Axon guidance: receptor complexes and signaling mechanisms

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The generation of a functional neuronal network requires that axons navigate precisely to their appropriate targets. Molecules that specify guidance decisions have been identified, and the signaling events that occur downstream of guidance receptors are beginning to be understood. New research shows that guidance receptor signaling can be hierarchical – one receptor silencing the other – thereby allowing navigating growth cones to interpret opposing guidance cues. Among the known intracellular signaling molecules shared by all guidance receptor families, Rho GTPases appear to be primary regulators of actin dynamics and growth cone guidance. Novel effector molecules complete the picture and suggest additional signaling mechanisms.

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Abbreviations

Abl	Abelson kinase
CNS	central nervous system
CST	corticospinal tract
DB	DCC-binding
DCC	deleted in colorectal cancer
DRG	dorsal root ganglion
Ena	Enabled
Ephexin	Eph-interacting exchange protein
FAK	focal adhesion kinase
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
HGF	hepatocyte growth factor
MAPK	mitogen-activated protein kinase
Mtl	*Mig-two-like*
OTK	off-track
PAK	p21-activated kinase
PDZ	PSD95/Discs large/ZO-1
RGS	regulator of GTPase signaling
Rnd1	Round 1
ROCK	Rho-associated kinase
Robo	Roundabout
SCG	superior cervical ganglion
SDF-1	stromal cell-derived factor 1
SFKs	Src family kinases
VASP	vasodilator-stimulated phosphoprotein

Introduction

Navigating axons express receptors for guidance molecules presented by neighboring cells. Activation of and subsequent signaling by these receptors determines whether or not the axon grows towards a target (attraction) or away from it (repulsion) [1]. Basic guidance principles are conserved throughout evolution, so that different approaches — genetic, cell and molecular biological, and biochemical — in different organisms — from nematodes to mammals — have synergized to push our understanding of axonal guidance forward at amazing speed. At least four conserved families of guidance molecules with important developmental functions have been identified: semaphorins, Slits, and netrins, which are all secreted molecules (except for some semaphorins), and ephrins, which are tethered to the plasma membrane (reviewed in [2–11]). The functions of these axon guidance molecules are not limited to axon guidance, as Slit plays important roles in mesodermal cell migration [12,13], ephrins are important in somitogenesis, vasculogenesis (reviewed in [9]), and synaptic plasticity [14–16], and semaphorins are crucial for heart and bone development [17]. Ligand activities are transduced by receptors and their signaling effectors. This review focuses on recent advances made in our understanding of receptor crosstalk and signaling mechanisms.

Semaphorins

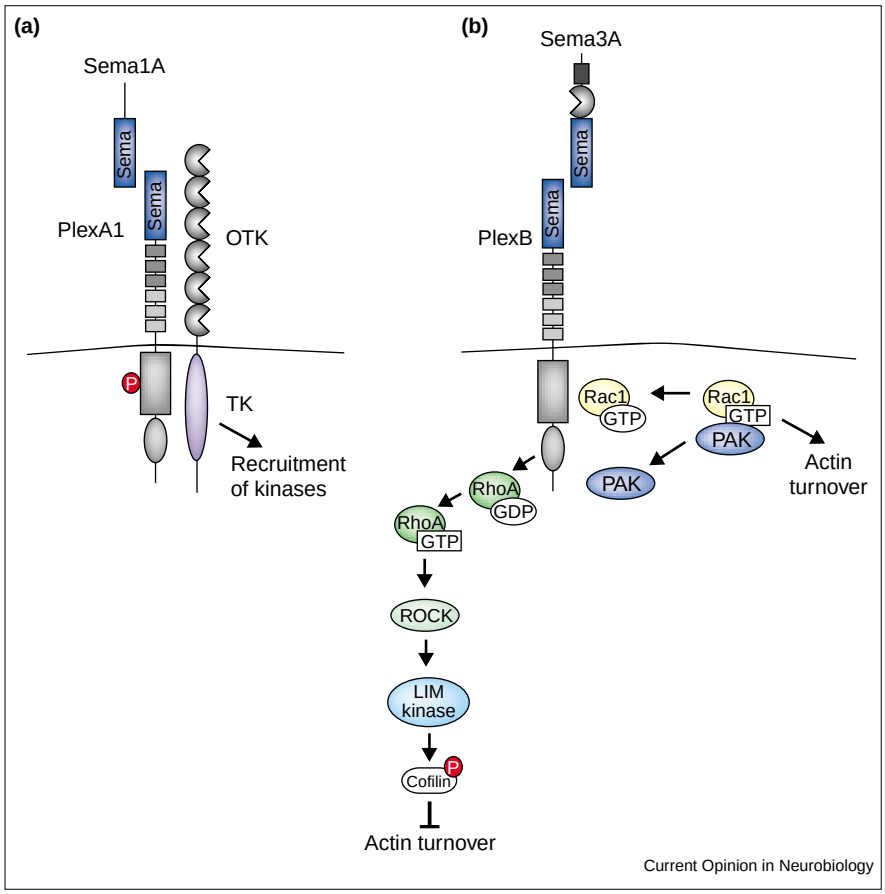
Semaphorins comprise a large, 20 member family of soluble and membrane-tethered molecules that are critically involved in axon guidance both in invertebrates and in vertebrates. They share a conserved, 500 amino acid long region: the 'Sema' domain. Many neuronal cells respond to semaphorins including sympathetic, motor, cerebellar, hippocampal, olfactory, corticospinal and dorsal root ganglion (DRG) neurons (reviewed in [18]).

