dynamics in fibroblasts, Rho guanosine triphosphatases (GTPases) were strong candidates to transduce guidance signals in the growth cone. A function for Rho GTPases in growth cone guidance was suggested from studies with dominant mutant isoforms (*81*) and was confirmed by the analysis of loss-of-function mutations in flies and worms (*82–85*). Biochemical links have also been made between several guidance receptors and Rho GTPases. For example, EphA receptors regulate the guanine nucleotide exchange factor (GEF) Ephexin (*86*); Robo receptors may act at least in part by regulating GTPase-activating proteins (GAPs) (*87*); and Plexins bind directly to Rho GTPases (*88*) and Rho GEFs (*39*), and may even have intrinsic GAP activity (*89*). Several downstream effectors of Rho GTPases have also been implicated in axon growth and guidance, such as Pak (*90*) and Rho kinase (*91*).

Genetic studies have also revealed important roles for Ena/VASP proteins in axon guidance (*92–94*). These proteins antagonize capping proteins to promote actin filament elongation (*95*). In motile fibroblasts, Ena/VASP proteins localize to the leading edge of lamellipodia. Depletion of Ena/VASP proteins from the leading edge leads to shorter, more highly branched filaments that generate greater protrusive force and increased motility. Conversely, increasing Ena/VASP levels at the leading edge results in longer, unbranched filaments and reduced motility (*95*, *96*). Genetic studies implicating Ena/VASP proteins in repulsive growth cone guidance by both Slit (*94*) and netrin (*92*) have been interpreted in light of this negative role in fibroblast motility. However, in growth cones, Ena/VASP proteins localize to filopodial tips (*97*), where actin filaments are normally unbranched and stable. Here, their activity would be expected to promote filopodial extension, making a role in attractive guidance equally plausible.

These considerations raise an important point. Migrating fibroblasts and axonal growth cones can have very different cytoskeletal organizations, and the location and action of molecules such as Ena/VASP proteins and Rho GTPases in growth cones cannot be inferred merely by analogy to fibroblasts. It will be important to determine precisely when, where, and how these proteins function in growth cones.

Calcium signaling may also play an important role in growth cone turning. In cultured *Xenopus* spinal neurons, turning in response to a netrin-1 gradient requires calcium influx through the plasma membrane, as well as calcium release from intracellular stores (*98*). Moreover, netrin-1 induces a transient $Ca^{2+}$ gradient in the growth cone (*98*), and the creation of such a gradient by local photolysis of caged $Ca^{2+}$ or release from intracellular stores is sufficient to induce turning in the absence of netrin-1 (*98*, *99*). Spontaneous calcium transients have also been observed in growth cones (*100*) and in filopodia (*101*). The frequencies of these transients appear to correlate negatively with growth cone extension rates, but compelling evidence of their involvement in growth cone turning in vivo is lacking.

## Plasticity of Guidance Responses

Axons can evidently differ in their response to the same cue, as they must if they are to follow divergent pathways. But even a single growth cone may need to respond to the same cue in different ways at different points along its journey. This is particularly true if the growth cone is to navigate through a series of intermediate targets before reaching its final goal, as many do. Specifying an axon's trajectory is therefore not just a simple matter of selecting the appropriate set of guidance receptors and delivering them to the growth cone. The growth cone must also be able to modulate its responsiveness en route. Some of the mechanisms underlying this plasticity have recently come to light.

*Modulation by cyclic nucleotides.* In vitro, the responses of *Xenopus* spinal axons can be modulated by changing the levels of cyclic nucleotides (*102–104*). Responses to some guidance cues, including netrin-1, are sensitive to levels of cAMP or protein kinase A (PKA) activity, while others, including Sema3A, are modulated by cGMP and protein kinase G (PKG). The general finding is that lowering cAMP or cGMP levels or inhibiting PKA or PKG, converts an attractive response to a repulsive one, whereas elevating cAMP or cGMP, or activating PKA or PKG, switches repulsion to attraction.

