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Signal transduction in axon guidance

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Abstract. Neurons establish specific connections by extending projections to contact their targets. Projections, such as axons, navigate to the target by sensing guidance cues in their environment and responding with directed movement and shape change. The recent identification of the molecular identities of many guidance cues and guidance receptors has demonstrated that axons are guided to their targets by combinations of cues that attract and repel them. The current challenge is to elucidate how these guidance cue/receptor interactions control navigation. This review focuses on recent progress in identifying the signaling pathways downstream of these receptors and in determining why an axon is attracted or repelled by a particular guidance cue.

Key words. cAMP; cytoskeleton; growth cone; IgCAM; tyrosine kinase; Rho-GTPase.

Axon guidance signaling pathways regulate growth cone motility

Axon navigation is carried out largely in the growth cone at the leading tip of the axon [1, 2]. The growth cone senses guidance cues in the environment through guidance cue receptors and responds by undergoing cytoskeletal changes that determine the direction of axon growth. These cytoskeletal changes are essential for navigation, as disrupting them disrupts guidance [3]. Because of the important role of cytoskeletal changes in guidance, the growth cone cytoskeleton is thought to be a major target of axon guidance signaling pathways.

The growth cone cytoskeleton is highly structured and dynamic. The growth cone's leading edge resembles a hand with multiple finger-like protrusions (filopodia) emerging from web-like veils of cytoplasm (lamellipodia). These structures are actin based: filopodia contain tight bundles of actin filaments, while lamellipodia contain a meshwork of actin filaments. Behind the leading edge, the base of the growth cone and the axon shaft are rich in microtubules which are used, at least in part, to consolidate guidance decisions made at the leading edge. As the growth cone navigates, filopodia and lamellipodia continually extend and retract as they sense and respond to guidance cues. Likewise, microtubules grow, shrink, and are moved as they explore the growth cone interior. The dynamic behavior of these filaments is central to guidance [3]. For example, contact between a filopodium and an attractive surface can trigger filopodium stabilization and expansion. Within minutes, the signals initiated by this single filopodial contact can serve to completely reorient the direction of growth cone movement as the stabilized filopodium is invaded by microtubules and transformed into an axon shaft [4]. The leading edge then moves on from this location to explore new regions. Guidance cues and receptors control guidance by controlling where and when such cytoskeletal remodeling occurs [5].

Actin dynamics are targets for guidance cue regulation

The dynamic behavior of filopodia and lamellipodia essential for axon guidance depends upon actin filament assembly and translocation (fig. 1). Recent work suggests that guidance cues can influence growth cone behavior by regulating these processes. For example, treating a growth cone with a high dose of a repulsive cue can cause it to collapse and retract, and this collapse has been associated with a loss of actin filaments from the leading edge [6]. Recent work shows that collapse stimulated by the Semaphorin III guidance cue is blocked by treatments which appear to interfere with the ability of Semaphorin III to influence actin dynamics [7].

Numerous cellular factors have been implicated in regulating actin filament assembly and disassembly, and some of these undoubtedly play important roles in growth cone motility. For example, one such set of molecules is the Ena protein family, initially implicated in axon guidance through genetics in *Drosophila*. A mouse homolog of Ena, Mena, promotes actin filament assembly when overexpressed in fibroblasts and interacts physically with the actin-monomer-binding protein profilin [8]. Such signal-responsive regulators of filament assembly make excellent candidates for downstream effectors of guidance receptor signaling cascades.

In addition to filament assembly, the translocation of actin filaments within the growth cone can also be regulated. One important aspect of actin filament movement is the phenomenon of retrograde flow: the tendency of actin filaments to translocate from the leading edge of the growth cone toward the central domain [9]. A possible linkage between retrograde flow and growth cone extension was suggested by the observation that slowing retrograde flow in aplysia bag cell growth cones using inhibitors of myosins stimulated the rapid extension of filopodia [10]. Although regulating retrograde flow would be an attractive way to control gowth cone motility, the degree to which it is used to control axon guidance decisions in vivo is not known. Forscher and coworkers have suggested that some guidance receptors could act by coupling actin filaments to the substrate on which the growth cone is traveling. This would slow filament translocation toward the growth cone base, promoting the net protrusion of filaments and thus growth cone extension [reviewed in ref. 11].