Repulsive signals elicited by semaphorins are exerted by two receptor families: neuropilins — NP1 and NP2 — and plexins — subdivided into four subfamilies: PlexA1–4, PlexB1–3, PlexC1, and PlexD1 (reviewed in [3]). Many semaphorins bind directly to plexins and activate cytoplasmic signaling cascades. The well characterized class 3 semaphorins (Sema3A–3F) require neuropilins as coreceptors for plexin signaling.

Plexins

Plexins are transmembrane proteins with no obvious catalytic properties themselves. Interestingly, plexins possess a Sema domain at their amino (N) terminus, in a manner similar to their semaphorin ligands. In addition to the 'Sema' domain, Plexins contain a cysteine-rich motif in their extracellular region and a conserved plexin-specific sex-plexin domain in their cytoplasmic tail (reviewed in [3]). Work from last year [19•] showed that the plexin Sema domain is autoinhibitory. Coexpression of PlexA1 or PlexA2, but not PlexA3, with the Sema3A/NP1/2 complex mediates a cellular response, such as contraction, of COS cells [19•]. Deletion mutants of PlexA1 lacking the Sema domain constitutively signal when introduced into COS cells or primary DRG neurons. Cotransfection of the Sema domain or addition of the purified PlexA1 Sema domain protein to the medium reversed this effect. This suggests that the intramolecular interaction of the Sema domain of

Figure 1



Possible scenarios on how semaphorins signal repulsion. **(a)** Drosophila Sema1A signals through PlexA1, which becomes phosphorylated and associates with a receptor tyrosine kinase renamed OTK. OTK has all the structural hallmarks of a tyrosine kinase (TK), but is catalytically inactive and may have to associate with other tyrosine kinases. **(b)** Sema3A can signal through PlexBs. RhoA seems to be the major signaling output. RhoA interacts with the more C-terminal part of the conserved cytoplasmic region of PlexB in its active (GTP-bound) and inactive (GDP-bound) state. Upon activation of RhoA, ROCK is activated, turns on LIM kinase, which in turn phosphorylates cofilin. The growth cone retracts. Rac1 signaling appears to be antagonized by sequestering Rac1 GTP away from PAK. Note, however, that earlier studies suggested that downstream of PlexAs, Rac may be the critical GTPase mediating growth cone collapse [22]. Arrows represent positive actions; T-bars represent inhibitory actions; P, phosphorylation.

PlexA1 with its ectodomain silences PlexA1 signaling. Autoinhibition is released by Sema3A ligand binding.

In a manner comparable to many other receptors for extracellular ligands, plexins become phosphorylated upon ligand binding. The obvious lack of a plexin kinase domain led Winberg *et al.* [20•] to investigate potential kinases responsible for this process. A candidate gene approach in *Drosophila melanogaster* hinted to a previously described protein tyrosine kinase receptor of unknown function, Dtrk, so-called because of its sequence homology to mammalian neurotrophin receptor tyrosine kinases of the Trk family [21]. In fact, Dtrk is not a homolog of TrkA and was subsequently renamed 'off-track' (OTK) due to the observed axon guidance defects in *otk* fly mutants [20•]. Winberg *et al.* [20•] showed that OTK receptors, when overexpressed in mammalian COS cells, constitutively associate with *Drosophila* and mammalian plexins, suggesting a role for OTK as a receptor for semaphorin (Figure 1a). The axon guidance defects observed in loss-of-function mutants of *otk* resemble those of PlexA and Sema1A and, most importantly, genetic suppression studies indicate that OTK functions downstream of Sema1A. However, even though first identified as a kinase [21], due to various mutations in conserved residues, OTK does not have an active kinase domain and may rather act as a docking protein for other kinases providing the link to phospho–tyrosine signaling.

Rho GTPases downstream of plexins

Rho GTPases provide another mode of signaling particularly appreciated in axon guidance. Several laboratories have provided biochemical and genetic evidence that Rho and its family members Rac1 and Cdc42 play distinct roles in semaphorin receptor signaling. Rho GTPases function as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound form. Early work gave the first indication that Rac1 may directly mediate F-actin reorganization downstream of Sema3A/PlexA1 [22]. In support of this finding, Fournier *et al.* [23] presented work showing that ligand-aggregated semaphorin receptors colocalize with Rac1 in growth cones during growth cone collapse (Figure 1b). Furthermore, more recent *in vitro* studies showed that the cytoplasmic domain of PlexB1 directly binds the Rac1 GTPase in a GTP-dependent manner ([24,25] and references therein; Figure 1b). Interestingly, Rac1 appears to selectively bind to mammalian PlexB1, *Drosophila* PlexB, and to a lesser extend to PlexB2, but fails to interact with PlexA2 or PlexD1.

A remarkable feature of Rho GTPases is their distinct impacts on the actin cytoskeleton of cultured cells.

