CHAPTER FOUR

New insights into the molecular mechanisms of axon guidance receptor regulation and signaling

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Abstract

As the nervous system develops, newly differentiated neurons need to extend their axons toward their synaptic targets to form functional neural circuits. During this highly dynamic process of axon pathfinding, guidance receptors expressed at the tips of motile axons interact with soluble guidance cues or membrane tethered molecules present in the environment to be either attracted toward or repelled away from the source of these cues. As competing cues are often present at the same location and during the same developmental period, guidance receptors need to be both spatially and

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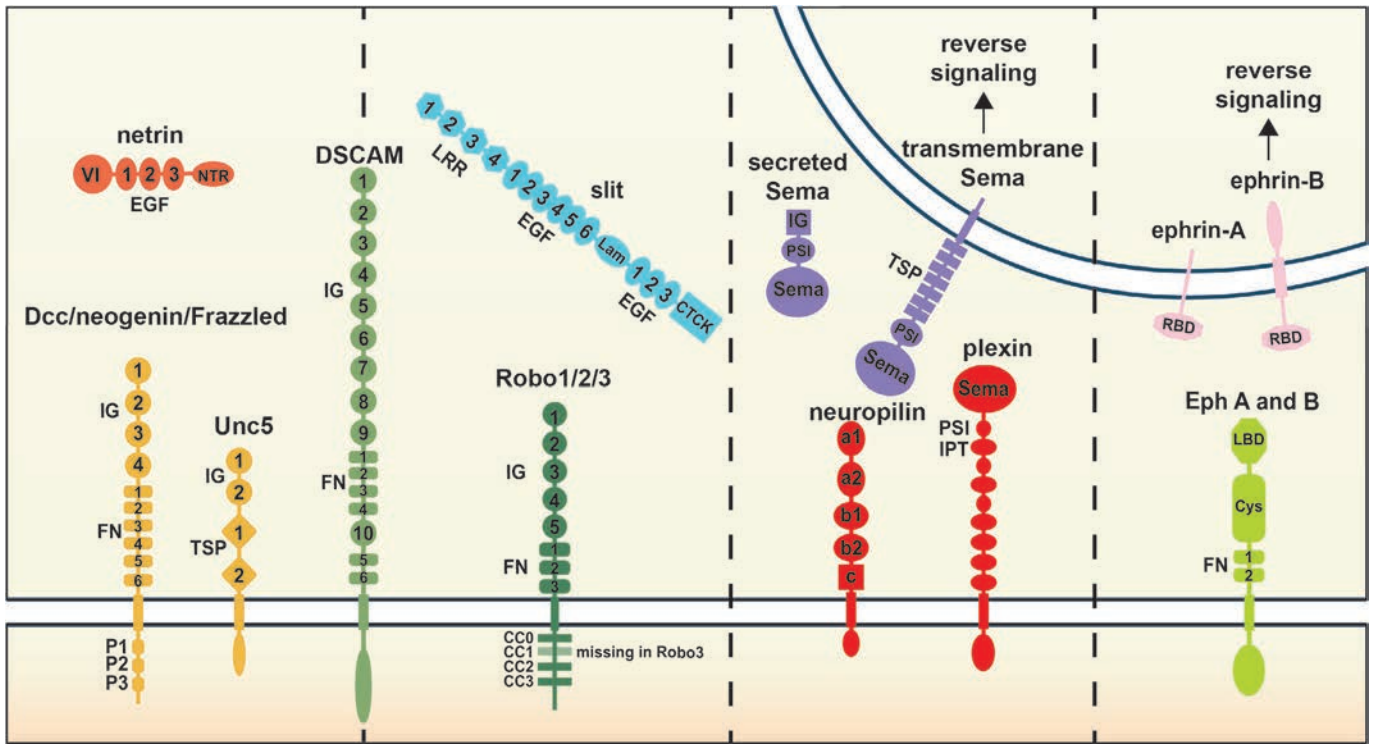
temporally regulated in order for the navigating axons to make appropriate guidance decisions. This regulation is exerted by a diverse array of molecular mechanisms that have come into focus over the past several decades and these mechanisms ensure that the correct complement of surface receptors is present on the growth cone, a fan-shaped expansion at the tip of the axon. This dynamic, highly motile structure is defined by a lamellipodial network lining the periphery of the growth cone interspersed with finger-like filopodial projections that serve to explore the surrounding environment. Once axon guidance receptors are deployed at the right place and time at the growth cone surface, they respond to their respective ligands by initiating a complex set of signaling events that serve to rearrange the growth cone membrane and the actin and microtubule cytoskeleton to affect axon growth and guidance. In this review, we highlight recent advances that shed light on the rich complexity of mechanisms that regulate axon guidance receptor distribution, activation and downstream signaling.

1. Introduction

More than 100 years ago, Ramon Y Cajal described the swellings at the tips of axons, which he named "growth cones" (Cajal, 1890). Based on observations of these structures in preparations of developing chick spinal cords, Cajal predicted that during development, growth cones would be dynamic and could respond to chemical cues released in the embryonic environment to be guided to the correct targets (Cajal, 1890). It was not until nearly a century later that conserved families of secreted and membrane-anchored cues and their neuronal receptors began to be identified (Tessier-Lavigne & Goodman, 1996). Initially, four major families of ligand receptor pairs were shown to influence axon growth and guidance in various contexts, including netrins and their Deleted in Colorectal Cancer (Dcc), neogenin and Uncoordinated-5 (Unc5) receptors (Kennedy, 2000), slits and their roundabout (Robo) receptors (Brose & Tessier-Lavigne, 2000), semaphorins and their plexin and neuropilin receptors (Pasterkamp & Kolodkin, 2003), and ephrins and their Eph receptors (Kullander & Klein, 2002) Fig. 1. The secreted and membrane-anchored cues were originally grouped into four rough categories, acting as attractants or repellents, at either short or long-range (Tessier-Lavigne & Goodman, 1996); however, these distinctions have blurred over the years, since it is now clear that many of these cues can trigger diverse and sometimes even opposite axon responses, depending on the receptor composition and intracellular properties of the responding growth cones (Bashaw & Klein, 2010). Nevertheless, in the majority of *in vivo* settings, slit-Robo, Sema-plexin and ephrin-Eph signaling result in axon repulsion, while netrin promotes attraction through its Dcc and neogenin



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receptors, and repulsion through its Unc5 family receptors. Additionally, it is now clear that many other families of ligand and receptors, including morphogens (BMP, Sonic Hedgehog, and Wnt), growth factors (VegF and Fgf) and their corresponding receptors also act to regulate axon guidance (Bashaw & Klein, 2010; Chedotal, 2019; Stoeckli, 2018).