Actin dynamics are likely to be a major target of guidance receptor signaling pathways. However, other potential targets within the growth cone may also be important, such as microtubules and the secretory machinery [5]. Furthermore, signaling need not be confined to the growth cone. For example, guidance cue receptor activation could also alter gene expression and change the repertoire of guidance machinery in the axon. This is especially intriguing given the well-established roles of both local and nuclear-mediated events in synaptic plasticity [12].

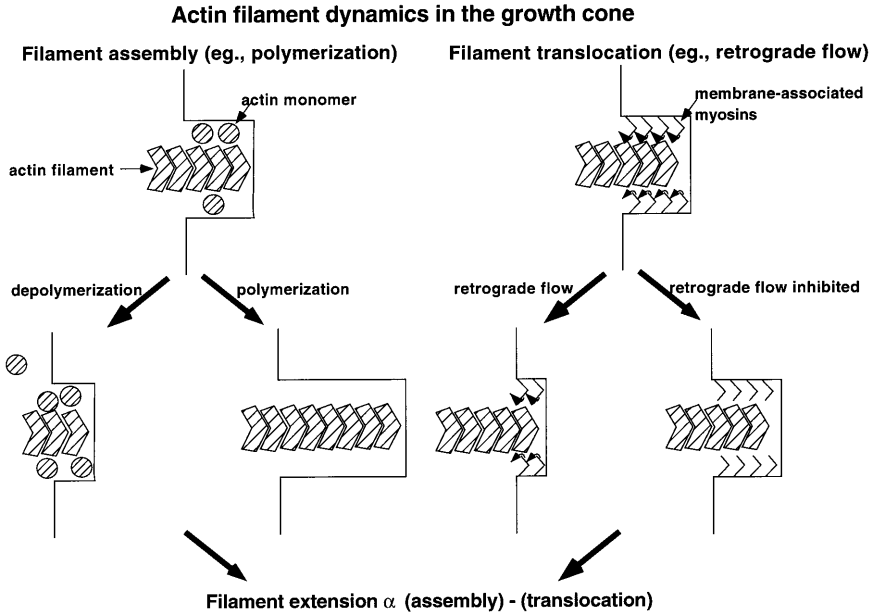


Figure 1. Actin dynamics in the growth cone involve both assembly and translocation of actin filaments. Depolymerization of actin filaments at the leading edge of the growth cone would favor its local retraction, whereas polymerization would favor extension. Likewise, the translocation of assembled actin filaments toward the growth cone base (e.g., by membrane-associated myosin motor proteins) would favor growth cone retraction, whereas an inhibition of retrograde flow (e.g., by myosin inhibition or coupling of the cytoskeleton to extracellular substrate) would not favor growth cone retraction. The combined effects of these two phenomena would serve to regulate the net extension of an actin filament with respect to the substratum.

Table 1. Types of signaling proteins implicated in axon guidance.

Guidance cues and receptors		Cytoplasmic signaling molecules
Cue	receptor	
Ephrins	Eph RTK: EphB2	Rho-family GTPases: Rho, Rac, Cdc42
Eph RTKs	Ephrin: EphrinB1	Rho-family GNEF: Unc-73
?	RPTP: DLAR	SH2/SH3 adapter protein: Dock/Nck
IgCAM (?)	IgCAM: L1, NCAM	Tyrosine kinases: Src, Abl
Semaphorins	Neuropilins	Tyrosine kinase substrate: Ena/Mena
Netrins	Unc-40/Unc-5	Serine/threonine kinase: PAK
		Actin-binding protein: profilin
		Actin-severing protein: gelsolin

Signal transducers linking the surface to the cytoskeleton

What signaling cascades link guidance receptors to the growth cone motility machinery? Many of the receptors and signal transducers are known, but there are not yet clear paths from the surface to the cytoskeleton (table 1). This review will focus initially on the molecules through which axon guidance signals appear to flow, then consider possible links from guidance receptors to these transducers, and finally discuss the role of cyclic nucleotide levels setting thresholds for guidance responses.

