Signaling from Axon Guidance Receptors

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Determining how axon guidance receptors transmit signals to allow precise pathfinding decisions is fundamental to our understanding of nervous system development and may suggest new strategies to promote axon regeneration after injury or disease. Signaling mechanisms that act downstream of four prominent families of axon guidance cues—netrins, semaphorins, ephrins, and slits—have been extensively studied in both invertebrate and vertebrate model systems. Although details of these signaling mechanisms are still fragmentary and there appears to be considerable diversity in how different guidance receptors regulate the motility of the axonal growth cone, a number of common themes have emerged. Here, we review recent insights into how specific receptors for each of these guidance cues engage downstream regulators of the growth cone cytoskeleton to control axon guidance.

Generating precise patterns of connectivity depends on the regulated action of conserved families of guidance cues and their neuronal receptors. Activation of specific signaling pathways can promote attraction, repulsion, result in growth cone collapse, or affect the rate of axon extension through signaling events that act locally to modulate cytoskeletal dynamics in the growth cone. Here, we review recent insights into how specific guidance receptors from each of the four "classic" guidance pathways engage downstream regulators of the growth cone cytoskeleton with a particular emphasis on Rho family small GTPases. We begin with a consideration of how events at the growth cone plasma membrane, including endocytosis and proteolytic processing, influence guidance receptor activation and signaling and then discuss how bidirectional links between receptors and cytoplasmic signaling molecules control axon guidance responses.

ENDOCYTOSIS

Endocytosis may be a necessary aspect of guidance receptor activation and signaling. In the case of membrane-associated ephrins, endocytosis of the ephrin-Eph complex is required for efficient cell detachment (parallel to proteolytic cleavage, see the following). Vav family guanine nucleotide exchange factors (GEFs) have been implicated as regulators of Eph receptor

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endocytosis and/or signaling. Neurons lacking Vav2 and Vav3 fail to respond to soluble ephrinA-Fc proteins with growth cone collapse and do not endocytose the ephrinA/EphA complex. $Vav2^{-/-}Vav3^{-/-}$ mice show defects in the retinogeniculate map consistent with a role as mediators of Eph repulsive signaling (Cowan et al. 2005). Vav proteins may trigger Eph internalization into signaling endosomes from where Eph receptors mediate dynamic changes of the actin cytoskeleton underlying growth cone collapse (Fig. 1A). Alternatively, Vav proteins may act in concert with other regulators of Rho GTPases to regulate Eph repulsive signaling independent of endocytosis (see later). For ephrinB-EphB-induced repulsive guidance, efficient cell detachment requires bidirectional endocytosis (Fig. 1B). Vav proteins may primarily promote cell detachment by mediating local Rac-dependent endocytosis of the ephrin-Eph complex and membrane. Neuronal growth cone collapse was observed, but detachment from a target cell was delayed when the target cell expressed a carboxy terminally truncated Eph. This mutant Eph receptor, unlike its full-length counterpart, failed to undergo forward ephrin-Eph endocytosis, resulting in inefficient removal of the ephrin-Eph complex from the cell surface (Zimmer et al. 2003).

Endocytosis may also be part of an adaptation process that resets the growth cone's sensitivity to a repulsive cue. Adaptation in *Xenopus* retinal growth cones to Sema3A or netrin-1 involves two processes: A fast desensitization,

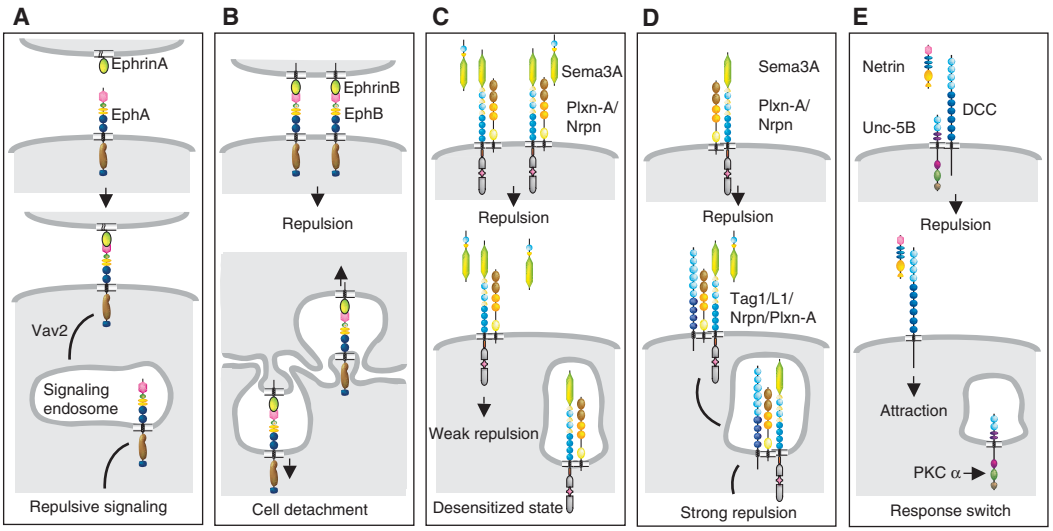

Figure 1. Regulation of guidance receptor activation and signaling by endocytosis. (*A*) In response to ephrin binding to Ephs, the Rho family GEF Vav2 is recruited to the activated Eph receptor. Vav family GEFs are required for EphA endocytosis and ephrinA1-induced growth cone (GC) collapse, suggesting a role for Vavs in trafficking Ephs into signaling endosomes. (*B*) EphrinB binding to EphBs in neuronal GCs leads to collapse triggered by Eph forward signaling. Bi-directional endocytosis is necessary to remove the Ephrin-Eph protein complex from the cell surface, thereby allowing efficient cell detachment. (*C*) Endocytosis is a mechanism by which GCs adapt to guidance cues. *Xenopus* retinal GCs undergo collapse in response to Sema3A binding to its receptors Plxn-A and Nrpn. Exposure to a low dose of Sema3A desensitized the GCs, because Sema3A receptors were rapidly endocytosed. (*D*) Neural cell adhesion molecules (CAMs) such as L1 and TAG-1 modulate the response of certain axons to Sema3A. L1 binding to Nrpn (shown) and TAG-1 binding to L1 (not shown) facilitate internalization of Sema3A receptors, thereby enhancing the sensitivity of the GCs to Sema3A. (*E*) Endocytosis switches chemorepulsion to chemoattraction. Chemorepulsion by netrin is mediated by a complex of DCC and Unc5 receptors, whereas chemoattraction of netrin is mediated by DCC alone. Activation of protein kinase Cα (PKCα) specifically promotes internalization of the repellent Unc5 receptor, thereby converting netrin-mediated repulsion to attraction.

which is dependent on receptor endocytosis, and a slower resensitization, which is dependent on protein synthesis (Fig. 1C). If background levels of a particular guidance cue increase, growth cones make use of this adaptation mechanism to tune their responsiveness appropriately to higher levels of the guidance cue riding on top of the background levels (Piper et al. 2005).