In the first part of this review, we focus on regulatory mechanisms including receptor–receptor interactions and proteolytic processing where recent studies have shed light on how these mechanisms can diversify signaling outputs. Additionally, we discuss in detail the major advances that structural biological approaches have brought to our understanding of the diverse conformational landscape of surface receptor assemblies, and how in turn, this knowledge is driving insight into receptor activation mechanisms. In the second half of the chapter, we turn our attention toward intracellular signaling mechanisms, with a particular focus on advances in understanding the spatial and temporal control of signaling events. In addition to discussing how guidance receptors impinge on actin and microtubule regulatory proteins, we also describe new insights into the role of endosomes as signaling hubs in axon guidance. Lastly, we discuss new technologies that have been developed to allow investigators to probe spatial and temporal regulation and signaling in axon guidance at even greater resolution.

2. Mechanisms of axon guidance receptor regulation

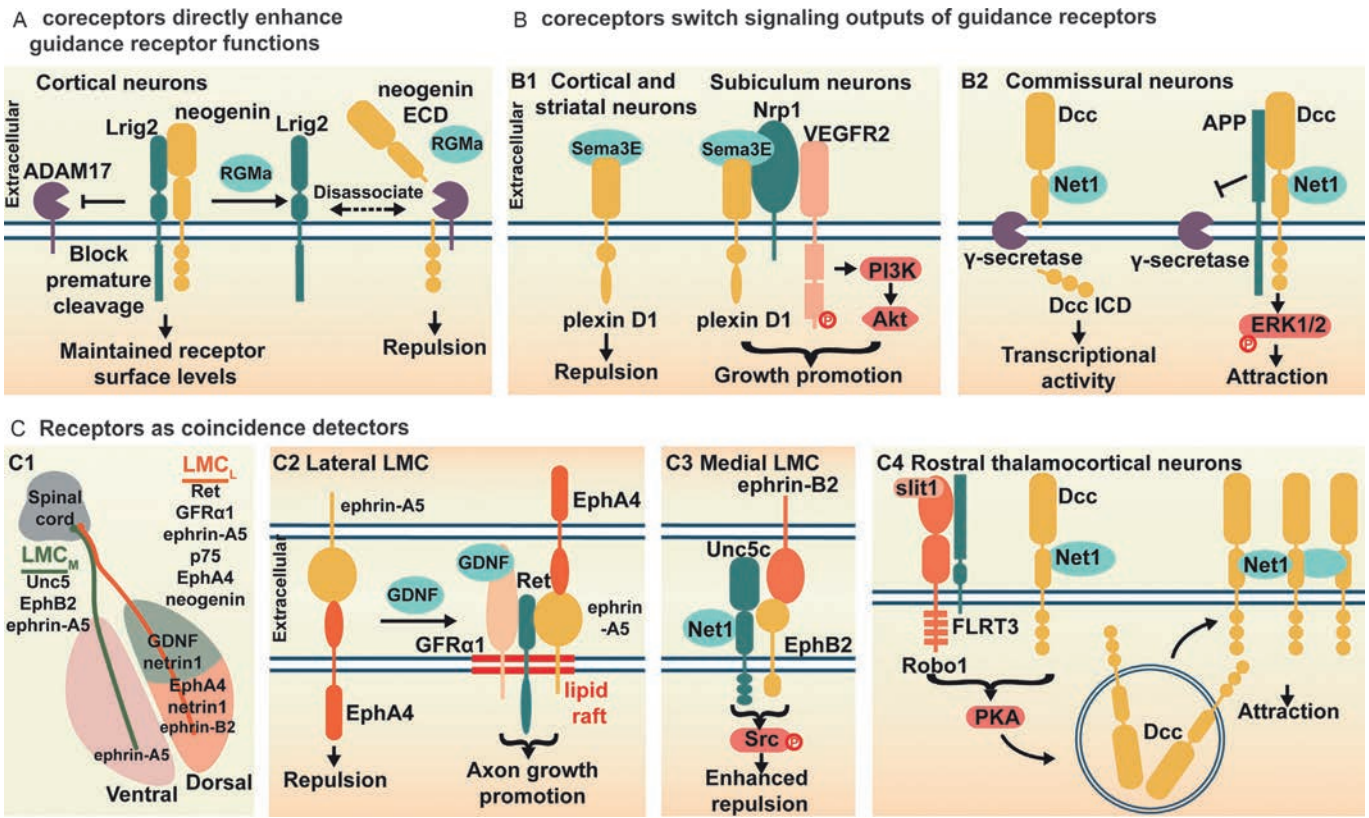
Axon guidance receptors can be regulated at multiple levels, including at the transcriptional, the translational and post-translational/protein levels to ensure the right complement of receptors are deployed at the right time and place. For example, the netrin receptor Dcc (Leggere et al., 2016; Saito et al., 2016), and all three of the Robo receptors for slit (Chen, Gore, Long,

Fig. 1 Domain structures of classical axon guidance receptors and their ligands. A schematic depicting axon guidance molecules discussed in this review: netrin and its receptors Dcc, neogenin, Frazzled and Unc5, slit and its receptors Robo1, 2, and 3, transmembrane and secreted semaphorins and their plexin and neuropilin receptors, and ephrin and its Eph receptors. DSCAM can interact with both netrin and slit. Transmembrane Semas and ephrins can also signal in reverse as receptors. CTCK, C-terminal cysteine knot; Cys, cysteine-rich domains; EGF, epidermal growth factor-like domains; FN, fibronectin type III domains; IG, immunoglobulin-like domains; IPT, immunoglobulin-plexin-transcription domains; Lam, laminin-type domains; LRR, leucine-rich-repeat domains; NTR, the netrin module; PSI, plexin-semaphorin-integrin domain; RBD, receptor binding domains; LBD, ligand binding domains; TSP, thrombospondin type 1 domains.

Ma, & Tessier-Lavigne, 2008; Johnson, Junge, & Chen, 2019; Zhuang et al., 2019) are alternatively spliced to produce receptor isoforms that may have distinct activities. In addition, microRNA and RNA binding proteins modulate the translation of guidance receptors, including Robo1, ephrin-B1 and neuropilin 1 (Nrp1) to control their availability (Arvanitis, Jungas, Behar, & Davy, 2010; Hornberg, Cioni, Harris, & Holt, 2016; Yang et al., 2018). Post-translational modifications also play key roles in regulating the surface distribution of receptors. For example, differential regulation of Robo receptor trafficking at the growth cone at low levels before and high levels after midline crossing precisely times axonal responses to slit to ensure the correct formation of midline circuits (Alther, Domanitskaya, & Stoeckli, 2016; Gorla et al., 2019; Kinoshita-Kawada et al., 2019). In another example, glycosylation of the Wnt receptor Frizzled3 regulates its trafficking to the growth cone surface, which mediates the anterior turning of post-crossing commissural axons at the floor plate in the developing mouse spinal cord (Onishi & Zou, 2017). These mechanisms of regulated receptor splicing, translation and trafficking to control axon responsiveness during guidance have recently been reviewed in detail (Gorla & Bashaw, 2020). In this section of the review, we instead focus on the regulation of axon guidance receptors at the post-translational/protein level, paying particular attention to the ways these intricate regulatory events impinge on the activation and downstream signaling pathways of axon guidance receptors.