The Rho family GTPases as transducers of information to the growth cone cytoskeleton

The Rho family of small GTPases are molecular switches which transmit information through protein: protein interactions with effectors as they cycle between the GTP-bound (active) state and the GDP-bound (inactive) state [13]. A now classic series of experiments in which activated mutants of the Rho family members Cdc42, Rac, and Rho were injected into fibroblasts implicated these proteins as cytoskeletal regulators [14–16]. These observations have been extended to growth cones using cultured neuroblastoma cells [17]. Injection of activated Rho induced neurite retraction and growth cone collapse, while Rac stimulated lamellipodia formation and Cdc42 stimulated lamellipodial and filopodial extension. Taken together, one can imagine guidance receptors generating specific responses by activating and/or inhibiting particular combinations of Rho family members. In fact, Rac-1 activation is implicated in mediating the collapsing effects of Semaphorin-III on chick dorsal root ganglion growth cones [7]. Note, however, that collapse is the opposite of the Rac effect in the neuroblastoma cell experiments. Thus, although it is clear that Rho family members are involved in cytoskeletal dynamics, how the specificity of the response is generated is unknown. Rho family proteins are also implicated in regulating other structures that could influence growth cone motility, including integrin-containing adhesion complexes [18] and cadherin-containing adherens junctions [19, 20]. Finally, other studies suggest Rho family GTPases can regulate gene transcription [21, 22]. Thus, these proteins could influence guidance through multiple mechanisms.

The activity of Rho family GTPases is regulated by a host of factors that influence their cycling between the GTP- and GDP-bound states [13]. Guanine nucleotide exchange factors (GNEFs) assist entry into the GTP-loaded effector-binding mode by stimulating nucleotide release, while GTPase-activating proteins (GAPs) help catalyze their exit from the GTP-loaded state. The role of guanine nucleotide dissociation inhibitors (GDIs) is unclear. GNEFs, GAPs, and GDIs are excellent candidates for transducing signals to Rho family GTPases.

Regulation of axon guidance by a Rho family GNEF

Recent work in *Caenorhabditis elegans* demonstrates the importance of Rho family GTPase regulators in axon guidance [23]. *unc-73*, a gene required for the navigation of many axons, contains two Rho family GNEF domains. Its GNEF activity appears important for guidance, as one *unc-73* loss-of-function allele contains a point mutation inactivating the first GNEF domain. This first GNEF domain functions as a Rac-specific GNEF in vitro, and promotes reorganization of the actin cytoskeleton when overexpressed in mammalian fibroblasts. The existence of two GNEF domains in Unc-73 suggests a simple way for one receptor to activate combinations of Rho GTPases although the first GNEF domain is sufficient to rescue the mutant. The upstream pathways feeding into Unc-73 are not yet known, but a vertebrate relative of Unc-73, Trio, was isolated as a protein that binds to the receptor tyrosine phosphatase LAR [24]. Intriguingly, the *Drosophila* LAR relative, DLAR, regulates guidance in the motor axon system and shows genetic interactions with dominant negative Rac mutants [25].

Potential effectors of Rho family GTPases

How do the Rho family GTPases carry out their functions? Numerous proteins that bind to GTP-bound Rho, Rac, and/or Cdc42 have been identified [26]. Many of these possess biochemical activities that implicate them in controlling the actin cytoskeleton. For example, binding of Cdc42 to the neuronally expressed

N-WASP protein stimulates N-WASP actin-depolymerizing activity [27]. However, functions of these potential effectors in axon guidance are not yet established. Rho family GTPases can also affect the cytoskeleton through second messenger signaling. Both Rho and Rac can stimulate production of phosphatidyl inositol-4,5 bisphosphate (PIP_2), a phospholipid that binds a variety of actin-associated factors, including profilin and gelsolin [28]. In human platelets, Rac-stimulated PIP_2 production catalyzes actin filament growth by promoting the uncapping of actin filaments [28].