Endocytosis of guidance receptors is also influenced by other membrane receptor systems and can lead to changes in responsiveness to the guidance cue. The cell adhesion molecules L1 and TAG-1 promote Sema3A activity through interaction and coendocytosis with its receptor neuropilin-1 (Nrpn-1). Cortical axons from L1-deficient mice no longer respond to Sema3A (Castellani et al. 2000) and sensory nociceptive neurons lacking TAG-1 or L1 show reduced sensitivity to ventral spinal cord chemorepellents, including Sema3A (Fig. 1D) (Law et al. 2008). Hence, CAMs represent cell–cell communication cues that can modulate responses to long-range diffusible molecules.

Activation of protein kinase Cα (PKCα) converts netrin-1-mediated chemorepulsion to chemoattraction by specifically internalizing the repellent UNC5A receptors from the cell surface; the remaining attractant DCC receptors mediate the chemoattractive response to netrin-1 (Fig. 1E) (Bartoe et al. 2006). Here, in contrast to desensitization, the endocytosis of UNC5 is not triggered by its own ligand. Instead, activation of the G protein coupled Adenosine 2B (A2b) receptor leads to the PKC dependent endocytosis of UNC5 (McKenna et al. 2008). Although the role of A2b in netrin signaling has been quite controversial (Bouchard et al. 2004; Corset et al. 2000; Shewan et al. 2002; Stein et al. 2001), in the context of UNC5 regulation, A2b acts independently of netrin, and its ability to regulate UNC5 surface levels support a role as a potent modulator of netrin responses.

PROTEOLYTIC PROCESSING AND RECEPTOR SIGNALING

Like endocytosis, proteolytic processing contributes to receptor activation and modulates guidance responses (Fig. 2). For instance, preventing the metalloprotease-dependent ectodomain shedding of DCC results in enhanced DCC expression and potentiates netrin-induced axon outgrowth (Galko and Tessier-Lavigne 2000). A number of studies have implicated Kuzbanian (Kuz)/ADAM10 family proteases in the signaling pathways of guidance receptors. For example, Kuz/Adam10 regulates the cleavage of Eph receptors and ephrinA2 ligands. Adam10 forms a stable complex with ephrinA2 and on Eph interaction with ephrinA2, the resulting ligand-receptor complex is cleaved by Adam10 (Hattori et al. 2000) (Fig. 2A). More recently, the mechanism that allows cleavage of only those ephrin ligands that are engaged by receptors has been elucidated (Janes et al. 2005). Ligand/receptor binding exposes a new recognition sequence for Adam 10, resulting in the optimal positioning of the protease domain with respect to the substrate (Janes et al. 2005). This mechanism explains how an initially adhesive interaction can be converted to repulsion and offers an efficient strategy for axon detachment and attenuation of signaling. The matrix metalloprotease family can also convert ephrinB/EphB adhesion into axon retraction by specific cleavage of the EphB2 receptor (Lin et al. 2008). Thus, both ephrin ligands and Eph receptors can be substrates for regulated proteolysis and these events are critical in mediating axon retraction. *Kuz* has also been implicated in *Slit-robo* mediated repulsion in *Drosophila*. Specifically, ectopic midline crossing of ipsilateral interneurons, a hallmark of defective midline repulsion, is observed in *kuz* zygotic mutant embryos and dose-dependent interactions between *kuz* and *slit* suggest that Kuz may be a positive regulator of Slit-Robo signaling (Schimmelpfeng et al. 2001). This raises the intriguing possibility that Kuz may regulate guidance by regulating the cleavage of Slit or Robo (Fig. 2D).

Kuz was identified in *Drosophila* for its role in regulating Notch signaling during neurogenesis (Pan and Rubin 1997; Rooke et al. 1996). Kuz cleavage of Notch releases the extra-cellular domain and triggers the subsequent cleavage and