2.1 Receptor-coreceptor and receptor-receptor interactions

The disparity between the relatively small number of guidance receptor-ligand pairs available and the overwhelming complexity of connections in the mature nervous system creates a major developmental challenge. One mechanism that has emerged to solve this dilemma is to employ coreceptors, which are transmembrane or membrane-anchored proteins that directly bind to guidance receptors, but not their ligands, to modulate and diversify receptor outputs (Fig. 2). Additionally, receptors with distinct ligands can interact with each other to modify and modulate signaling activity. Binding to coreceptors or other receptors can induce additional downstream signaling events or activate the same effectors but at different levels, to adjust guidance receptors' responses to extracellular stimuli in a context-specific way. Such accurate spatial-temporal control of guidance events is paramount for the proper assembly of neural circuits.



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Some coreceptors strengthen the signaling outputs of the guidance receptors with which they interact. For example, Leucine rich repeats and immunoglobulin like domains 2 (Lrig2) binds to neogenin in the developing mouse brain (Fig. 2A). Lrig2 is required for repulsive guidance molecule a (RGMa) induced growth cone collapse by blocking premature proteolytic processing of neogenin to maintain neogenin surface levels (van Erp et al., 2015). Binding of the RGMa induces dissociation of the Lrig2–neogenin complex, suggesting that ligand binding can regulate receptor–coreceptor interactions. Lrig2-dependent regulation of neogenin signaling controls cortical neuron migration and optic nerve regeneration, yet its significance in neogenin directed axon guidance in development remains to be seen.

Guidance receptors and their coreceptors can also undergo rapid and local phosphorylation or dephosphorylation, to allow for precise regulation and integration of guidance receptor signaling. In vertebrates, the divergent Robo3 receptor is essential for commissural axons to cross the midline. In *Robo3* knockout (KO) mouse embryos, dorsal commissural axons in the developing spinal cord display a complete failure of midline crossing, and initial explant culture and genetic analysis suggested that Robo3 promotes

Fig. 2 Regulation of axon guidance receptors via receptor-coreceptor interactions. (A) Coreceptors can enhance guidance receptor functions. In cortical neurons, in the absence of RGMa, Lrig2 binds to neogenin to block premature ADAM17-mediated proteolytic cleavage of the ECD of neogenin. Binding of RGMa initiates the dissociation of Lrig2 and neogenin, allowing neogenin ECD shedding and RGMa-induced neurite growth inhibition to occur. (B) Coreceptor binding can switch signaling outputs of guidance receptors. (B1) In cortical and striatal neurons, which do not express Nrp1, Sema3E acts as a repellent interacting with plexin D1. In subiculum neurons, Nrp1 recruits VEGFR2 to mediate a growth promoting response to Sema3E via activating the PI3K/Akt pathway. (B2) In spinal cord commissural neurons, APP binds to Dcc to inhibit γ-secretase dependent proteolytic cleavage of the Dcc ICD and its potential transcriptional activity, and to enhance netrin1-induced attraction via increasing the activation of ERK1/2. (C) Receptor-receptor interactions can also function as coincidence detectors to induce synergistic effects from two guidance cues. (C1) A schematic depicting the spinal cord, the lateral motor column spinal motor neurons that target the limb and the developing limb bud divided into ventral and dorsal halves. Expression of some genes either in the motor axons or the dorsal limb are shown. (C2) In medial LMCs that express EphA4, ephrin-A5 acts as a repellent. In lateral LMCs that express ephrinA5, co-detection of GDNF and EphA4 by the GFRα-Ret-ephrin-A5 receptor-coreceptor complex mediates axon growth promotion. (C3) Co-stimulation with netrin1 and ephrin-B2 leads to enhanced phosphorylation of Src kinase, which strengthens repulsion in medial LMCs mediated by the Unc5c-EphB2 complex. (C4) In rostral thalamocortical neurons, Robo1 binding to its coreceptor FLRT3 enables co-detection of slit1 and netrin1, which activates PKA to increase surface Dcc levels via enhanced vesicular transport, to facilitate attraction.

midline crossing by antagonizing Robo1-Robo2-slit mediated repulsion (Friocourt & Chedotal, 2017; Sabatier et al., 2004). Interestingly, Robo3's ability to inhibit repulsion is not, contrary to the initial models, due to competitive binding of slit, because mammalian Robo3 binds to slit2 with orders of magnitude lower affinity compared to Robo1 (Zelina et al., 2014). Moreover, *Robo3/slit1/slit2* triple KO and *Robo1/2/3* triple KO only partially rescue the Robo3-dependent loss of midline crossing, indicating that Robo3 likely functions in additional pathways independently of Robo1 and 2 to promote midline crossing (Jaworski, Long, & Tessier-Lavigne, 2010; Sabatier et al., 2004). Indeed, the Robo3 receptor forms a complex with Dcc to potentiate netrin-Dcc signaling, which requires tyrosine-phosphorylation of Robo3 in its conserved intracellular CC0 domain by Src-family kinases upon netrin1 stimulation (Zelina et al., 2014). *Robo3* mutants show migratory defects in precerebellar neurons, which can only be rescued by full-length Robo3 but not by Robo3 variants that cannot bind to Dcc or be tyrosine phosphorylated. This observation demonstrates the functional significance of Robo3 as a Dcc coreceptor; however, how netrin1 stimulation triggers Robo3 phosphorylation and what signaling machineries are assembled downstream remain unknown. Robo3 KO spinal cord explants also show significantly decreased axon outgrowth in response to bath application of netrin1 (Zelina et al., 2014). While this suggests that Robo3's ability to potentiate netrin1 signaling is important for axon outgrowth, whether Robo3 is also responsible for axon turning and axon guidance in this context has not been investigated.

Down syndrome cell adhesion molecule (DSCAM) is best known for its role in controlling dendritic self-avoidance through homophilic interactions mediated by a remarkable pool of diverse splice variants (Sachse et al., 2019). More recently another mode of action for Dscam has emerged from studies of *Drosophila* mechanosensory neurons (MSNs), where dephosphorylation of Dscam1 by the Receptor protein tyrosine phosphatase 69D (RPTP69D) is required cell-autonomously for the consolidation and extension of axon collaterals (Dascenco et al., 2015). Interestingly, RPTP69D's ability to interact with and regulate Dscam1's function is enhanced upon slit binding to Dscam1. Here slit might function independently of Robo, as Robo1 or Robo2 null mutants show no defect in MSNs axon collateral formation, although it remains possible that Robo1 and 2 might function redundantly. In addition, RPTP69D acts as a coreceptor for Robo3 to enhance its repulsive function by increasing Robo3 surface levels in *Drosophila* mushroom body small lateral neurons (Oliva et al., 2016). Interestingly, RPTP69D interacts

with Robo3 in a dephosphorylation-independent pathway, which suggests that RPTPs have two distinct modes of function in brain development, acting as either phosphatases or coreceptors for different guidance receptors.