Overexpression and clustering of PlexB1 in Swiss 3T3 cells induces a RhoA-like phenotype, rather than the expected Rac1-like phenotype, suggesting that RhoA is activated downstream of ligand-bound PlexBs [24]. Furthermore, using specific RhoA inhibitors, all cytoskeletal effects of PlexB1 were blocked. RhoA activation may require direct interaction with the PlexB intracellular domain. In fact, using sequential mutagenesis of the PlexB carboxyl (C) terminus, a larger amino acid stretch than that required for Rac1 binding was identified, which appears to directly recruit RhoA but, when deleted, leaves Rac1 recruitment unaffected [26••]. Interestingly, whereas PlexB selectively associates with $Rac1^{GTP}$, its binding to RhoA is GTP-independent. *In vivo*, PlexB signaling seems to downregulate Rac1 activity, as overexpression of PlexB enhances the dominant-negative Rac1 phenotype in motor axon guidance in the fly. On the other hand, genetic data suggests that Rho GTPases are activated via PlexB, thereby leading to a decrease in motility of the growth cone. In a model, Hu *et al.* [26••] illustrate how the relative balance between Rac1 and RhoA activities may give semaphorins fine control over the actin regulatory machinery. The high affinity of PlexB for Rac^{GTP} results in local sequestering of Rac^{GTP} and inactivation of its major output, the p21-activated kinase (PAK), which was previously implicated in axon guidance (reviewed in [27]). Inactivation of PAK may suppress lamellipodia formation at the semaphorin contact site. At the same time, PlexB binds RhoA and enhances its output by an as yet unknown mechanism (Figure 1b).

A link between Rac1/RhoA and the actin cytoskeleton may exist via LIM kinase, a serine/threonine kinase, which phosphorylates and inactivates cofilin, an actin-depolymerizing factor (reviewed in [28]; Figure 1). Interference with binding of LIM kinase to cofilin by a synthetic cell-permeable peptide suppresses Sema3A-induced growth cone collapse of DRG neurons. Furthermore, expression of a dominant interfering LIM kinase, which fails to be activated by either PAK or Rho-associated kinase (ROCK), suppresses the collapsing activity of Sema3A [29•]. Therefore, RhoA recruitment to Plexins may activate ROCK, which phosphorylates and thereby activates LIM kinase, which in its activated state suppresses cofilin-mediated actin depolymerization (Figure 1b).

Mouse mutant studies

Drosophila plexin mutants show clear and well-characterized axon guidance defects. Vertebrates possess nine plexins and the first genetic evidence implicating PlexA3 in axon guidance in mammals was recently reported [30]. In contrast to earlier studies in COS cells, in which PlexA3 failed to mediate class 3 semaphorin responses [19•], Cheng *et al.* [30] found that sympathetic superior cervical ganglion (SCG) neurons derived from *plexA3* mutant mice completely lost their repulsive responses to Sema3F, but not to Sema3A, *in vitro*. This suggests that PlexA3 is an essential component of the Sema3F receptor complex for guidance of SCG axons. In the same experiments [30], the responses of other neurons to Sema3F and Sema3A were partially impaired, indicating that PlexA3 is a required component in class 3 semaphorin function. *In vivo*, PlexA3 is crucial for proper targeting of a subset of hippocampal afferents, but less important for guidance of peripheral axons of the SCG and DRG. Experiments using chimeric receptors, consisting of the cytoplasmic domain of PlexA3 and the extracellular domain of the Met receptor expressed in *Xenopus laevis* spinal neurons, showed that PlexA3 signaling is sufficient to induce growth cone repulsion when stimulated with the Met ligand hepatocyte growth factor (HGF) [30]. This is in contrast to the normal attractive response of HGF mediated by wild-type Met.