The PAK family of serine/threonine kinases may also function in the Rac and Cdc42 pathway [29], and a subset of PAK proteins are found in axons [30]. Binding of PAK proteins to GTP-loaded Rac and Cdc42 stimulates PAK kinase activity [29]. This initially suggested PAK kinases were downstream effectors. However, the discovery that a PAK associates with PIX, a GNEF for Rac and Cdc42, suggested that PAK proteins could work upstream of Rho family GTPases [31]. Indeed, when PAK is overexpressed, its association with PIX appears necessary for the induction of Rac-dependent morphological changes [32]. PAK kinase activity is not required in these assays; whether PAK simply activates Rho family GTPases or whether it also activates downstream targets or feeds back on upstream components remains an open question.

Adapters and cytoskeleton-binding proteins implicated in axon guidance

Genetic screens have identified several axon guidance regulators with molecular properties making them good candidates for intermediaries in growth cone signal transduction. The Dreadlocks SH2/SH3 adapter protein is required for axon guidance in *Drosophila* and is a homolog of the vertebrate adapter Nck [33]. Nck can bind activated receptor tyrosine kinases, including guidance receptors of the Eph family, through its phosphotyrosine-binding SH2 domain [34] and it can interact with PAK and WASP proteins through its SH3 domains [35, 36]. Thus, it constitutes a potential bridge from the cell surface to the cytoskeletal signaling machinery.

The cytoplasmic tyrosine kinases of the Src family are implicated in cytoskeletal control [37], and a series of studies in *Drosophila* provide genetic evidence for the Src-related kinase D-abl in axon guidance. D-abl is required for nervous system morphogenesis [38], and recent data indicate that D-abl functions antagonistically with the receptor protein tyrosine phosphatase DLAR in motor axon guidance [39]. Additional genetic interactions of D-abl with Ena and Disabled suggest this cohort of genes functions in cytoskeletal control [40, 41]. Members of the Ena family, as mentioned above, interact with regulators of actin polymerization [8]. Disabled is an adapter protein that can bind tyrosine-phosphorylated proteins (through its PTB domain) [42]. While not yet demonstrated to play a role in axon guidance, a vertebrate Disabled homolog is important for neuronal migration in the cortex [43].

A number of additional axon guidance signal transducers have been identified in *C. elegans* through mutations affecting locomotion. The Unc-51 protein is a serine/threonine kinase and interacts with a novel protein, Unc-14 [44]. The Unc-115 protein has an actin-binding domain as well as LIM protein:protein interaction domains [45], and the Unc-44 protein has similarities to ankyrin, which interacts with the actin cytoskeleton via spectrin [46]. Genetic and protein:protein interaction approaches should soon place these proteins within signaling pathways.

Starting at the top: proximal targets of guidance receptors (table 1)

A number of different families of guidance receptors have been identified, and the kinds of signals many of these receptors send are not yet known. However, a subset of receptors contains recognizable signaling motifs, such as tyrosine kinase and tyrosine phosphatase domains, that have guided efforts toward identifying their initial targets.

Eph receptor tyrosine kinases: receptors, kinases, and more

The Eph's are a large family of receptor tyrosine kinases, many of which play important roles in axon guidance [47]. Eph receptors are remarkable in that they and their ligands, the ephrins, are often distributed in gradients where they function in the establishment of topographic maps [48, 49]. Like other receptor tyrosine kinases, ligand-binding stimulates the kinase activity of Eph receptors, permitting them to signal by phosphorylating other proteins and by phosphorylating themselves to create docking sites for other proteins. Eph receptors require oligomerization of their ligands [50, 51]; this sensitivity to oligomerization could make their responses to changes in ligand concentration highly nonlinear, and potentially help them sense the small changes in ligand concentration encountered in gradients.