Instead of enhancing or inhibiting the function of guidance receptors, some coreceptors introduce novel functional outputs by switching responses to guidance cues. For example, Nrp1, the coreceptor for plexin D1, acts as a molecular gate to switch semaphorin 3E (Sema3E)-mediated repulsion to growth promotion in the subiculo-mammillary axons in descending forebrain axon tracts in mice (Chauvet et al., 2007). Nrp1 promotes growth by recruiting and inducing the phosphorylation of another transmembrane receptor, vascular endothelial growth factor receptor type 2 (VEGFR2), leading to the activation of the phosphatidylinositol-3 kinase (PI3K) pathway downstream of VEGFR2 (Bellon et al., 2010). Similarly, in the mouse visual system, Ng-CAM-related cell adhesion molecule (Nr-CAM) forms a complex with plexin A1 that shifts the Sema6D response from repulsion to attraction in the contralateral projecting RGCs to facilitate chiasm crossing (Kuwajima et al., 2012). Unexpectedly, the chiasm cells and the contralateral projecting RGCs both express plexin A1 and Nr-CAM, and their expression in both cell types is important for switching the repulsive response of Sema6D. Finally, in the developing mouse spinal cord, amyloid precursor protein (APP) can interact with Dcc to enhance the activation and phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2), which are known Dcc effectors that mediate netrin-induced axon growth and turning (Rama et al., 2012). Biochemical experiments using rat neuroblastoma cell lysates overexpressing APP and Dcc indicates that APP might also inhibit γ-secretase cleavage of Dcc, a step essential for the transcriptional function of the *Drosophila* Dcc homologue Frazzled (Fra) (Neuhaus-Follini & Bashaw, 2015). Although yet to be tested *in vivo*, this observation raises the intriguing possibility that in some commissural neurons, APP could switch the Dcc response from the transcriptional regulation of target genes to the canonical Netrin-dependent regulation of the cytoskeleton.

Most studies to date only focus on the function of one ligand-receptor pair in a given guidance scenario. However, during normal development, most projecting axons encounter many different extracellular stimuli simultaneously at guidance choice points. To help navigating axons resolve and integrate the multitude of extracellular information, some receptors act as coincidence-detectors to elicit synergistic effects from two or more guidance signals, resulting in significantly elevated growth cone responses that are distinct from simple additive effects of two parallel events (for a review on the

interaction between different types of axon guidance pathways, see Morales & Kania, 2017). Such coincidence-detection reduces targeting errors, increases fidelity, and also significantly reinforces correct guidance decisions. This is perhaps best illustrated in motor axon targeting in the vertebrate limb. During development, motor axons projecting from the medial and lateral regions of the spinal cord lateral motor column (LMC) bifurcate at the base of the limb bud to form ventral and dorsal limb nerves, respectively (Fig. 2C1). The RTK Ret acts as a coreceptor for both GFRα1, the receptor for glial-derived neurotrophin factor (GDNF), and ephrin-A5, the reverse signaling receptor for EphA4, and Ret is essential for the targeting of lateral LMC axons *in vivo* (Bonanomi et al., 2012). In growth cone turning assays, co-stimulation with low levels of both GDNF and EphA4 can promote turning, whereas low levels of either GDNF or EphA4 alone produce no response. GDNF stimulation can recruit Ret to lipid rafts, suggesting that GDNF-GFRα1 signaling potentiates ephrin-A5 reverse signaling by bringing the co-receptor Ret within close proximity to the GPI-anchored ephrin-A5. Furthermore, in stripe assays, ephrin-A5 application sensitizes netrin responses in chick LMC explants by enhancing the protein levels of both its repulsive receptor EphA4 and the netrin receptor neogenin, suggesting a mechanism where a repulsive guidance cue potentiates the responses of an attractive guidance cue (Croteau, Kao, & Kania, 2019). The intracellular domain of EphA4 is dispensable for this function, yet it remains to be seen if the extracellular domains or transmembrane domains of EphA4 can directly bind to neogenin or if EphA4 indirectly increases neogenin levels. In medial LMC axons, co-stimulation of both netrin1 and ephrin-B2 can induce complex formation between their respective receptors (Unc5c and EphB2) and amplify repulsion through greater and prolonged activation of their common downstream effector Src kinase (Poliak et al., 2015). However, Src inhibition only partially blocks this growth cone collapse evoked by simultaneous netrin1 and ephrin-B2 application, indicating that additional unknown mechanisms exist to synergize Unc5c and EphB2 signaling. Further illustrating the point, in rostral thalamocortical axons, simultaneous detection of slit1 and netrin1 activates attraction via synergistic action of Robo1, its coreceptor fibronectin leucine rich transmembrane protein 3 (FLRT3) and the netrin receptor Dcc (Leyva-Diaz et al., 2014) (Fig. 2C4). FLRT3 forms a complex with Robo1 and transduces slit1 and netrin1 co-stimulation into an increase in Dcc surface levels, likely by promoting PKA-dependent mobilization of intracellular pools of Dcc.

The control of guidance receptor signaling by direct binding to coreceptors provides an exciting model for how developing neurons expand the functional outputs of guidance receptors. These recent studies demonstrate that besides recruiting additional intracellular effectors (Fig. 2B, C3, C4), coreceptors can also modulate the proteolytic processing (Fig. 2A, B2) and the localization (Fig. 2C2) of guidance receptors. Being transmembrane or GPI-anchored affords coreceptors regulatory capacity on both sides of the plasma membrane, so that coreceptors are uniquely poised to regulate guidance receptor signaling in a context dependent manner.

2.2 Structural insights into axon guidance receptor signaling: dimerization, oligomerization and clustering

Receptor activation entails far more than a one-to-one binding between a receptor and a ligand, but instead often calls for the recruitment of other receptor molecules to form complexes or clusters. In the last decade, integrated structural studies that offer crystallographic analysis coupled with cell-based or *in vivo* functional assays, have provided valuable insights into the 3D organizations of receptor complexes and how these structural features modulate guidance receptor activation and function. Here, we focus on four prominent families of axon guidance receptors, the plexins, Robos, Ephs and netrin receptors Dcc/neogenin/Unc5, as examples to describe how clustering and the oligomerization state of guidance receptors at the surface of the cell plays a pivotal role in relaying information across the membrane.