Modulation of netrin-1 responsiveness by cAMP levels may play an important role in pathfinding of *Xenopus* retinal axons to the tectum (*105*). These axons are first attracted out of the eye by netrin-1 at the optic nerve head, become indifferent to it as they then grow through the ventral diencephalon, and finally are repelled by netrin-1 once they reach the tectum. These changes correlate with a gradual decline in cAMP levels and can be reversed by artificially raising cAMP levels. An intriguing variation on this theme has been documented in the mammalian cortex (*106*). Sema3A attracts the apical dendrites of pyramidal neurons toward the cortical plate but repels their axons away from it. Interestingly, a guanylyl cyclase is specifically localized in dendrites, implying that cGMP levels may be higher in dendrites than in axons.

*Local translation in the growth cone.* Applying netrin-1 or Sema3A to cultured *Xenopus* retinal axons induces local protein synthesis within the growth cone, and blocking translation inhibits the turning but not the growth of these axons (*107*). Induced protein synthesis is rapid enough to contribute directly to growth cone steering, but work on *Xenopus* spinal axons suggests a more subtle role: Growth cones might need to synthesize new proteins to maintain their sensitivity as they migrate up or down a ligand gradient (*108*). Spinal growth cones undergo consecutive phases of desensitization and resensitization to netrin-1 in vitro, and resensitization requires protein synthesis. Inhibiting translation in spinal axons does not block turning toward the netrin-1 source, as it does in retinal axons (*107*), but actually causes turning away from it (*108*). This is difficult to explain if translation has a direct role in growth cone turning, but could be explained by a role in resensitization: If desensitization is more rapid on the side of the growth cone facing toward the source, then a failure to synthesize the new proteins needed for resensitization could result, paradoxically, in a stronger attractive signal on the side facing away from the source.

Local translation might also be used to completely switch the growth cone's responsiveness to specific cues once it reaches an intermediate target. Evidence for such a mechanism comes from the finding that the 3′-untranslated region of the *EphA2* mRNA contains a sequence that confers selective translation in the distal segments of commissural axons, after they have crossed the midline (*109*). This could explain why the EphA2 receptor is only expressed at high levels in the segments of these axons that extend beyond the midline. The implication is that commissural axons might become sensitive to the ephrinA ligands in the spinal

cord only after crossing, although this remains to be tested.

*Switching responses at the midline*. To reach their targets on the contralateral side of the CNS, commissural axons must first grow toward the midline, but then leave it again on the opposite side and never turn back. Experiments in rodents, chicks, and flies have suggested a simple model for this behavior, in which commissural growth cones switch their sensitivity to midline attractants and repellents as they cross (Fig. 3). Before crossing, commissural axons are attracted to the midline by netrin (*3*, *4*) but are insensitive to the midline repellents Slit and, in vertebrates, certain class 3 semaphorins (*17*, *26*). After crossing, these axons are insensitive to netrins (at least in the vertebrate hindbrain) (*110*) but are repelled by both Slits and semaphorins (*26*). What turns attraction off and repulsion on at the midline?

One way in which netrin attraction could be turned off is by exposure to Slit. This is suggested by studies on cultured *Xenopus* spinal neurons (*111*). Young spinal axons in vitro, like precrossing commissural axons in vivo, are attracted by netrin and are unresponsive to Slit. However, when both cues are applied simultaneously, netrin can still stimulate axon growth but not turning. This is not just a simple matter of repulsion canceling out attraction, because these axons are not repelled by Slit at all, and other attractive responses are not affected. Thus, Slit specifically silences attraction by netrin. This silencing effect is mediated by a direct interaction between the cytoplasmic domains of the Robo and DCC receptors (*111*).

This could explain how attraction by netrin is shut down at the midline, but what turns Slit repulsion on? In flies, Robo receptors are expressed at high levels on commissural axons only after crossing, even though *robo* mRNA is expressed early on (*14*). Robo protein is also synthesized before crossing, but an intracellular sorting receptor (Comm) apparently prevents it from being delivered to the growth cone, targeting the newly synthesized Robo instead for lysosomal degradation (*112, 113*). Once a commissural axon has crossed the midline, Comm appears to be inactivated, possibly by both transcriptional and posttranscriptional mechanisms. This allows Robo to be delivered to the growth cone, thereby conferring sensitivity to Slit. Thus, it is not the local synthesis or activity of the Robo receptor that is regulated in *Drosophila* commissural axons, but rather its intracellular trafficking. This mechanism may also apply to Robo2 and Robo3. These two Slit receptors are also down-regulated during midline crossing, but must be up-regulated after crossing for axons to select their appropriate pathways on the contralateral side (*24*, *25*).