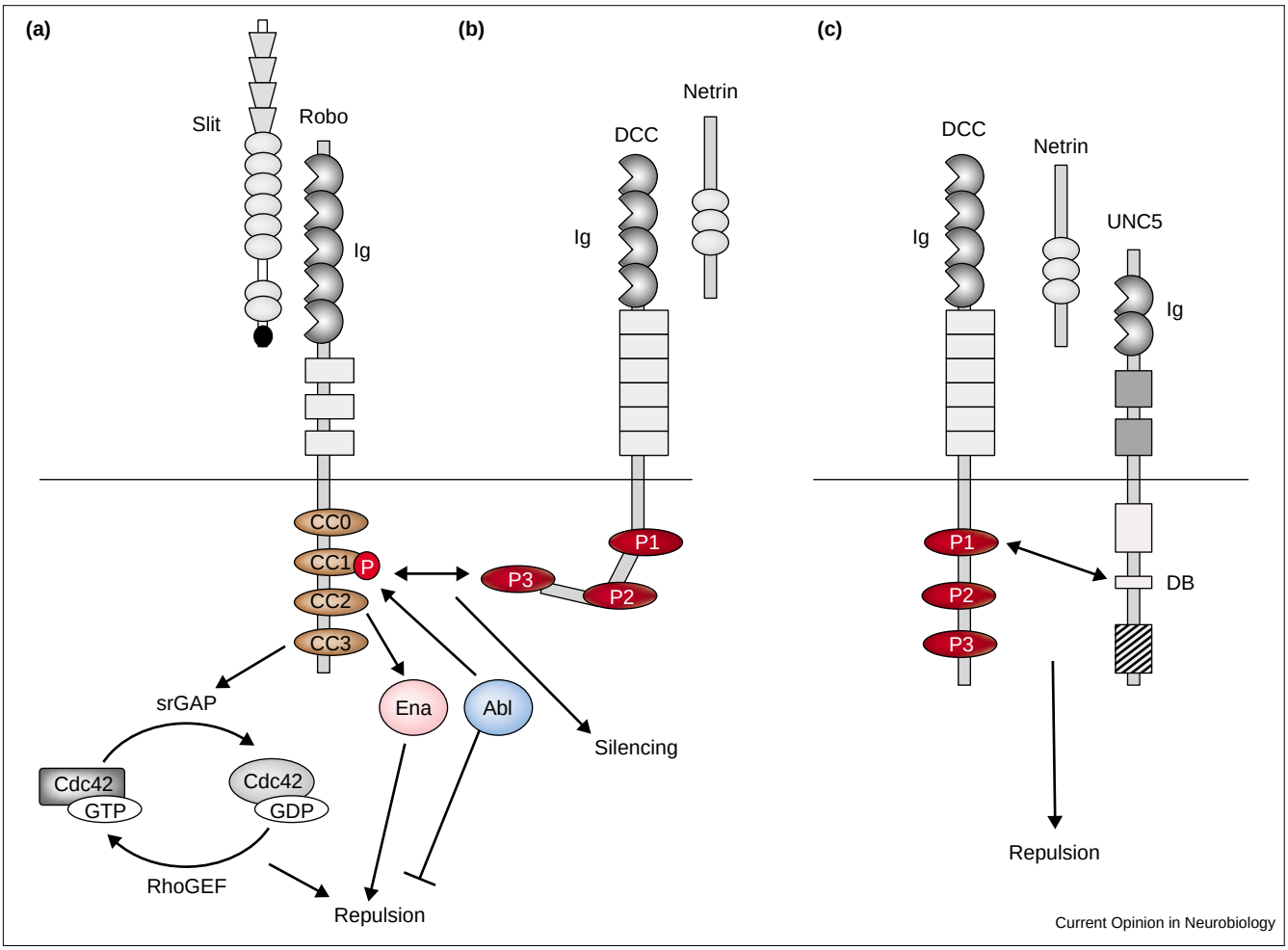
As we have mentioned earlier, the semaphorin receptors NP1 and NP2 are required for mediating the repulsive effects of class 3 semaphorins (reviewed in [2,3]). More recent work indicates that NP1/2 and their semaphorin ligands control neuronal migration in the central nervous system (CNS) [31]. However, the composition of functional NP/Plexin receptors is not known, because of the early lethality of mutant mice or the functional redundancy between family members. New genetic tools are required to dissect the components of semaphorin signaling in the mammalian nervous system.

Slit/Robo system

Neuropilin receptors are expressed by commissural neurons and are required to navigate commissural axons across the midline of the CNS to their rostral targets after midline crossing [32]. In this system, class 3 semaphorins act in concert with another class of repellent proteins, the Slits, to prevent commissural axons from recrossing or lingering at the midline. *Drosophila* Slit is a large extracellular matrix protein expressed by midline glia cells and acts as a long-range chemorepellent for axons and migrating muscle precursors ([33], see also [13]). Mammals have three *slit* genes (*slit1–3*) whose products also repel axons (reviewed in [5]). Slit2 is a heparan sulfate binding protein [34], which can also induce axon branching ([35], reviewed in [36]).

Slits act via Roundabout (Robo) receptors named after *robo* mutant flies, in which commissural axons cross and recross the midline in the manner of a roundabout. In a series of landmark papers published a couple of years ago, the laboratories of Dickson and Goodman showed that three Robo receptors in *Drosophila* are coexpressed in different combinations — the 'Robo code' — by axons after midline crossing. This code determines the medial–lateral position of axons within longitudinal tracts (reviewed in [4,6]). The *robo* gene family and the midline repulsion role of Robo protein are conserved from invertebrates to vertebrates (reviewed in [5]). Recent work has added novel functions for Robo2 in anterior–posterior axon guidance in the visual system in zebrafish [37]. Interestingly, the nematode *Caenorhabditis elegans* has only one Robo receptor (SAX-3), which appears to have both Slit-dependent and Slit-independent functions in development [38•].

Figure 2



Signaling by Slit and netrin receptors. **(a)** Slit-engaged Robo recruits Ena to CC2, which regulates actin dynamics and mediates repulsion. The Abl tyrosine kinase phosphorylates CC1 and antagonizes Ena. srGAPs are recruited to CC3 and antagonize Rho GEFs by transforming active Cdc42–GTP into Cdc42–GDP, thereby promoting repulsion of migratory cells. **(b)** Netrin binding to DCC receptors causes DCC clustering via its P3 domain and promotes attraction (not shown). In the presence of both Slit and netrin, Robo and DCC associate via CC1 in Robo and P3 in DCC. This heteromerization silences DCC-mediated attraction. **(c)** DCC also interacts with UNC5 via its P1 domain and the DB domain of UNC5. The DCC–UNC5 receptor complex signals repulsion. Arrows represent positive actions; T-bars represent inhibitory actions; P, phosphorylation.