Plexins form autoinhibitory *cis*-homodimers to prevent premature activation in the absence of semaphorin binding. Crystal structures of the Sema-binding regions of plexin B1, A2 and C1 in complex with their respective ligands Sema4D, 6A, and 7A show essentially identical domain architectures in which a Sema dimer binds to two plexin molecules (Janssen et al., 2010; Liu et al., 2010; Nogi et al., 2010). The formation of this semaphorin-plexin interface is essential for plexin-mediated membrane collapse in COS-7 cells and growth cone collapse in chick dorsal root ganglion neurons. PlexA1, A2 and A4 form autoinhibitory homodimers in "closed" ring-like structures, which are mediated by the intermolecular interface between the Sema domain located at the tip of one plexin A "ring" and the ECD 4 and 5 situated at the "stalk" of another plexin A receptor (Kong et al., 2016; Marita et al., 2015). This structure remained undetected until the crystal structures of the entire extracellular domains (ECDs) of plexin As were determined, underscoring the idea that structural data involving fragments of molecules should be

interpreted with caution. Disrupting this plexin A-plexin A interaction in the absence of exogenous Sema stimulation, induces membrane collapse in COS-7 cells and dentate gyrus granule cell growth cones. Conversely, preserving this interaction eliminates collapse, supporting a model where plexin As are normally autoinhibited by *cis*-homodimerization (Kong et al., 2016). The amino acid residues at this interface are only highly conserved among vertebrate plexin As, but it remains possible that other classes of plexins employ similar autoinhibitory *cis*-interactions via alternative interfaces. In addition to preventing inappropriate activation of receptors, the pre-signaling association between two plexin As could potentially serve to position receptors within close proximity to each other to prepare for rapid and precise responses upon ligand binding.

The Robo receptor, on the other hand, autoinhibits its dimerization and downstream signaling by engaging in a compact "closed" conformation (Fig. 3B). The Ig4 domain in both Robo1 and 2 mediates receptor dimerization and is required for the Robo2 overexpression-induced membrane collapse phenotype in COS-7 cells (Aleksandrova et al., 2018; Yom-Tov et al., 2017). Crystal structures of the intact Robo2 ECD uncovered a "closed" hairpin-like conformation which blocks access to the Ig4 homodimerization interface (Barak et al., 2019). Additionally, it is also revealed that the Ig5 domain of Robo2 interacts with its Ig1 and 2 domains *in trans*, potentially mediating trans-inhibition via stabilizing the "closed" conformation of an opposing Robo2 receptor (Fig. 3B). These observations support a model in which slit2 binding to the Ig1 domain of Robo2 relieves trans-inhibition and shifts Robo2's conformation from "closed" to "open" to facilitate Robo2 homodimerization and signaling. This model is inferred by crystallographic analysis of superimposed slit-binding and trans-interacting Robo2 interfaces and has not been confirmed by co-crystals of Robo2 and slit2. An inhibitory trans-interaction between Robo receptors has also been observed in *Drosophila*, where Robo2 expressed on midline cells can bind to Robo1 expressed on crossing commissural axons to inhibit slit-mediated repulsion (Evans, Santiago, Arbeille, & Bashaw, 2015). However, whether the formation of this heterodimer also relies on the same Ig5-Ig1/2 binding interface remains unclear. While *Drosophila* Robo2 shares similarities with both vertebrate Robo1 and 2, equivalent trans-homo or -hetero dimers of Robo receptors have not been identified in vertebrates. The ECD of the divergent vertebrate Robo3 receptor, which has identical domain structure with that of Robo1 and 2, assumes a fully extended "open" conformation and exists exclusively in monomeric state in the absence of ligand binding (Pak et al., 2020).

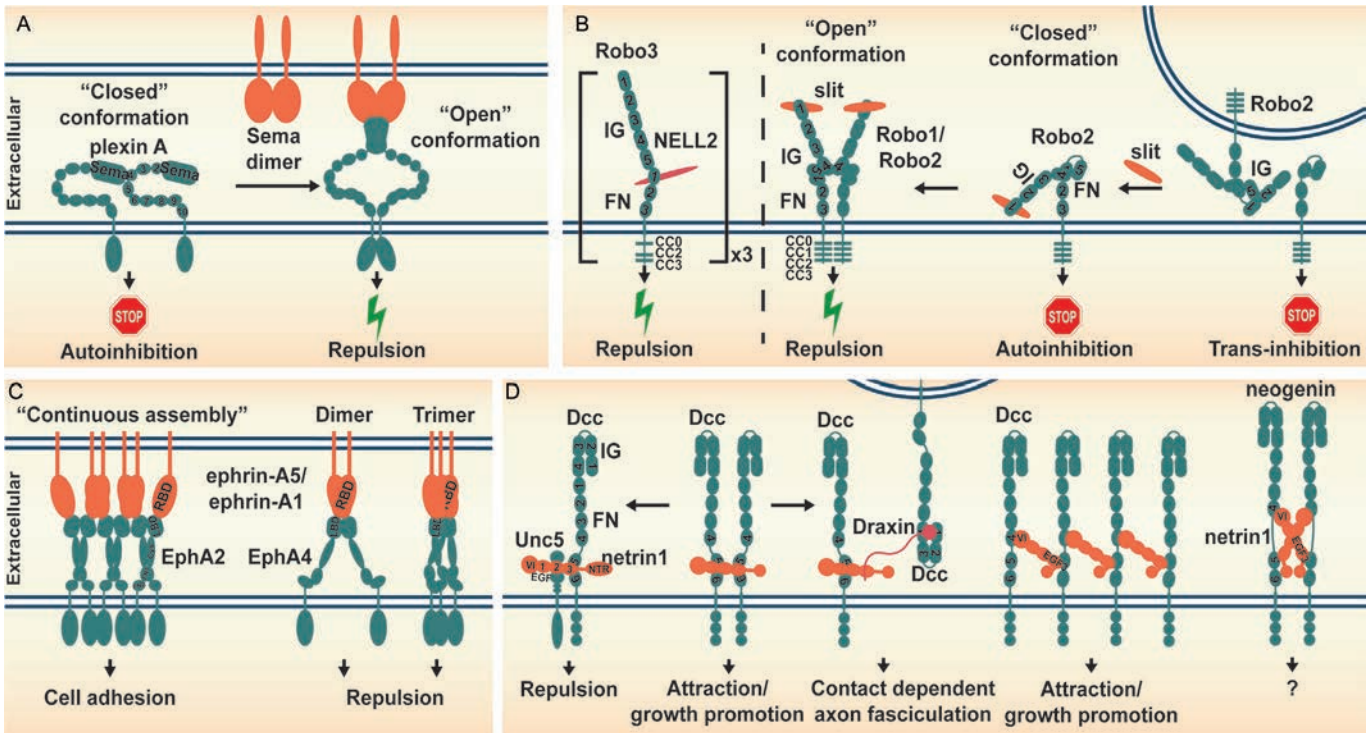

Fig. 3 See figure legend on next page.