A number of well-established approaches have been implemented to search for molecules influenced by the tyrosine kinase activity of the Eph receptors. Two conserved tyrosine residues in the juxtamembrane region domain of the Eph receptor become phosphorylated in response to Eph ligand exposure, and a number of

proteins that bind these sites or are recruited into signaling complexes in response to kinase activation have been identified [52–54] . It is especially noteworthy that these proteins include signaling proteins discussed above, the Src family tyrosine kinases Fyn, Src, and Yes, and the SH2/SH3 adapter Nck. The functional importance of interactions between Eph receptors and these proteins has not been established, but these analyses provide important candidates for further examination.

Although Eph receptors likely function in many cases as ligand-activated receptor tyrosine kinases, not all activities depend on their function as kinases or as receptors. This initially emerged from genetic analysis of Eph receptor function in the mouse. While a null mutation in the Eph receptor EphB2 disrupts axon navigation in the brain anterior commissure, these defects are not observed in a mutant where only the EphB2 kinase domain is lacking [55]. Thus, EphB2 has kinase-independent functions. Furthermore, EphB2 expression is not detected in the mistargeted axons, but rather in cells contacted by these axons. The axons instead express ligands for EphB2, the transmembrane class B ephrins. This suggested that EphB2 could function as a ligand for its ephrinB partners. Consistent with this proposal, the addition of soluble forms of EphB2 to ephrinB-expressing cells stimulates the tyrosine phosphorylation of ephrinB cytoplasmic domains [56]. The mechanism of ephrinB phosphorylation is unknown, but Src kinases phosphorylate ephrinB intracellular domains efficiently [56]. Bidirectional signaling between Ephs and ephrin would provide an elegant way to coordinate processes in two cells in contact.

Signaling from immunoglobulin-domain-containing cell adhesion molecules

The immunoglobulin-domain-containing cell adhesion molecules (IgCAMs) are a large family of transmembrane and GPI-linked proteins abundantly expressed on growth cones. Although loss-of-function mutants in individual IgCAMs often show subtle axon-targeting effects, combining these mutations with mutations in other IgCAMs and guidance receptors demonstrates their important role in guidance [57, 58]. Interest has increasingly focused on the ability of these cell adhesion molecules to transduce signals to the cytoskeleton [59].

Recent papers suggest that IgCAMs L1 and NCAM may influence growth cone motility by functioning through the fibroblast growth factor receptor (FGF-R) [60, 61]. FGF-R has been implicated in axon guidance, as a dominant-negative FGF-R lacking the kinase domain disrupts *Xenopus* retinal axon targeting [62]. Links between the FGF-R and L1 and NCAM were suggested by the ability of the dominant-negative FGF-R to block L1- and NCAM-stimulated outgrowth in PC12 cells [61]. Consistent with a direct involvement of L1 and NCAM in the activation of the FGF-R, addition of soluble L1 and NCAM to PC12 cells leads to rapid (≤ 10 min) FGF-R tyrosine phosphorylation. There is also evidence that these proteins share downstream effectors. The outgrowth-promoting effects of FGF-R, L1, and NCAM are all blocked by a phosphopeptide derived from the site on the FGF-R that interacts with the SH2 domain of phospholipase C-gamma (PLC-γ) and inhibits PLC-γ activation [61]. This data leads the authors to propose that L1 and NCAM activate PLC-γ through the FGF-R. Activated PLC-γ would then catalyze the conversion of PIP_2 to IP_3 and diacyl glycerol, decreasing PIP_2 levels and increasing calcium levels. These second messengers would then stimulate cytoskeletal changes via PIP_2 and calcium-binding regulators of actin polymerization like gelsolin and profilin. It will be of interest to address the contribution of FGF-R signaling to IgCAM function during guidance decisions in vivo.

A second way in which IgCAMs could regulate growth cone motility is through direct interactions with cytoskeletal proteins [11]. For example, L1 can bind to ankyrin [63], which is linked to the actin cytoskeleton through spectrin, and ankyrinB mutant mice show nervous system phenotypes similar to L1 mutants [64]. The physical coupling of IgCAMs to actin filaments could influence guidance by slowing retrograde flow and propelling the growth cone across the substrate membrane protrusion [65].