How does Robo mediate the repulsive activity of Slit? The cytoplasmic tails of *Drosophila* and human Robo1 contain four short blocks of conserved cytoplasmic regions termed CC0–CC3, whereas human Robo2 and Robo3 only have two such repeats (CC0 and CC1). By studying genetic interactions in *Drosophila*, Bashaw *et al.* [39] found that the cytoplasmic Abelson (Abl) tyrosine kinase (reviewed in [40]) antagonizes Robo-mediated repulsion, most likely by phosphorylating and thereby modulating Robo function (Figure 2a). Mutation of the conserved Abl phosphorylation site in CC1 generates a hyperactive Robo receptor. Genetic studies also showed that two CNS-specific receptor tyrosine phosphatases are positive regulators of Slit/Robo repulsive signaling [41]. This is consistent with a suppressive role for tyrosine phosphorylation of Robo. Whether these phosphatases interact directly with Robo or Abl is not known. Bashaw *et al.* [39] also found that Abl and its substrate Enabled (Ena) have complementary roles in Robo signaling. Ena and vasodilator-stimulated phosphoprotein (VASP) belong to the proline-rich Ena/VASP protein family that regulates actin dynamics in response to signaling from the cell membrane (reviewed in [42]). Ena binds to the Robo CC2 repeat, which is absent in Robo2 and Robo3 [39]. Loss of *ena* function in *Drosophila* partially disrupts Robo signaling, indicating that Ena enhances Robo function, but it is not Robo's sole signaling output.

Recent work implicates Rho GTPases in Robo signaling, in a manner similar to plexins. As mentioned above, Rho GTPases cycle between an active, GTP-bound state, and an inactive, GDP-bound state. This cycle is regulated by GTPase-activating proteins (GAPs), which convert the GTP-bound forms to GDP-bound forms, and guanine nucleotide exchange factors (GEFs), which do the opposite.

Wong *et al*. [43••] identified a new family of GAPs, termed Slit–Robo GAPs (srGAPs) that directly interact with the CC3 region of Robo (Figure 2a). Intriguingly, overexpression of a dominant-negative srGAP1 blocked Slit-induced repulsion of migratory cells from neuronal explants. Mechanistically, Slit binding to Robo appears to enhance Robo/srGAP1 interaction and thereby specifically inactivates Cdc42 and RhoA, but not Rac1. Hence, expression of a dominant-negative srGAP1 suppresses this inactivation and blocks Slit-induced repulsion.

Netrins

The CNS midline not only expresses repulsive factors, such as Slits and semaphorins, but also a small family of secreted proteins, termed netrins, which attract commissural axons before midline crossing. Netrin-induced attraction is mediated by the DCC (deleted in colorectal cancer) family of receptors that include Frazzled in *Drosophila*, UNC40 in *C. elegans*, and DCC and neogenin in vertebrates [11]. Their cytoplasmic domains contain three regions of high sequence homology across species, named P1–P3. Netrin engagement causes multimerization of DCC receptors, mediated by the association of P3 regions, and leads to attraction and stimulation of axonal growth in an *in vitro* assay (reviewed in [7]). In an elegant study, Stein and Tessier-Lavigne [44••] demonstrated that Slit signaling via Robo can also silence netrin attraction via DCC receptors. They showed that the direction of axon navigation is not simply the result of the integration of attractive and repulsive signals across the growth cone, as was commonly proposed, but that Robo–DCC receptor association silences the netrin response. Activation of Robo by Slit induces association with DCC involving CC1 in Robo and P3 in DCC (see also [7]; Figure 2b). Exactly how this heteromeric interaction causes silencing is unclear. CC1 in Robo is also the site of Abl phosphorylation; it will be interesting to see whether Abl regulation of Robo activity affects Robo–DCC interactions or vice versa.

Switching the functions of guidance receptors by way of heteromerization appears to be a common theme. *In vitro*, netrin attraction via DCC receptors can be switched to repulsion, by the interaction of netrins with a second class of receptors of the UNC5 family, including *C. elegans* and *Drosophila* UNC5 and their three vertebrate homologues (UNC5H1–H3; reviewed in [45]). In this case, P1 in DCC and the DCC-binding (DB) motif in UNC5 mediate the association (Figure 2c). In the absence of DCC, UNC5 alone is able to mediate netrin repulsion. But there may be a qualitative difference between the signal elicited by UNC5 alone versus that elicited by UNC5 and DCC together. Recent genetic experiments in *Drosophila* by the Dickson laboratory suggest that long range chemorepulsion of netrins is mediated by UNC5/DCC receptor complexes, whereas UNC5 in the absence of DCC acts at short range [46].