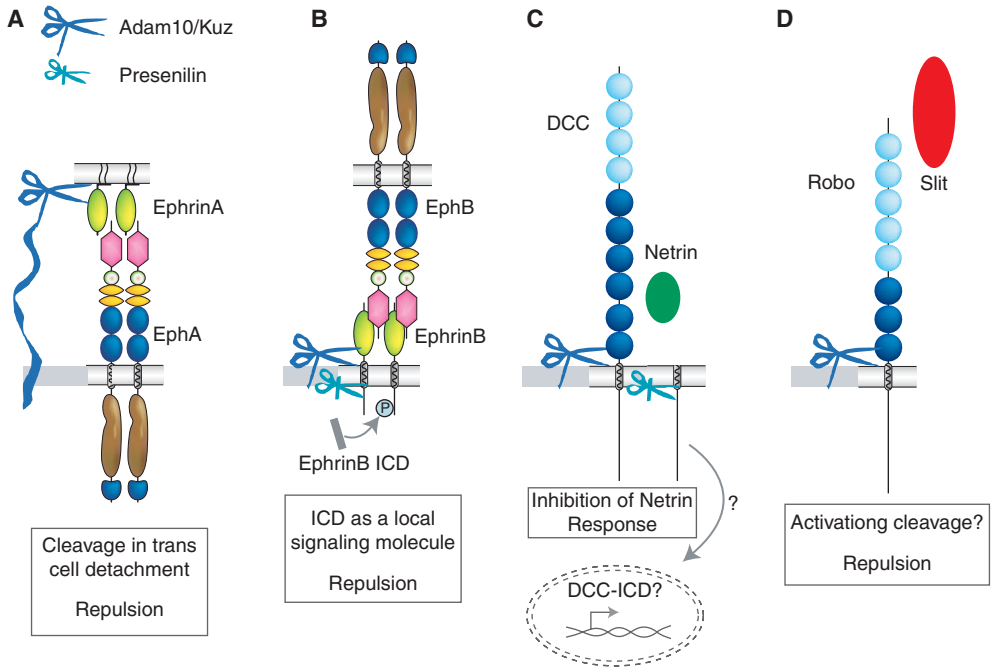

Figure 2. Regulation of receptor activation and signaling by proteolytic processing. (*A*) Following ligand-receptor complex formation, ADAM10 cleaves the ephrinA5 ligand. This regulated proteolytic event both leads to release from the initial cell–cell adhesion, allowing for growth cone retraction, and is necessary for the transduction of the EphA3 forward signal. (*B*) Processive cleavage of ephrinB leads to the release of the ephrinB intracellular domain (ICD), which may activate SRC-family kinases to contribute to reverse signaling. On the other hand, cleavage of the EphB2 receptor, in this case by matrix metalloproteases (not pictured), is required for receptor activation in vitro. (*C*) Regulated proteolysis of DCC occurs by ADAM10-mediated creation of a carboxy-terminal fragment (CTF), followed by γ-secretase mediated intramembrane cleavage releasing DCC ICD. This ICD is competent to translocate to the nucleus when fused with Gal4. The cleavage event by ADAM10 leads to attenuation of neuritogenesis in vitro. (*D*) Kuzbanian appears to act positively in the Slit-Robo signaling pathway. Based on genetic observations and the abnormal presence of Robo protein on the commissural portions of axons in *kuz* mutants, we speculate that Kuz may cleave Robo to regulate receptor activity.

release of the Notch intracellular domain (ICD) by the γ-secretase complex. This second cleavage event releases Notch ICD from the membrane, allowing it to translocate to the nucleus, where it acts as a transcriptional regulator (Mumm and Kopan 2000). Interestingly, a number of in vitro studies have shown that DCC (Parent et al. 2005; Taniguchi et al. 2003) and several ephrin ligands and Eph receptors appear to undergo a similar Adam10/γ-secretase sequential proteolysis (Georgakopoulos et al. 2006; Litterst et al. 2007; Tomita et al. 2006) and that preventing these processing events leads to deficits in the in vitro activities of these guidance molecules. Several observations hint at potentially important regulatory activities of released receptor ICDs. For instance, a DCC ICD fused with a Gal4 DNA binding domain can initiate γ-secretase-dependent transcription, suggesting that the DCC ICD could regulate transcription (Taniguchi et al. 2003) (Fig. 2C), whereas ephrin's ICD can bind to and locally activate Src family kinases (Georgakopoulos et al. 2006) (Fig. 2B). If and how these processing events contribute to in vivo receptor function will be an important area of future research.

SECOND MESSENGERS AND AXON GUIDANCE RECEPTOR SIGNALING

Calcium (Ca^{++}) and cyclic nucleotides (cAMP and cGMP) can act in vitro to directly mediate guidance receptor signaling and also can modulate the strength and valence of guidance responses. Disruption of Ca^{++} and cyclic nucleotide signaling leads to guidance defects in many systems and in some cases direct links have been made to specific guidance receptor signaling pathways. These two signaling systems show extensive cross talk in the regulation of growth cone guidance: Ca^{++} signaling can promote the production of cyclic nucleotides through activation of soluble adenylyl cyclases and nitric oxide synthase (NOS) and cyclic nucleotides can regulate cellular calcium concentration by controlling the activity of plasma membrane Ca^{++} channels as well as through the regulation of calcium-induced calcium release (CICR) from internal stores (Gomez and Zheng 2006; Zheng and Poo 2007). This positive feedback could potentially play a role in amplifying responses to shallow gradients of guidance cues.

CALCIUM

Exposure of growth cones to in vitro gradients of guidance cues can induce a corresponding gradient of Ca^{++} elevation (Gomez and Zheng 2006; Henley and Poo 2004). These asymmetric changes in Ca^{++}concentrations appear to be instructive signals to direct growth cone turning, because focal elevation of Ca^{++} is sufficient to induce turning responses (Zheng 2000). Increases in Ca^{++} influx and CICR can be triggered by guidance cues and the outcome for growth cone behavior (either attraction or repulsion) can be influenced by the magnitude of the Ca^{++}elevation, the slope of the Ca^{++} gradient, and potentially the specific source of the Ca^{++}as well (Gomez and Zheng 2006; Zheng and Poo 2007). In general, moderate amplitude increases in Ca^{++}(often involving CICR) favor attraction, whereas high or low amplitude increases favor repulsion, although differences in neuron type, growth substrate, and resting Ca^{++}concentrations can affect growth cone responses. Although much of the strongest evidence for the importance of Ca^{++} signaling during chemotropic axon guidance comes from studies of cultured neurons, examples are accumulating that support the in vivo significance of a number of the key in vitro observations.

Electrophysiological recordings from growth cones indicate that attractive and repulsive guidance cues trigger rapid and reciprocal changes in membrane potential; attractants such as BDNF and Netrin lead to membrane depolarization and repellants such as Slit and Semaphorin lead to hyperpolarization (Henley et al. 2004; Li et al. 2005; Nishiyama et al. 2008; Wang and Poo 2005) (Fig. 3). Moreover, the polarity of the change in membrane potential determines attraction versus repulsion (Nishiyama et al. 2008). For Netrin and BDNF mediated attraction, transient receptor potential (TRP) Ca^{++} channels contribute to membrane depolarization, and Ca^{++} influx through these channels is required for chemoattraction (Li et al. 2005; Wang and Poo 2005) (Fig. 3A). Although earlier work had shown that Netrin induces Ca^{++} influx in part through voltage-dependent calcium channels (VDCCs) (Hong et al. 2000), it remained unclear how Netrin stimulation results in adequate membrane depolarization to activate these channels. RNA interference and pharmacological manipulations in cultured *Xenopus* neurons indicate that BDNF and Netrin, through engagement of their respective TrkB and DCC receptors lead to Ca^{++} release from internal stores and activation of TRP channels: The subsequent TRP channel-dependent membrane depolarization is sufficient to activate VDCCs and the resulting Ca^{++} influx is essential for attractive turning. Importantly, TRP channels mediate axon guidance in vivo as well; morpholino antisense knockdown of TRPC1 reduces contralateral projections of *Xenopus* commissural interneurons, likely reflecting a requirement in Netrin-dependent midline crossing (Shim et al. 2005). A role for Semaphorins in activation of cyclic nucleotide gated (CNG) calcium channels strengthens the case