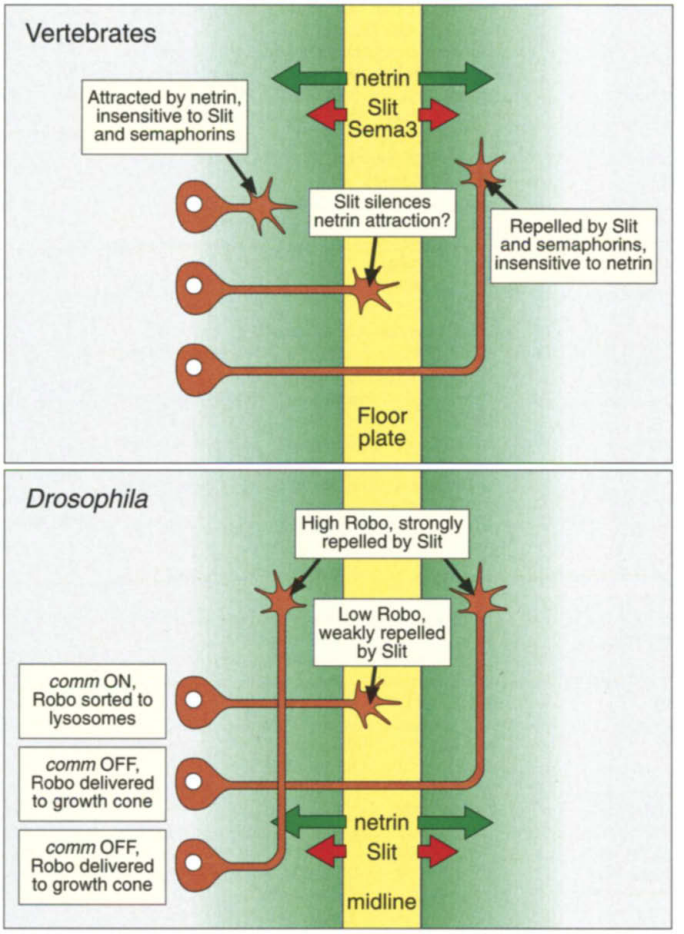


**Fig. 3.** Switching sensitivity at the midline. As they cross the floor plate, vertebrate commissural axons lose sensitivity to the midline attractant, netrin, and acquire sensitivity to Slit and semaphorin repellents. This switch may be mediated in part by silencing of netrin attraction by Slit. *Drosophila* commissural axons also become sensitive to Slit only after crossing. This appears to reflect Comm's role in regulating the intracellular trafficking of Robo.

## Concluding Remarks

Netrins, Slits, semaphorins, and ephrins are not the only guidance cues we know of, and many more undoubtedly still await discovery. Nevertheless, members of these four families have turned up repeatedly in various genetic and biochemical assays and have been found to control a wide range of guidance decisions in vivo. How can so few molecules contribute so much to the correct wiring of the nervous system? Two related principles emerging from these studies seem to be important. First, guidance cues are multifunctional. A single cue can either attract or repel axons, at short or long range, and may even elicit other responses such as branching or an altered sensitivity to other cues. Second, growth cone responses are remarkably plastic, subject to modulation by both instrinsic and extrinsic factors. Together, these mechanisms may underlie much of the diversity in growth cone behavior.

What are the major challenges that still lie ahead? One will be to identify more guidance factors, in particular those that may have more specialized functions, and to figure out how they work. Another challenge will be to gain a better picture of how guidance cues steer growth cones. We now have a few tantalizing glimpses, but are still a long way from a coherent view of growth cone turning. Also, having learned that the outcome of a particular signaling event is essentially unpredictable, the need is now greater than ever to push ahead with the analysis of guidance mechanisms in vivo. We need to know, for example, how the distributions of the various guidance molecules are controlled in space and time, and how each growth cone knows when and how to respond to these cues. The ultimate challenge, after all, is to find out how a comparatively small number of guidance molecules generate such astonishingly complex patterns of neuronal wiring.

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