Robo3 interacts with its ligand NELL2 via its FN1 domain. Exchanging the FN1 domain of Robo1 with that of Robo3 converts the predominantly dimeric Robo1, typically in a more compressed conformation, into a strictly monomeric molecule with an elongated "open" shape, indicating that the FN1 domains of Robos determine both their oligomerization and conformational characteristics. Future analysis that identifies the functionally relevant residues and interfaces within the FN1 domain of Robo3, that are not conserved in Robo 1 and 2, will provide important information on how different Robo receptors are regulated.

Different Eph receptors form disparate assemblies at the membrane to instigate distinct signaling pathways. Crystal structures suggest that full length EphA2 ECDs, either alone or in complex with the receptor binding domain of ephrin-A5 or -A1, could cluster to form a highly ordered oligomer that exists as a continuous array on the membrane (Himanen et al., 2010; Seiradake, Harlos, Sutton, Aricescu, & Jones, 2010). Restricting lateral movement of EphA2 using a physical barrier eliminates the formation of EphA2 arrays and blocks the cellular responses to ephrin-A1, as indicated by the decreased recruitment of signaling effectors and altered cytoskeletal morphology (Salaita et al., 2010). Thus, the ephrin-induced array-like arrangement of EphA2 is functionally important for signal propagation. However, large signaling arrays are not detected for all Ephs. EphA4 and EphB2, which mediate repulsion instead of cell-adhesion like EphA2, form smaller clusters

Fig. 3 The dynamic conformational landscape and multimerization state of axon guidance receptors at the membrane modulate their signaling outputs. (A) Plexin A receptors form an auto-inhibitory dimer, which can shift into an "open" shape upon binding to a Sema dimer, to mediate repulsion. (B) Slit binding can potentially break trans-inhibitory interactions between two Robo2 receptors on opposing cells, by inducing a conformational change of Robo2 from a "closed" auto-inhibitory conformation to an "open" conformation. The following *cis*-dimerization of Robo2 receptors generates a repulsive response. The Robo3 receptor instead assumes an elongated shape and interacts with its ligand NELL2 in a 3:3 trimer to mediate axon repulsion. For simplicity, the NELL2-Robo3 interaction is depicted as a monomeric interaction. (C) Upon binding to ephrin-A5 or -A1, EphA2 receptors cluster to form a continuous assembly at the membrane to mediate cell adhesion. EphA4 forms smaller multimers, such as dimers or trimers, to transduce repulsion. (D) Netrin binds to two Dcc receptors to generate attraction but can also mediate repulsion when the receptor binding interface on the EGF2/3 domains of netrin1 is occupied instead by UNC5. Draxin can interact with both netrin1 and Dcc to potentially stabilize trans-interaction between two Dcc receptors to mediate contact dependent axon fasciculation. netrin1 can also interact with the FN4 domains of Dcc and neogenin to form either an extended array or a 2:2 tetramer with the receptors, depending on the length of the linker region between the FN4 and 5 domains of the receptors.

such as dimers or trimers (Schaupp et al., 2014; Seiradake et al., 2013). It is currently unclear what mechanisms prevent the lateral expansion of these small multimers. One possibility is that the coalescence of small EphB2 oligomers into larger assemblies could terminate signaling, likely through accelerating endocytosis of large receptor aggregates (Ojosnegros et al., 2017).

Finally, netrin can employ distinct interfaces to form unique signaling complexes with different netrin receptors, and the composition of those complexes can determine the signaling outputs to be either attraction or repulsion. Several recent studies revealed three potential receptor binding sites on netrin1. First, crystal structures suggest that the EGF-1 and -2 domains of netrin1, when binding to the fibronectin type III (FN) 5 and 6 domains of Dcc, are required for chemoattractive multimerization of Dcc (Fig. 3D). Importantly, when the same binding site on netrin1 is occupied instead by Unc5A, the netrin response is switched from attraction to repulsion, as demonstrated in mouse spinal cord neurons (Finci et al., 2014). It is important to note that the netrin-Unc5 interaction still awaits structural characterization. Secondly, the interaction between the EGF-3 domain of netrin1 and the FN5 domain of the receptor is shared among Dcc and neogenin 1 (Neo1; (Finci et al., 2014; Xu et al., 2014). This binding interface is required for Dcc-mediated attraction, but its functional significance in Neo1 has not been demonstrated. Additionally, the novel Dcc ligand Draxin can also bind to netrin1 in the same region on EGF-3 as Dcc, with even higher affinity (Gao et al., 2015; Liu et al., 2018). This suggests that Draxin could function by connecting Dcc-bound netrin1 to an opposing Dcc receptor, to facilitate contact-dependent axon fasciculation (Liu et al., 2018). Lastly, the N-terminal laminin-like domain of netrin1 can bind to the FN4 domain of both Dcc and Neo1, yet may lead to the differential formation of either a continuous netrin1-Dcc assembly or a 2:2 netrin1-Neo1 tetramer, due to differences in the linker length connecting the FN4 and 5 domains of the receptors (Xu et al., 2014). The double mutant phenotype in the developing mouse spinal cord suggests that Neo1 and Dcc collaborate to mediate netrin1-dependent attraction in commissural axons. Whether and how differences in the structure of Dcc and Neo1 signaling complexes determine their relative contribution to midline crossing have not yet been explored.

Despite significant recent progress, our understanding of the structural characteristics of guidance receptor complexes is still largely incomplete. Indeed, continued progress is hindered by the challenge of crystalizing full-length transmembrane receptors, which are more likely to represent

their native membrane-bound conformations than fragments of the receptors. Nevertheless, structures of the receptor ECD fragments in complex with their ligands have shed some light on the 3D organization of receptor signaling assemblies and suggest potential mechanisms to explain unique features of each axon guidance pathway. However, it has been difficult to test the significance of these assemblies *in vivo* due to a lack of genetic manipulation tools in vertebrates. Fortunately, the development of widely applicable new techniques for genome editing, such as the CRISPR/Cas-9 system, will enable investigators to test the functions of receptor assembly interfaces in relevant physiological contexts in the future.

2.3 Proteolytic processing of guidance receptors

Proteolytic processing controls the levels and functions of axon guidance receptors and is increasingly appreciated as one of the main mechanisms that regulates and diversifies their signaling outputs. Receptor cleavage can either downregulate surface levels of full-length receptors to terminate their signaling, or generate various receptor fragments that are active signaling molecules, or achieve both at the same time (Fig. 4). These receptor fragments can localize to different extracellular and intracellular spaces to initiate downstream pathways that are often distinct from the ones mediated by full-length receptors. As such, the contributing proteases act as molecular switches to change the neuron's responses to guidance cues. In this section of the review, we will focus on deciphering the involvement of proteases (often referred to as "sheddases"), which release receptor ECDs into extracellular space, and the intramembrane protease γ-secretase, which cleaves within the transmembrane domain to generate soluble receptor ICDs. It is important to note that in addition to functioning separately, sheddases and γ-secretase often target the same receptors in sequential cleavages, and in many cases are coupled with transcriptional activities of the receptor ICDs.