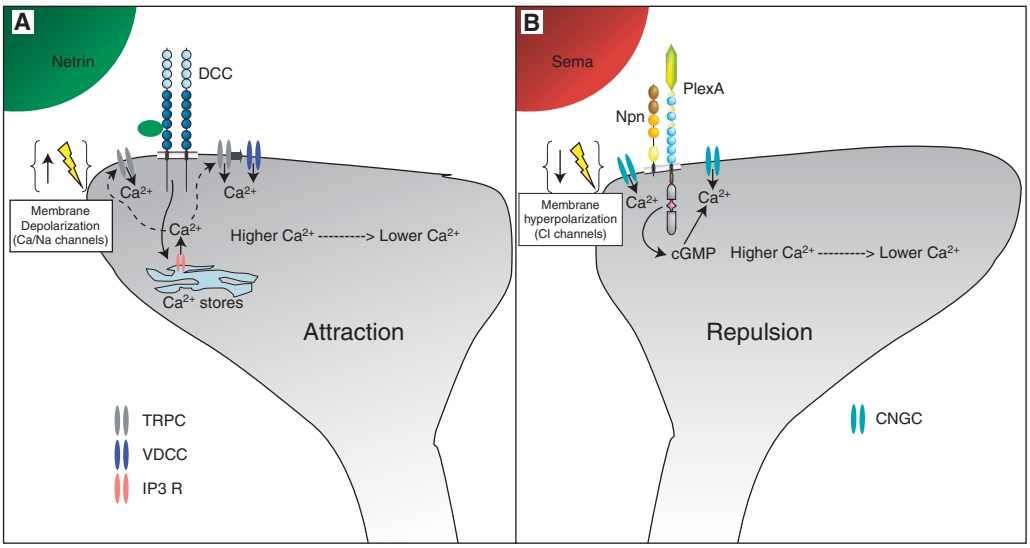

Figure 3. Second messengers and guidance receptor signaling. (*A*) In vitro application of chemoattractants like Netrin and BDNF (not pictured) leads to rapid membrane depolarization and triggers an asymmetric elevation of intracellular calcium on the side of the growth cone facing the source of the chemoattractant. Activation of DCC (or TRK receptors for BDNF) leads to the release of calcium from intracellular stores through IP3 receptors, which in turn activates plasma membrane TRP channels and voltage-dependent calcium channels (VDCC). The resulting gradient of intracellular calcium directs growth cone attraction. (*B*) In vitro application of chemorepellants like Semaphorin and Slit (not pictured) leads to rapid membrane hyperpolarization and a local elevation of intracellular calcium. Activation of PlexA receptors leads to the production of cGMP (likely through nitric oxide synthase), which in turn activates cyclic nucleotide gated (CNG) channels in the plasma membrane. In this case, the gradient of calcium and cGMP across the growth cone leads to growth cone repulsion.

for the specific regulation of calcium influx through plasma membrane channels and points to the importance of cross regulation of cyclic nucleotide and calcium signaling (Togashi et al. 2008) (Fig. 3B). Here, Sema signaling through Plexin receptors stimulates the production of cyclic GMP (cGMP), which in turn is required for membrane hyperpolarization, the activation of CNG channels, and growth cone repulsion (Nishiyama et al. 2008; Togashi et al. 2008). It will be interesting to see to what extent CNG channels contribute to repulsive axon guidance in vivo.

CYCLIC NUCLEOTIDES

Like calcium signaling, cyclic nucleotides (cAMP or cGMP) can have profound effects on growth cone responses to guidance cues. The levels of cyclic nucleotides, specifically the ratio of cAMP to cGMP, can determine whether the response to a guidance cue will be attractive or repulsive, with high cyclic nucleotide levels (or high cAMP/cGMP ratios) favoring attraction and low levels (or low cAMP/cGMP ratios) favoring repulsion (Nishiyama et al. 2003; Song et al. 1998; Song et al. 1997). Although a clear demonstration that cyclic nucleotide signaling can convert receptor responses from attraction to repulsion and vice versa in vivo is still lacking, cyclic nucleotide signaling can clearly modulate the strength of receptor responses. For example, during motor axon guidance in *Drosophila*, cAMP signaling through protein kinase A (PKA) can modulate axon repulsion. Specifically, the *Drosophila* A-kinase anchoring protein (AKAP), Nervy, links the Plexin-A receptor to PKA to inhibit sema repulsion (Terman and Kolodkin 2004). Together with a number of recent studies in zebrafish and in cultured

neurons that suggest a similar inhibitory effect of cAMP/PKA on the strength of repulsive guidance signals, the role of *nervy* in Sema signaling suggests that cyclic nucleotide signaling reduces the strength of guidance outputs (Chalasani et al. 2003; Chalasani et al. 2007; Dontchev and Letourneau 2002).

In these examples, the signals mediating changes in cyclic nucleotide levels are independent of the guidance receptors whose outputs they regulate; however, more direct roles for cAMP or cGMP signaling downstream of specific guidance signals have been suggested. For instance, as mentioned previously, Sema-Plexin signaling leads to the production of cGMP, and cGMP plays an instructive role in promoting repulsion by regulating membrane hyperpolarization and the influx of Ca^{++} through CNG channels. Genetic studies of motor axon guidance in *Drosophila* support an in vivo requirement for cGMP signaling during Sema-Plexin repulsion; mutations in a receptor guanylyl cyclase Gyc76C lead to motor axon guidance defects that are similar to those observed in *sema* and *plexin* mutants, and genetic interactions indicate that Gyc76C acts in the Sema-Plexin pathway (Ayoob et al. 2004). In the case of Netrin, pharmacological manipulations in cultured *Xenopus* neurons have shown that Netrin outgrowth and attraction require DCC (or A2b)-mediated elevation of cAMP and activation of PKA (Corset et al. 2000; Hopker et al. 1999). More recently, soluble adenylyl cyclase has been shown to regulate the production of cAMP in response to Netrin in rat DRG neurons (Wu et al. 2006). However, other studies in rodent commissural neurons support a different role for cAMP/PKA in contributing to Netrin responses and have shown: (1) that Netrin does not lead to elevations in cAMP levels or activation of PKA, (2) that PKA is not required for Netrin attraction, but instead regulates the Netrin response through promoting DCC recruitment to the plasma membrane, and (3) that mutations in soluble adenylyl cyclase do not result in commissural axon guidance defects in mice (Bouchard et al. 2004; Moore and Kennedy 2006; Moore et al. 2008b). Although these observations do not preclude a role for direct Netrin-dependent cAMP signaling in other cellular contexts, such as in vitro steering of cultured *Xenopus* neurons, they do argue against the generality of this mechanism for Netrin-directed axon path finding in vivo. Together, the available evidence favors an important role for cyclic nucleotides in modulating the strength of guidance responses in vivo rather than switching the polarity of responses.