Specificity in axon guidance signaling

Signal transduction pathways controlling axon guidance have much in common with signaling pathways regulating growth and differentiation. They use many similar components (e.g., Ras superfamily GTPases), and many of the same molecules (e.g., FGF-R). In all these signaling pathways, there must be specificity. In the case of axon guidance, two major questions concerning the specificity of responses to guidance cues are: (i) what determines whether an axon is attracted or repelled by a guidance cue? and (ii) how are multiple guidance cues integrated to generate a coherent decision? Although there are not yet definitive answers to either question, a recent series of experiments has provided insight into the kinds of signal processing involved.

The thin line between attraction and repulsion: second messengers switch guidance responses

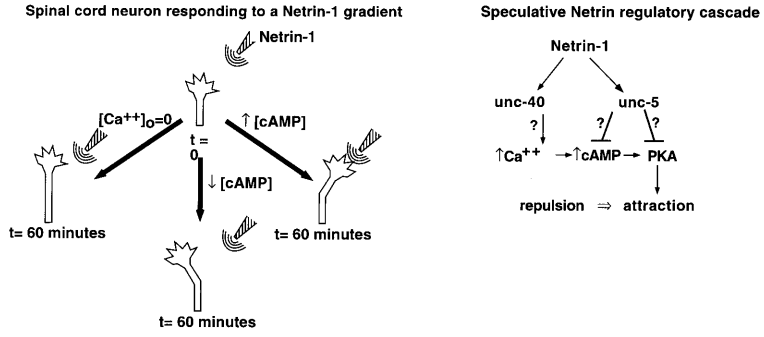
An initial insight to come from the identification of guidance cues was that a given cue can attract one

neuron and repel another [66]. In the simplest case, there could be multiple receptors, some mediating attraction and others repulsion. For the Unc-6/netrin guidance cues, there are indeed multiple receptors, Unc-40/DCC and Unc-5 [67–69]. But while Unc-5 is required for the repulsive response to Unc-6, Unc-40/DCC is involved in both attraction and repulsion [70]. Does Unc-40/DCC serve simply as a coreceptor for the Unc-5 repulsive receptor and an unknown attractive receptor, or does Unc-40 signal in both situations, with the interpretation of the signal influenced by Unc-5?

The use of cultured neurons exposed to gradients of defined guidance cues has provided strong evidence that the mechanisms of attraction and repulsion are closely related. *Xenopus* spinal cord neurons are attracted toward a pipette dispensing Netrin-1 [71]. However, when the level of cAMP-dependent signaling is decreased through the use of an inhibitor of protein kinase A (PKA) or a competitive analog of cAMP, these growth cones are repelled by Netrin-1 [70] (fig. 2A). The switch in behavior is dramatic and relatively rapid, taking place within 60 min of application of the inhibitors, and it is reversible. Although the battery of guidance receptors present on the growth cone surface could be altered by cAMP signaling, when taken together with other effects of cAMP on growth cone turning, it appears likely that cAMP alters the performance of the signal transduction pathway. Whether cAMP alters activity at the level of the receptor or the downstream signaling cascade is still an open question.

Netrin signaling involves not only cAMP, but also calcium. Instead of playing a modulatory role like cAMP, the presence of extracellular calcium is absolutely required for both repulsion and attraction [70]. In the current scheme, receipt of the netrin signal by DCC would locally elevate calcium levels (this has not yet been observed), which would stimulate calcium/calmodulin-activated adenylyl cyclase increasing cAMP levels. Calcium and cAMP would then work in conjunction to carry out cytoskeletal changes. The increase in calcium would mobilize cytoskeleton-remodeling enzymes that would cause either retraction or extension depending on the level of PKA activity. As noted above, the Unc-5

A Second messenger regulation of Netrin-1 response



B Responses to different guidance cues share second messenger sensitivities

	cAMP-sensitive, Ca^{++}-dependent	cGMP-sensitive, Ca^{++}-independent
Guidance cues	Netrin-1, MAG, BDNF, Acetylcholine	NT-3, Sema III
Nucleotide effect	↑ cAMP ⇒ attraction ↓ cAMP ⇒ repulsion	↑ cGMP ⇒ attraction ↓ cGMP ⇒ repulsion