The β-site amyloid precursor protein cleaving enzyme 1 (BACE1), sometimes referred to as β-secretase, is best known for its central role in the proteolytic processing of APP (Lichtenthaler, Lemberg, & Fluhrer, 2018; Saftig & Bovolenta, 2015). BACE1, together with γ-secretase, cleaves APP to produce the pathogenic Aβ peptide, which forms the amyloid plaques that are pivotal in the pathogenesis of Alzheimer's disease. In addition to APP, BACE1 also cleaves the neural cell adhesion molecule close homolog of L1 (CHL1), which functions downstream of the Nrp1-plexin A receptor complex (Barao et al., 2015). In mouse embryonic thalamic

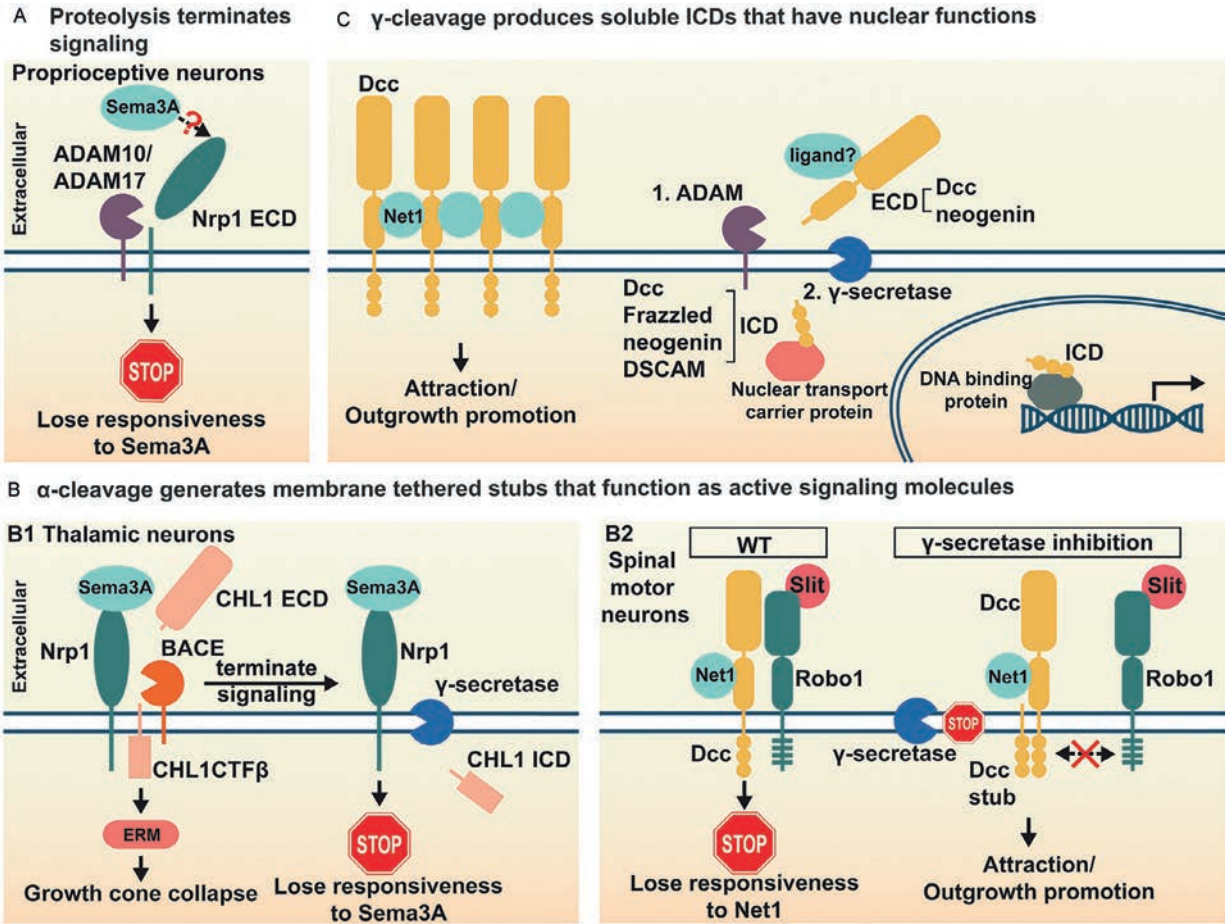

Fig. 4 See figure legend on next page.

neurons, BACE1 mediates Sema3A-induced growth cone collapse specifically via the membrane-tethered C-terminal fragment generated by BACE1 (CHL1CTFβ; Fig. 4B1). CHL1CTFβ recruits downstream effectors that can directly regulate actin polymerization in growth cone filopodia. Intriguingly, signaling is also terminated by proteolysis, when γ-secretase cleaves the CHL1 ICD containing the effector binding domain. How do neurons prevent premature γ-secretase cleavage of CHL1? The answer might lie in the differential subcellular localization of BACE1 (active in the trans-Golgi network and endosomes) and γ-secretase (active in endosomes and at the plasma membrane; (Lichtenthaler et al., 2018)). Aside from APP and CHL1, however, very few BACE1 substrates have been described, and even less is known about the functional significance of most BACE1-mediated cleavages. Candidate BACE1 substrates correspond to 19% of the neuronal proteins that are targeted by sheddases, as identified in a mass spectrometry-based screen, making BACE1 one of the major sheddases in mouse embryonic neurons (Kuhn et al., 2012).

Fig. 4 Receptor proteolytic processing regulates downstream signaling outputs. (A) Proteolytic cleavages of receptors can terminate their signaling by downregulating the surface levels of functional full-length receptors. To grow past a repulsive Sema3A barrier, Rat proprioceptive axons lose responsiveness to Sema3A as ADAM10 and ADAM17 cleave the Sema3A receptor Nrp1. It is unclear if this cleavage is induced by Sema-3A binding or if it happens constitutively without ligand stimulation. (B) ADAM-cleavage releases the ECDs of guidance receptors to form membrane-tethered C-terminal stubs that can function as active signaling molecules. (B1) In mouse thalamic neurons, CHL1 is recruited by Nrp1 upon Sema3A stimulation. BACE sheds the ECD of CHL1 to generate CHL1CTFβ which can interact with ezrin-radixin-moesin (ERM) proteins to mediate growth cone collapse. The signaling cascade is terminated when CHL1CTFβ is further cleaved by γ-secretase. (B2) In WT mouse spinal motor neurons, slit-activated Robo1 binds to full-length Dcc to silence netrin1 response. When γ-secretase function is inhibited, the accumulated Dcc stubs preferentially bind to full-length Dcc to protect them against slit-Robo1 mediated inhibition of netrin1 responsiveness. (C) γ-secretase cleavages of guidance receptors produce soluble ICD fragments that can function inside the nucleus. In canonical Dcc signaling pathways, netrin1 binding triggers the formation of a continuous netrin1-Dcc assembly which induces local cytoskeletal rearrangements to mediate attraction or axon growth promotion. In certain subpopulations of neurons, potential ligand binding initiates sequential proteolytic cleavages of Dcc/Frazzled, neogenin and DSCAM, first by ADAMs and then by γ-secretase. This process releases the ICD fragments that can translocate into the nucleus to regulate gene transcription. The function of ICDs might require assistance from nuclear transport carrier proteins (such as importin beta IPO5, which binds to the DSCAM ICD) and DNA binding proteins (such as the transcriptional regulator LMO4, which interacts with the Neo1 ICD).