How do changes in intracellular Ca^{++} and cyclic nucleotide signaling result in directed growth cone turning? Although a full discussion of the many downstream effectors of calcium and cyclic nucleotide signaling are well beyond the scope of this article, key targets include (1) kinases such as PKA, PKC, Src, and CamKinaseII, (2) calcineurin and PP1 protein phosphatases, (3) Calpain proteases, and 4) Rho-family small GTPases. Here, we emphasize the role of Rho GTPases in guidance receptor signaling because they are implicated as key players downstream of all of the "classical" axon guidance receptors, because considerable progress has been made in establishing direct links between Rho GTPases and guidance receptors, and because there is strong evidence from many model systems for the in vivo significance of Rho GTPase function in axon guidance receptor signaling.

RHO FAMILY SMALL GTPases

In this section, we discuss how guidance receptors are linked to activation of Rho GTPases through guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), and how these pathways in turn result in modulation of cytoskeletal dynamics to achieve specific guidance outcomes. For more extensive discussion of the role of Rho GTPases in neuronal development, we refer the reader to Hall and Lalli (2010).

Netrins, Semas, ephrins, and Slits all regulate the activity of Rho-family GTPases. Netrin, through DCC, increases Rac activity in fibroblasts (Li et al. 2002), increases Rac and Cdc42 activity in rat commissural neurons (Shekarabi et al. 2005), and inhibits RhoA activity (Moore

et al. 2008a). Sema treatment via plexin-B1 results in activation of RhoA (Swiercz et al. 2002) and sequestering of active Rac (Hu et al. 2001); however, Sema3A via plexin-A activates Rac, but not RhoA (Turner et al. 2004). Ephrins, through Eph receptors, result in increased RhoA activity as well, but also cause transient, decreased Rac activity in retinal ganglion cells (RGCs) (Jurney et al. 2002; Wahl et al. 2000), whereas Eph-ephrin reverse signaling activates Rac and Cdc42 to direct repulsive axon pruning (Xu and Henkemeyer 2009). Slits, acting through Robo receptors, lead to Rac and Rho activation and inhibition of Cdc42 (Fan et al. 2003; Wong et al. 2001). Thus, there is no clear consensus for how Rho GTPases mediate repulsion, because these repulsive guidance pathways each influence RhoA, Rac, and Cdc42 activity in distinct ways. Considerable progress has been made in understanding how these diverse guidance signals are coupled to the Rho GTPases to affect their activity.

NETRIN-DCC

Genetic analysis in *Caenorhabditis elegans* supports a role for Rac downstream of DCC-mediated attraction; mutations in the Rac gene *ced-10* partially suppress defects caused by expression of a constitutively active form of the DCC receptor homolog, Unc-40 (Gitai et al. 2003). In vitro, Netrin induces the rapid activation of Rac1, Cdc42, and Pak1, which results in profound changes in growth cone morphology, leading to increased surface area and a greater number of filopodia (Li et al. 2002; Shekarabi and Kennedy 2002; Shekarabi et al. 2005). Netrin increases the amount of a nonhydrolyzable GTP analog, GTPγS, bound to Rac and Cdc42 in commissural neurons, suggesting that one or more GEFs may be associated with the DCC receptor to drive the observed increases in Rac and Cdc42 activity. In certain cell types, the Trio GEF may fulfill this function. Trio is an important regulator of axon guidance decisions in several contexts (Liebl et al. 2000; Newsome et al. 2000). Trio positively contributes to midline axon crossing in the embryonic CNS in *Drosophila* and can physically interact with Frazzled (Forsthoefel et al. 2005) and with mammalian DCC (Briançon-Marjollet et al. 2008), though in the latter case the interaction is likely mediated through binding to PAK-1. In contrast to wild-type, Netrin stimulation of cortical neurons from Trio−/− mice does not result in Rac activation and Trio−/− mice also display guidance defects, which partially overlap with defects seen in netrin or DCC−/− mice (Briançon-Marjollet et al. 2008). However, the commissural axon guidance defects in Trio−/− mutants are considerably milder than those in netrin or DCC−/− mutants, indicating that additional factors must act in commissural neurons to transmit Netrin signals.

The CZH family GEF, DOCK180 also contributes to netrin-DCC attraction in mouse cortical and commissural neurons by mediating Rac activation. DOCK180 is required for dissociated cortical neuron outgrowth in response to netrin and for commissural neuron turning in explant assays (Li et al. 2008), and knockdown of DOCK180 in chick spinal cords also reduces commissural axon midline crossing. Netrin can induce both axon outgrowth and attractive axon turning, therefore it is unclear in these assays whether commissural neuron turning defects are a secondary consequence of defects in axon outgrowth (Li et al. 2008). Also unclear is whether Trio and DOCK180 act in the same or in a parallel pathway to mediate netrin-dependent Rac activation downstream of DCC. Both Trio and DOCK180 can interact with DCC, thus either may be sufficient to activate Rac after its recruitment to the receptor.