Figure 2. (*A*) Left panel. Responses of cultured *Xenopus* spinal cord neurons to a gradient of Netrin-1 from a pipette. When extracellular calcium is removed, the growth cone ignores the Netrin-1 gradient. When signaling through the cAMP signaling pathway is inhibited, the response to Netrin-1 is converted from attractive to repulsive. Right panel. Speculative regulatory scheme suggesting that the effects caused by a reduction in cAMP signaling in vitro is mechanistically related to the effect of signaling through the Unc-5 receptor in vivo. In this model, an increase in calcium mobilizes factors which promote repulsion, unless they are modified by PKA phosphorylation, in which case they promote attraction. In one highly speculative scenario, one could imagine that calcium stimulates a protein to bind to actin monomers, decreasing polymerization by sequestering monomers. However, PKA modification of this same protein could cause it to catalyze the addition of the monomer to an existing filament; in this form the protein would stimulate polymerization and repulsion would be converted to attraction. Many alternative schemes are possible and the molecular identities of components and the linkages between them remain to be identified. (*B*) Summary of the two classes of guidance signals that generate responses with shared sensitivities to second messengers (MAG, myelin-associated glycoprotein; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3).

receptors mediate repulsive responses to netrins in vivo. Unc-5 could serve this function by inhibiting the PKA pathway, possibly by regulating cAMP levels. Whether PKA turns off repulsive signaling pathways to permit attraction or more subtly alters the properties of pathway components awaits the identification of its targets.

Second messengers are shared by diverse guidance receptors

The initial work on cAMP-mediated regulation of netrin responses has now been expanded to show that a variety of guidance cues can be readily converted between attractive and repulsive by changing levels of cyclic nucleotides [72]. The cues fall into two classes. Calcium and cAMP affect one class, which includes Netrin-1, brain-derived neurotrophic factor (BDNF), myelin-associated glycoprotein (MAG), and acetylcholine, while cGMP affects the other class, which includes Semaphorin-III and Neurotrophin-3 (NT-3) (fig. 2B). In each case, elevation of cyclic nucleotide levels converts repulsion to attraction. This suggests that guidance cues in each class activate common signaling pathways and that second messenger modulation of responses to the guidance cue is a general phenomenon.

Implications for the specificity of axon guidance

These observations also have several potentially important implications for the logic of axon guidance signaling. First, using cyclic nucleotide levels to guide the nature of the guidance response would provide an elegant solution to the problem of integrating information from many diverse receptors into a coherent choice [for a nice example of this problem in vivo, see ref. 58]. If information is assembled through cyclic nucleotide levels, many classes of receptors could converge on a few common enzymes. Second, the ability to rapidly switch between attraction and repulsion would permit a growth cone to change its responses based on experience. For example, commisural axons are attracted to the floor plate by chemoattractants, but lose their responsiveness to these factors after they cross it [73]; this presumably helps them move on toward their targets. A decrease in cAMP levels stimulated by close contact with the target would suffice to generate this change. In addition, cAMP gradients within the growth cone stimulate turning [74], suggesting a way for growth cones to respond to gradients of guidance cues.

It is well documented that an axon path depends on its repertoire of guidance receptors [75]. One can now imagine how differences in targeting specificity between neurons can result from differences in the repertoire of downstream effectors, e.g., levels or types of cAMP pathway components. There is ample precedent for this in cell fate signaling during development, where the transcription factors present in the responding cell provide much of the specificity of the response.

Summary

Many of the molecular components of axon guidance signaling are now in hand. With the powerful embryological, genetic, and biochemical tools to study them, a series of links will soon be made between the surface and the cytoskeleton. As recent work on the role of cyclic nucleotides indicates, the field is now moving from a period of identifying components to understanding how they function together to generate the exquisite specificity of neuronal connections.

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