Ephrins

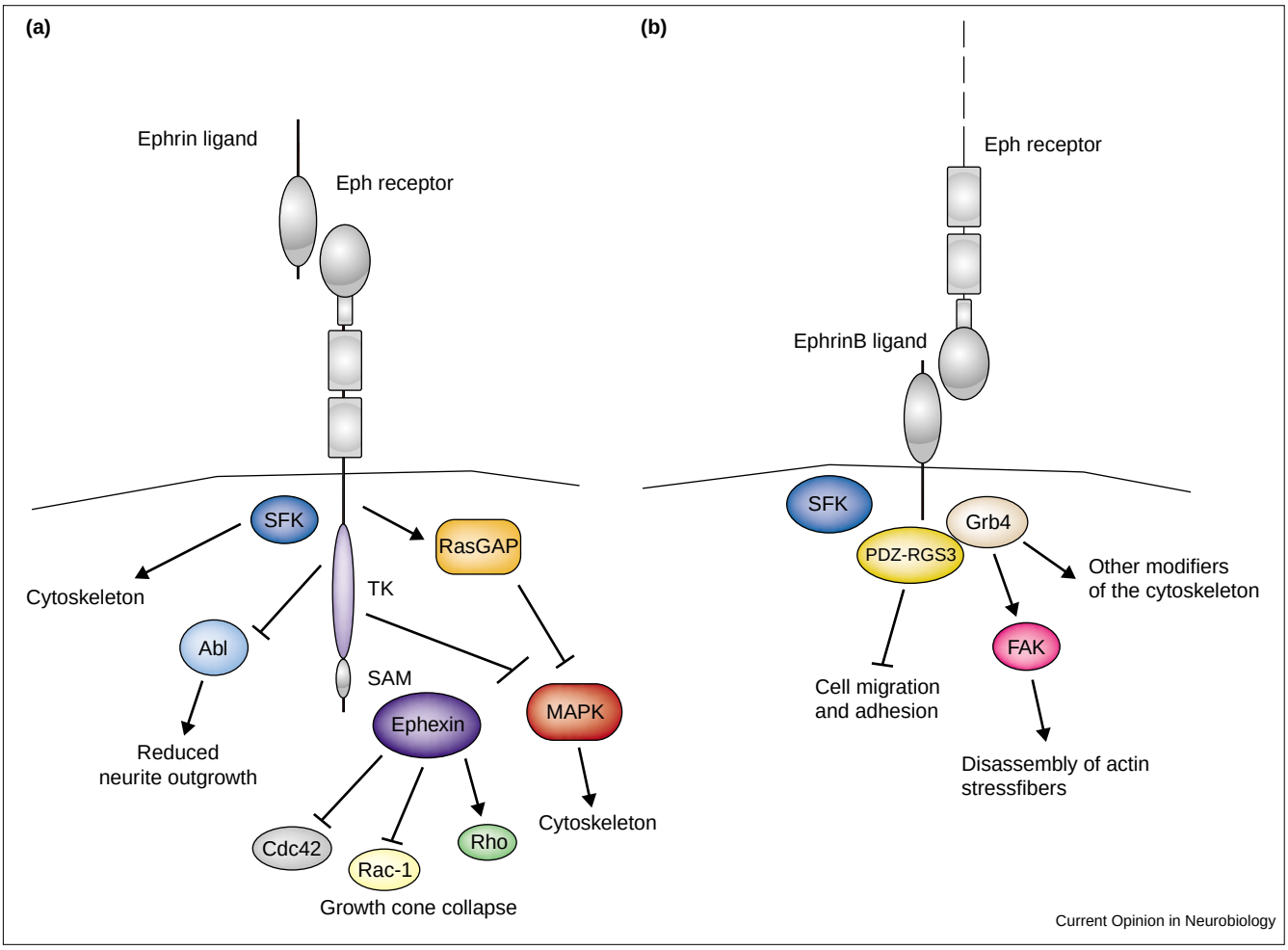
Ephrin ligands are tethered to the plasma membrane either by a glycosylphosphatidylinositol (GPI) anchor (all invertebrate ephrins, vertebrate ephrinA1–A5) or by a transmembrane segment (vertebrate ephrinB1–B3). Whereas invertebrates have only one receptor for ephrins, vertebrate ephrin receptors fall into two subclasses: EphA receptors (EphA1–A8) and EphB receptors (EphB1–B4), depending on whether they interact with A-type or B-type ephrins (reviewed in [9]). EphrinB ligands and EphB receptors are capable of eliciting 'bidirectional signaling' — the contact of a receptor-expressing cell with a ligand-expressing cell triggers signaling events in both cells, mediated by EphB forward signaling and ephrinB reverse signaling. This concept was introduced some time ago, as a result of the study of *EphB2* knockout mice [47] and the results of *in vitro* assays showing that ephrinB ligands become tyrosine phosphorylated upon binding to their respective receptors [48,49]. To date, however, we have not identified the crucial molecules for axon guidance downstream of Ephs and ephrins *in vivo*. In 2001, studies using different approaches [50,51•,52–55,56••,57–60,61•] critically advanced our understanding of how Eph receptors and ephrins may be regulated and how, in turn, they may regulate downstream events.

Mutant studies

EphA4 knockout mice jump like kangaroos, moving their hindlimbs simultaneously rather than in an alternating fashion [50]. This defective parallel walk correlates with deficient guidance of axons of the corticospinal tract (CST), a pathway that originates in the motor cortex, crosses the midline in the hindbrain (medulla), and projects into the spinal cord. In *EphA4*-null mice, axons aberrantly recross the midline freely and motor control is impaired. Knockin of kinase-deficient signaling mutants showed that EphA4 kinase activity is required for proper guidance of these axons [51•]. The knockout of ephrinB3, an EphA4 ligand, provided strong evidence that EphA4 interacts with ephrinB3, expressed at the midline, to prevent CST axons from aberrantly recrossing the midline [52,53]. Consistent with its cell autonomous role in the CST, EphA4 is expressed on CST axons, whereas axons of the anterior commissure (a pathway in the forebrain connecting the two hemispheres) express ephrin ligands. The anterior commissure is defective in *EphA4*-null mice, but is rescued by the kinase-dead version of *EphA4* mutants, suggesting that EphA4 can be both ligand and receptor *in vivo* [51•]. These experiments further suggest that ephrin reverse signaling is sufficient for anterior commissure formation, although at this point the identity of the EphA4 ligand involved in this process is not known [52,53].