SEMA-PLEXIN

Genetic analysis of motor axon guidance in *Drosophila* indicates that PlexinB mediates repulsion in part by binding to active Rac. At sites where growth cones are exposed to Semaphorins, PlexinBs are clustered and sequester active Rac, thereby preventing Rac from binding to and activating p21-activated kinase (PAK) and inducing lamellipodia extension (Hu et al. 2001). Work in cultured cells suggests that the interaction between PlexinB and Rac is

bi-directional: Active Rac enhances the affinity of Plexin-B1 for Sema4D and its localization at the cell surface (Vikis et al. 2002). Genetic evidence also indicated that PlexinB requires RhoA (Hu et al. 2001) and in cultured cells Plexin-B1 was shown to bind the Rho guanine nucleotide exchange factors PDZ-RhoGEF and LARG via its carboxy-terminal PDZ binding site (Swiercz et al. 2002). Binding of Sema-4D to plexin-B1 stimulates PDZ-RhoGEF/LARG activity, resulting in activation of RhoA. Dominant–negative forms of PDZ-RhoGEF/LARG block Sema4D-induced growth cone collapse in hippocampal neurons, thereby demonstrating the necessity of this pathway in primary neurons. How well-used this pathway is in vivo will have to await future genetic studies.

In contrast to Plexin-Bs, Plexin-A-induced growth cone repulsion requires the activation of Rac (Jin and Strittmatter 1997). Recent work in cultured sensory neurons delineated the pathway downstream of the Plexin-A/Npn-1 complex that mediates Sema3A-induced suppression of neurite outgrowth (Toyofuku et al. 2005). The FERM domain-containing GEF protein, FARP2, associates with the Plexin-A1/Npn-1 complex. Sema3A binding to Npn-1 causes FARP2 to dissociate from Plexin-A1, activating FARP2's GEF activity and raising the levels of Rac-GTP in the cell. Through a combination of dominant–negative and siRNA knockdown experiments, it was shown that activation of Rac triggers Rnd1 binding to Plexin-A1, thereby activating Plexin-A1's intrinsic RasGAP activity. Activated Plexin-A1 down-regulates R-Ras activity, which may lead to inhibition of integrin function and growth-cone repulsion. Whether FARP2 directly regulates cytoskeletal dynamics and whether FARP2 is a required effector for Plexin-A1-mediated axon guidance function in vivo remains to be shown. Interestingly, the related FERM domain-containing GEF protein, FARP1, is enriched in the dendrites of lateral motor column neurons that innervate the limbs, where it specifically mediates dendritic growth downstream of transmembrane Sema6A and Plexin-A4 (Zhuang et al. 2009).

EPHRIN-EPH FORWARD SIGNALING VIA THE DBL FAMILY GEF EPHEXIN1

In the absence of ephrin stimulation, nonphosphorylated ephexin1 is bound to EphA4 and activates RhoA, Rac1, and Cdc42, leading to a balance of GTPase activation that promotes axonal growth (Fig. 4A) (Sahin et al. 2005). Eph tyrosine kinase activity is required, but not sufficient to promote ephexin1 phosphorylation; instead, ephrin-induced clustering of Ephs appears to promote ephexin1 phosphorylation (Egea et al. 2005), probably involving Src tyrosine kinase. Tyrosine phosphorylation of ephexin1 shifts its exchange activity toward RhoA, thereby causing growth cone collapse in vitro (Sahin et al. 2005). This provides a model of how ephexin1 may steer growth cones: When Ephs are activated in a portion of the growth cone, tyrosine phosphorylated ephexin1 may tip the local balance of GTPase activity toward RhoA, thereby causing actin depolymerization and local retraction (Fig. 4B). In other regions of the growth cone that have not made contact with ephrins, ephexin1 promotes growth by activating RhoA, Rac1, and Cdc42. The in vivo role of vertebrate ephexin1 and the other four members of this family (ephexin2-5) are largely unexplored. But, this pathway appears to be evolutionarily conserved: the single *Drosophila* Eph receptor mediates synaptic homeostasis at the neuromuscular junction via ephexin (Frank et al. 2009).

EPHRIN-EPH FORWARD SIGNALING VIA α2-CHIMAERIN

The Rac-specific GAP α2-chimaerin is an essential mediator of ephrinB3/EphA4 forward signaling in vivo. Loss of α2-chimaerin impairs repulsion of EphA4-expressing CST axons at the spinal cord midline and the formation of the spinal central pattern generator (CPG) (Beg et al. 2007; Shi et al. 2007; Wegmeyer et al. 2007; Iwasato et al. 2007). The association of α2-chimaerin with Eph receptors appears to be direct or mediated by the Nck2 (Grb4) adaptor protein. The latter mechanism is supported by the observation that mice lacking Nck1 and

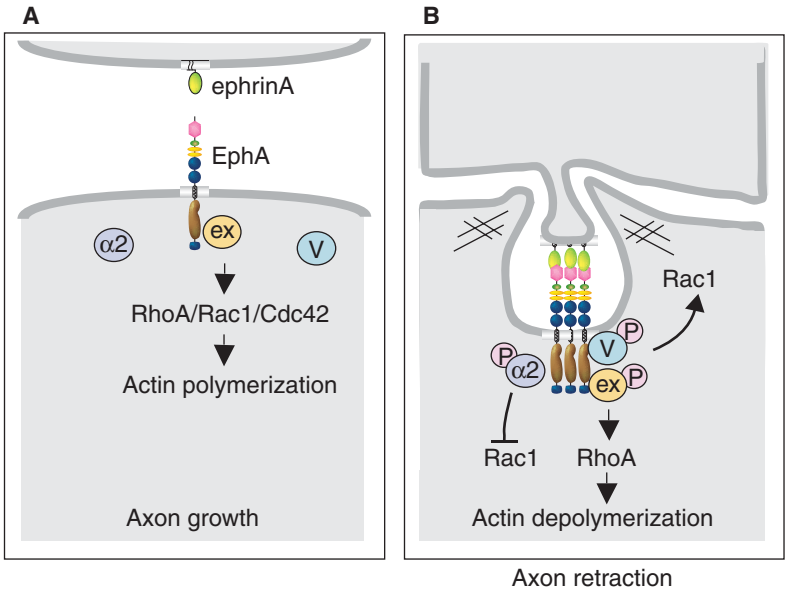

Figure 4. Eph forward signaling via GEFs and GAPs. (*A*) In the absence of ephrin stimulation, ephexin1 (ex) is bound to Eph receptors and activates RhoA, Rac1, and Cdc42, leading to a balance of GTPase activation that promotes actin polymerization and axonal growth. α2-chimaerin (α2) and Vav proteins (V) do not bind unclustered Ephs. (*B*) Upon ephrin-induced clustering and autophosphorylation of Ephs, ephexin1 is tyrosine phosphorylated (P), which shifts its exchange activity toward RhoA. α2-chimaerin is recruited to the Eph cluster and becomes tyrosine phosphorylated. This modification activates its intrinsic GAP activity, causing inactivation of Rac1. RhoA activation and Rac1 inactivation promote actin depolymerization and axon retraction. The specific role of Vav-mediated Rac1 activation is currently unclear. It may be linked to Vavs role in Eph endocytosis and may help to polymerize actin near the plasma membrane, where the endocytic vesicle forms.

Nck2 in the nervous system display similar defects in CST and CPG formation as α2-chimaerin and EphA4 null mice (Fawcett et al. 2007). The interaction with EphA4 activates the intrinsic GAP activity of α2-chimaerin and this leads to inactivation of Rac1 (Fig. 4B). The cooperative action of α2-chimaerin in reducing Rac1-mediated actin polymerization and ephexin1 in enhancing RhoA-mediated actin depolymerization appears to induce efficient axon retraction. Vav2/3 and α2-chimaerin have opposing effects on Rac1and yet both are mediators of EphA forward signaling. How can this paradoxical situation be explained? Unlike Vav2/3, α2-chimaerin does not influence Eph receptor endocytosis (Wegmeyer et al. 2007). It is possible that the activated Eph receptor first activates α2-chimaerin to induce axon retraction and then activates Vav2/3 to locally activate Rac1-dependent endocytosis to allow cell detachment.

EPH-EPHRIN REVERSE SIGNALING

Reverse signaling by receptor-like ephrinB proteins has been implicated in axon guidance (Henkemeyer et al. 1996). Following interactions with cognate Ephs, ephrinB proteins become clustered and signaling is initiated either by Src-mediated tyrosine phosphorylation of the ephrinB cytoplasmic tail or by recruitment of PDZ domain-containing effectors (Palmer et al. 2002). Nck2 is recruited to the phosphorylated ephrinB protein and is essential for several ephrinB-mediated processes, including spine formation (Cowan and Henkemeyer 2001; Segura et al. 2007). Nck1/2-deficient mice fail to develop a normal posterior branch of the anterior commissure, a phenotype previously attributed to defective ephrinB reverse signaling (Fawcett et al. 2007). Tyrosine phosphorylation-dependent ephrin-B3 reverse signaling controls the stereotyped

pruning of exuberant mossy fiber axons in the hippocampus and Nck2 has been implicated in this process. Nck2 appears to couple ephrinB3 to Dock180 and PAK, leading to activation of Rac1 and Cdc42 and downstream signaling that results in axon retraction/pruning (Xu and Henkemeyer 2009).

SLIT-ROBO

Although inhibition of Rac can accompany repulsive guidance decisions, activation of Rac may also be involved in mediating responses to repulsive cues exemplified by Eph-dependent growth cone retraction and Ephrin-dependent axon pruning. In the context of Slit-Robo, mediated repulsion activation of Robo receptors by Slit leads to activation of Rac (Fan et al. 2003; Hu et al. 2005; Wong et al. 2001; Yang and Bashaw 2006), and limiting Rac function reduces the efficiency of Slit-Robo signaling (Fan et al. 2003; Hakeda-Suzuki et al. 2002). In *Drosophila*, specific Rac GAPs and GEFs directly link the Robo receptor to the regulation of Rac activity during axon repulsion at the midline, as well as during tracheal cell migration. Genetic analysis indicates that the conserved Rac GAP, Vilse/CrGAP, contributes to Slit-dependent guidance decisions in both CNS axons at the midline and in tracheal cells (Hu et al. 2005; Lundstrom et al. 2004). Interestingly, in axons, high levels of Vilse overexpression causes similar defects to *robo* loss of function and low levels of overexpression cause dosage-dependent defects in Slit-Robo repulsion similar to loss of function of *vilse* (Hu et al. 2005). Thus, the consequences of increasing or decreasing *vilse* function are similar: Both lead to a compromise in the efficiency of Slit-Robo midline repulsion, suggesting that the precise levels or spatial distribution of *vilse* Rac GAP activity may be instructive for Robo repulsion.

Although regulation of the GAP activity of Vilse/crGAP could potentially account for the observed increase in Rac-GTP following Robo receptor activation, *vilse* mutants lead to only subtle defects in midline repulsion. Considering that Rac activity is required for midline repulsion in the *Drosophila* CNS, additional regulators should link Robo to Rac activation in these neurons. The dual Ras-Rho GEF Sos is a likely candidate, based on its CNS expression, genetic interaction with *slit* and *comm* mutants (Fritz and VanBerkum 2000), and its interaction with the adaptor protein Nck (Dock in *Drosophila*), which binds to Robo and recruits Pak in *Drosophila* (Fan et al. 2003). Indeed, genetic evidence supports a role for *sos* in Robo signaling and Sos interacts biochemically with Robo via binding to the adaptor, Dock. In response to Slit treatment, the normally cytoplasmic Sos is recruited to the plasma membrane in cultured human 293T cells, where it colocalizes with Robo and initiates membrane ruffling and lamellopodia formation (Yang and Bashaw 2006).

Based on this work and that described for forward Eph receptor signaling and reverse Ephrin ligand signaling, there are considerable parallels in how these repulsive guidance pathways regulate Rac activity. Both the Slit-Robo and the forward Eph pathway use a Rac GAP (Vilse for Robo and α-chimaerin for Ephs) and a Rac GEF (Sos for Robo and Vav for Ephs) to mediate repulsion. A comparison of Robo signaling with ephrinB reverse signaling during axon pruning reveals that a complex of proteins including the adapter Nck (Dock in *Drosophila*), Pak, and Rac are recruited to the receptors to mediate repulsive signaling. Coordinated action of GEFs and GAPs may promote the cycling of Rac activity, which may be more important than the overall levels of Rac-GTP in a responding growth cone. Alternatively, these GAPs and GEFs may act in distinct signaling steps, as in the example of Eph receptors in which Vav-family GEFs mediate endocytosis of the ligand-receptor complex through Rac activation (Cowan et al. 2005). Rac activation in the case of Robo receptors may precede internalization as well, as intracellular accumulations of Sos and Robo have been observed in cultured cells (Yang and Bashaw, unpubl.).