Engagement of Eph receptors by ephrins causes receptor tetramerization [54] and activation of their intrinsic tyrosine kinase activity, followed by autophosphorylation of distinct tyrosine residues in their cytoplasmic domains, thereby serving as docking sites for effectors with phospho–tyrosine binding domains. Mutation of the two juxtamembrane tyrosines in EphB2 and EphA4 abolishes the kinase activity of the receptor, suggesting that these residues are somehow

Figure 3



Bidirectional signaling via Eph receptors and ephrin ligands. (a) Eph receptor activation through its ephrinA or ephrinB ligand leads to dimerization and subsequent tetramerization in a stochiometric ratio with the ephrin ligand. The clustered Eph receptor undergoes autophosphorylation, which leads to the binding and activation of downstream signaling molecules. Autophosphorylation of two juxtamembrane tyrosine residues releases an intramolecular interaction of the juxtamembrane domain with the tyrosine kinase (TK) domain, which is required for full receptor activation. Src family kinases are recruited to the juxtamembrane tyrosines and thereby activated. SFKs regulate cytoskeleton dynamics, which may be required for growth cone collapse. On the other hand, Abl tyrosine kinase activity is counteracted by the active Eph receptor thereby inhibiting Abl's positive activity on neurite outgrowth. Ras GAP, an inactivator of Ras GTPase, is recruited to Ephs and may in part be responsible for inhibition of MAPK activity, which reduces actin cytoskeleton motility. Ephexin, a novel Rho GTPase-specific GEF, is recruited in a ligand-independent fashion. Ephexin action counteracts Cdc42 and Rac but activates RhoA selectively. In concert, these signaling mechanisms induce collapse of an outgrowing growth cone. (b) B-type ephrins become tetramerized and tyrosine-phosphorylated upon binding to their EphB receptor (see Update). Tyrosine phosphorylation by SFKs allows recruitment of Grb4 and subsequent activation of a variety of modifiers of the actin cytoskeleton, including FAK, whose activation leads to the disassembly of actin stress fibres. The PDZ-binding motif of ephrinBs recruits PDZ–RGS3 in a receptor independent fashion. PDZ–RGS3 inhibits cell migration and adhesion. A-type ephrins may also be competent in reverse signaling by activating the Fyn TK [69] (not shown). Arrows represent positive actions; T-bars represent inhibitory actions; P, phosphorylation; SAM, sterile α motif.

involved in the regulation and activity of the Eph kinase domain [55]. X-ray crystallography of the EphB2 cytoplasmic domain showed that the EphB2 juxtamembrane region in its unphosphorylated state adopts a helical conformation, which distorts the small lobe of the kinase domain and thereby keeps it in an inactive state. Only upon phosphorylation of these juxtamembrane tyrosines do the loop folds open, relieving the repression and consequently allowing full activation of the kinase domain [56••]. As a consequence, mutating tyrosine residues *in vitro* and *in vivo* not only influences the range of molecules that are activated downstream of the EphB2 receptor but also critically changes the regulation of kinase activity [51•].

Eph effectors

Previous work, mainly using overexpression systems, identified a wide array of potential downstream targets of Eph receptors, including Src family kinases, RasGAP, phospholipase Cγ and others (reviewed in [49]). A unique feature of Eph among the family of receptor tyrosine

kinases is their inability to stimulate cellular growth and proliferation. A potential molecular basis of this phenomenon has recently been identified [57]. Using different cell lines and primary cells, Miao *et al.* [57] demonstrated that stimulation of EphA with ephrinA causes downregulation of mitogen-associated protein kinase (MAPK) levels, after it had previously been stimulated with other growth factors such as platelet-derived growth factor and vascular endothelial growth factor (Figure 3a). Because MAPK signals to the cytoskeleton, inhibition of MAPK may play a role in axon guidance. Similarly, yeast-two hybrid screening [58] identified Arg and Abl tyrosine kinases as new binding partners for EphB2 and EphA4. Stimulation of endogenous EphB2 with clustered ephrinB1 caused suppression of Abl activity, thus providing a conceivable explanation of why Abl promotes neurite outgrowth whereas Eph receptors cause growth cone collapse (Figure 3a).

Furthermore, again using the yeast-two hybrid assay, Shamah *et al.* [59••] identified Ephexin (Eph-interacting exchange protein), a novel GEF, which interacts with EphA receptors. As expected, Ephexin activated Rho family GTPases *in vitro*. However, using cultured embryonic neurons stimulated with clustered ephrinA, the authors found that although EphA activated RhoA GTPase, it conversely inhibited the activity of Cdc42 and Rac1 (Figure 3a). Overexpression of a dominant-negative form of Ephexin in retinal ganglion cells significantly inhibited ephrinA-induced growth cone collapse, suggesting that Ephexin signaling may be a crucial component of Eph receptor-mediated axon guidance.