KINASE CASCADES

Src family kinases (Src, Fyn, Yes, and others; collectively known as SFKs) are nonreceptor

protein tyrosine kinases that have emerged as essential mediators of various guidance receptors. SFKs are critical for in vitro repulsive guidance of retinal ganglion and cortical axons downstream of EphA receptors, possibly mediating tyrosine phosphorylation of ephexin (Knöll and Drescher 2004; Zimmer et al. 2007). SFKs appear to be required in motor (LMC) axons for limb trajectory selection. Redirection of LMC axons by overexpressing either EphA4 or EphB2 was attenuated by inhibition of SFK function, suggesting that SFKs are critical for Eph forward signaling in vivo (Kao et al. 2009). Recruitment and activation of SFKs have been documented downstream of reverse signaling via GPI-anchored ephrinA ligands (Davy et al. 1999). Work in the moth *Manduca sexta* has shown that repulsion of migratory enteric neurons at the enteric midline is controlled by reverse signaling via the single GPI-anchored ephrin and that this process requires SFK function (Coate et al. 2009). The link between GPI-anchored ephrinAs and SFKs may be provided by transmembrane proteins such as $p75^{NTR}$ and TrkB (Lim et al. 2008; Marler et al. 2008). At least in stably transfected cells, the presence of $p75^{NTR}$ enhanced ephrinA reverse signaling as witnessed by elevated phosphotyrosine and Fyn protein levels (Lim et al. 2008).

SFKs and focal adhesion kinase (FAK) are also essential mediators of Netrin signaling. On Netrin binding to DCC/Neogenin, Src/Fyn and FAK are recruited to DCC, become activated by autophosphorylation, and induce tyrosine phosphorylation of the DCC cytoplasmic tail. Suppression of SFKs or FAK impaired neurite outgrowth and attractive turning responses of spinal cord and cortical neurons (Li et al. 2004; Liu et al. 2004; Meriane et al. 2004; Ren et al. 2004). Because SFKs and FAK have pleiotropic roles downstream of many receptor systems, the in vivo relevance for these observations remains to be established. At least SFKs have been placed in the Netrin signaling pathway controlling cell migration by genetic studies in *C. elegans* (Itoh et al. 2005). An important SFK substrate, $p130^{CAS}$ (Crk-associated substrate), is required for Netrin-induced neurite outgrowth and axon attraction of cortical and spinal cord neurons (Liu et al. 2007). $p130^{CAS}$ appears to act downstream of SFKs and upstream of the small GTPases Rac1 and Cdc42 (Liu et al. 2007). Fyn and Cdk5 also mediate Plexin-A/Npn1 signaling for growth cone collapse in DRG neurons. Moreover, the defects in cortical dendrite projections observed in Sema3A −/− mice were enhanced in Sema3A −/−; fyn −/− mice, suggesting that a signaling complex of PlexinA/SFKs/Cdk5 mediates Sema3A-induced guidance of cortical projections (Sasaki et al. 2002). Mutant *Drosophila* flies lacking endogenous SFKs (Src64B and Src42A) display commissure formation defects similar to those observed in *Wnt5* and *RYK/Derailed* mutants. Complex formation between Derailed and Src leads to the activation of Src kinase activity and Derailed phosphorylation, suggesting that SFKs are mediators of Wnt5/Derailed signaling (Wouda et al. 2008). The morphogen Sonic hedgehog (Shh) mediates cell fate specification and axon guidance in the developing nervous system by two distinct pathways. Cell fate specification by Shh is mediated by the receptor Patched (Ptc) via the canonical pathway requiring the Gli family of transcription factors. In contrast, axon guidance by Shh is mediated by SFK in a Smoothened-dependent manner via a rapidly acting, noncanonical signaling pathway not requiring transcription (Yam et al. 2009).

UPSTREAM REGULATORY ROLES FOR SECOND MESSENGERS AND RHO GTPases

In addition to their critical contributions to downstream signaling, many recent studies have shown that second messengers and Rho GTPases can also influence guidance responses by regulating the surface localization and activation of guidance receptors (Allen and Chilton 2009). For instance, netrin stimulation leads to an increase in DCC surface levels and this effect is enhanced by protein kinase A (PKA) activation. Blocking either adenylate cyclase, PKA activity, or exocytosis prevents the increase in DCC surface levels and blunts netrin-induced axon outgrowth (Bouchard et al. 2004). In

addition to PKA's role in regulating DCC, recent findings indicate that netrin-dependent inhibition of Rho activity also contributes to DCC membrane localilization (Moore et al. 2008a). Furthermore, genetic approaches in *C.elegans* have established that the trafficking and polarized localization of netrin and Slit receptors is critical for proper direction of axon outgrowth. Specifically, mutations in either the UNC-73 Trio-family RacGEF, its substrate the MIG-2 Rac small GTPase, or the VAB-8 kinesin-related protein disrupt the normal localization of the SAX-3 (Robo) and UNC-40 (DCC) receptors (Levy-Strumpf and Culotti 2007; Watari-Goshima et al. 2007). These perturbations in normal receptor localization lead to significant defects in Slit and netrin-dependent posterior oriented cell and growth cone migration, and further emphasize important upstream regulatory roles for Rho GTPases in the control of axon guidance receptor localization (Levy-Strumpf and Culotti 2007; Watari-Goshima et al. 2007). This unexpected upstream regulatory role must be carefully weighed when considering the outcome of Rho GTPase manipulations on axon guidance, especially because many earlier studies have assumed that by their very nature as regulators of the actin cytoskeleton that the Rho GTPases function exclusively as effectors of guidance signaling.

CONCLUDING REMARKS

The past decade has seen substantial progress in describing the mechanisms required to transmit axon guidance receptor signals. Although details of signaling pathways continue to emerge, our understanding of the key ligand-regulated events that control receptor activation and signaling is still fragmentary. Future progress will require the development of biochemical and optical strategies to uncover the dynamic changes in multi-protein signaling complexes that are triggered by guidance receptor activation. For instance, genetically encoded optical reporters for active forms of Rho GTPases, their effectors, and other signaling molecules will be instrumental in understanding the spatial and temporal dynamics of receptor signaling in vivo. It is also clear that many signaling and additional regulatory components await discovery, and biochemical, molecular, and genetic approaches in diverse experimental systems will continue to fill in the gaps in our knowledge.

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