EphrinB reverse signaling

Genetic data point to a crucial role for ephrinB reverse signaling *in vivo*, for example for commissure formation in the forebrain and for endothelial cell communication during angiogenic remodeling [47,51•,60]. The molecules downstream of ephrinB reverse signaling, however, have until recently remained elusive. EphrinB ligands have a 33 amino acid cytoplasmic tail with a PSD95/DLG/ZO-1 (PDZ) binding motif and five to six tyrosines, which become phosphorylated when Eph receptors bind ephrins (Figure 3b). PDZ–RGS3 (regulator of GTPase signaling 3), a novel regulator of heterotrimeric G-protein signaling, interacts with the ephrinB PDZ binding domain [61•]. Coinjection of ephrinB1 and PDZ–RGS3 into *Xenopus* embryos led to de-adhesion of the cells in a PDZ domain dependent manner (Figure 3b). Furthermore, migration of cerebellar granule cells by stromal cell-derived factor 1 (SDF-1), a ligand of the G-protein coupled receptor CXCR4, was inhibited by adding clustered EphB2–Fc receptor bodies. EphrinB signaling may downregulate the critical GEF activity required for efficient signaling of CXCR4. Recently, using a modified version of the yeast-two hybrid assay allowing screening for binding partners of phosphorylated ephrinB1, Cowan and Henkemeyer identified Grb4 (Nck2) as the first interactor of ephrins, whose interaction via its SH2 binding domain was dependent on tyrosine phosphorylation [62••] (Figure 3b). In addition to its SH2 domain, Grb4 also contains three SH3 domains, which bind to a group of proteins including those implicated in cytoskeleton regulation. Indeed, stimulation of cells overexpressing ephrinB1 activates focal adhesion kinase (FAK), causing cells to round up and F-actin stress fibers to disassemble (Figure 3b). Thus, ephrinB1 may exert its functions at least in part via recruitment of Grb4 and subsequent activation of proteins such as PAK, FAK, β-catenin, Paxillin and others [62••].

Conclusions and future directions

A variety of receptors and their respective ligands act in concert or against each other to achieve finely tuned axon guidance. Among the signals downstream of these receptors, Rho GTPases seem to have a crucial role in the regulation of actin dynamics. Several new molecules have been identified and add extra layers of intricacy to axon guidance mechanisms. However, at the same time, these new molecules hint to novel mechanisms for fast, local, and very precise responses of growth cones in different cell systems. Further studies and new creative approaches are required to reach the final goal of understanding how all these players dynamically act together to lead an axon to its distant targets.

Update

As described in more detail above, the binding of activated Rac^{GTP} to PlexB1 sequesters Rac and inhibits its binding to and subsequent activation of PAK [24,26••]. A more recent report by Vikis *et al.* [63] confirms these observations for the mammalian system downstream of Sema4D-activated PlexB1. Using overexpression experiments in COS and HEK293 cells, these authors demonstrate that the binding of activated Rac^{GTP} to PlexB1 additionally increases the cell surface expression of PlexB1 and leads to a slightly enhanced binding affinity for the Sema4D ligand. Thus, Rac could be involved in modulating plexin responsiveness to different semaphorins during axon guidance *in vivo*. PlexA receptors do not bind to Rac^{GTP} directly. An affinity screen using glutathione-*S*-transferase (GST)-fusion proteins identified RhoD and Round 1 (Rnd1) as interactors of PlexA1 [64]. The interaction of PlexA1 with Rnd1 induces growth cone collapse; the interaction with RhoD, in contrast, blocks this Rnd1-triggered effect. It is possible that Rnd1 versus RhoD recruitment fine-tunes the balance of RhoA versus Rac activity to induce growth cone collapse [64].

Rac GTPases regulate the actin cytoskeleton not only downstream of axon guidance receptors. In the fly, dominant mutant isoforms implicate Rac in many developmental processes involving actin cytoskeleton rearrangements. Two recent papers address the potential specificity of the three *Drosophila Rac* genes, *Rac1*, *Rac2*, and *Mtl* (*Mig-two-like*) [65••,66••]. Using loss-of-function mutants of the three genes, a specific requirement of Rac1 and Rac2 in myoblast fusion, Mtl in midline axon guidance, and Rac1

in mushroom body axon guidance is demonstrated [65••,66••]. Interestingly, upstream and downstream signaling of Rac GTPases varies depending on the developmental process in which a specific Rac GTPase is involved. The GEF Trio activates all three GTPases *in vitro*. *In vivo*, despite its widespread expression, Trio appears to act mainly upstream of Rac1 and Rac2 in axon guidance [65••]. A similar specificity is shown for the downstream signaling cascade; thus, PAK binding to Rac^{GTP} appears not to be required for axon growth but may play a role in guidance and branching of mushroom body axons [66••].

A fourth member of the Robo receptor family (Robo4) has recently been identified in the human and mouse genomes [67]. Its expression is predominant in endothelial cells, suggesting Slit/Robo interactions in angiogenesis.

Src family kinases (SFKs) have recently been shown to be positive regulators of ephrinB tyrosine phosphorylation and to be required for ephrinB reverse signaling, at least during ephrinB-mediated angiogenic sprouting *in vitro* [68••]. Furthermore, ephrinB ligands can recruit, with delayed kinetics, the cytoplasmic PDZ domain-containing protein tyrosine phosphatase PTP-BL. PTP-BL antagonizes the action of SFKs and dephosphorylates ephrinB. These data suggest the presence of a switch mechanism that allows a shift from phospho–tyrosine/SFK-dependent to PDZ-dependent ephrinB signaling.

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