ADAMs (a disintegrin and metalloproteases) are another family of transmembrane proteases. ADAMs are highly expressed in the developing nervous system with distinct spatial and temporal profiles, which suggests that they may have diverse roles in neurodevelopment (Hsia et al., 2019). ADAMs share a common domain structure consisting of the autoinhibitory pro-domain, the catalytic metalloprotease domain, the disintegrin domain, the cysteine-rich region, the EGF-like repeat domain (that is absent in ADAM10 and 17), the transmembrane domain and a shorter, less well-defined cytoplasmic tail (Lambrecht, Vanderkerken, & Hammad, 2018). In general, ADAMs function by cleaving the extracellular juxtamembrane region of type I transmembrane proteins, which leads to the shedding of the cleaved ECDs into extracellular space. For the purpose of this review, we will focus on discussing recent discoveries implicating ADAM10 and ADAM17, two of the most studied members of the ADAM family, in the modulation of axon guidance receptors. Phylogenetic and molecular evolution analyses demonstrate that a major diversification event separates ADAM10 and ADAM17 from all other members of the ADAM family, and as a result ADAM10 and ADAM17 are highly homologous, well-conserved and share many of the same substrates (Long et al., 2012). Unfortunately, global KOs of *Adam10* are embryonic lethal at E9.5 and global KOs of *Adam17* are perinatal lethal, and both mutants exhibit wide-spread developmental defects, complicating efforts to decipher their roles in neurodevelopment and to identify novel neuronal substrates (Hsia et al., 2019). In addition, compared to most other proteases, sheddases rely more on amino acid motifs or secondary structures to recognize their targets, rather than stringent substrate sequences (Lichtenthaler et al., 2018). Indeed, no consensus ADAM cleavage sequence has been identified to date (Lambrecht et al., 2018). This results in frequent failure in generating cleavage-resistant forms of ADAM targets, adding to the difficulties of predicting and verifying potential ADAM substrates. Conditional KO mutants for *Adam10* (Jorissen et al., 2010; Prox et al., 2013) and *Adam17* (Horiuchi et al., 2007), complemented by the development of novel proteomic strategies (Muller, Scilabra, & Lichtenthaler, 2016), will facilitate their study and help us gain mechanistic insights into their roles in specific axon guidance pathways.

Receptors of all four classical axon guidance cues-slits, netrins, semaphorins and ephrins-are physiological substrates for ADAMs during neurodevelopment, pointing to ADAM-mediated shedding as a universal mechanism in regulating guidance receptor signaling (Bai & Pfaff, 2011). In *Drosophila*, the ADAM10 homologue Kuzbanian cleaves Robo1 and is required cell-autonomously in neurons for slit-Robo1 mediated repulsion

(Coleman, Labrador, Chance, & Bashaw, 2010). Additionally, in cell lines, human ROBO1 undergoes sequential cleavages by ADAMs and γ-secretase to generate soluble ICDs that can localize to the nucleus, although the functions of the ICDs remain unexplored (Seki et al., 2010). Interestingly, the levels of ROBO1 ICDs are strongly enhanced by the ADAM17-specific activator phorbol 12-myristate 13-acetate (PMA), and also largely suppressed by the ADAM17-specific inhibitor TAPI-1, indicating that ADAM17 could be responsible for cleaving ROBO1 in this context. Although it remains unclear how ADAM cleavages facilitate Robo1 signaling, suppressing ADAM10 blocks ROBO1 cytosolic region-mediated recruitment of the Ras/Rac GEF Son of Sevenless (Sos), suggesting that the cytosolic domains of Robo1 are important for ADAM-dependent Robo1 signaling (Coleman et al., 2010). During rat development, proprioceptive axons (PAs) lose their sensitivity to the repellent Sema3A as ADAM10 and ADAM17 cleave its receptor Nrp1, and this process is required for the proper targeting of PAs in the spinal cord as they grow through a region of high Sema3A expression (Romi et al., 2014). Interestingly, in *Adam10* and *Adam17* double KO explants and spinal cords, PAs regain insensitivity to Sema3A after a one-day delay, indicating that Nrp1 cleavage by ADAMs might not be the only mechanism preventing Sema3A responsiveness in PAs. In primary rat cortical neuronal cultures, EphB2 is cleaved mainly by ADAM10 and then further processed by γ-secretase, although whether this proteolytic cascade regulates EphB2 in the context of axon guidance has not been investigated (Litterst et al., 2007). Instead of targeting the guidance receptor Eph, ADAMs regulate ephrin-Eph signaling in axon guidance by processing the ligand ephrin and the co-receptor neural cell adhesion molecule (NCAM). In HEK293 cells, the formation of the ephrin-A5-EphA3 complex activates ADAM10 (which is constitutively associated with EphA3) to cleave ephrin-A5 *in trans*, severing ephrin-A5-EphA3-mediated cell contact and enabling subsequent growth cone retraction (Janes et al., 2005). More recently, as shown in mouse cortical neurons, ephrin-A5 also induces ADAM10 cleavage of NCAM *in cis* (Brennaman, Moss, & Maness, 2014), which is required for ephrin-A5-EphA3-dependent growth cone collapse, possibly by terminating EphA3 clustering and signaling (Sullivan, Kumper, Temple, & Maness, 2016).

γ-Secretase is a multi-subunit intramembrane protease complex that mostly functions to release the ICDs of its target transmembrane proteins and can have major influences on the physiological signaling properties of guidance receptors (Bai & Pfaff, 2011). Multiple lines of evidence suggest that Dcc and neogenin are critically involved in orchestrating the netrin

response in neurons (Chedotal, 2019), and both go through sequential proteolytic cleavages by ADAMs and γ-secretase (Fig. 4C). In such cleavage events, a membrane receptor is first processed by sheddases to release its ECD. The membrane-tethered stub, with only a small fragment of ECD remaining, can then successfully pass through a gate formed by the γ-secretase component nicastrin to enter the active site for cleavage (Wolfe, 2019). In *presenilin 1* (*Ps1*, the catalytic subunit of the γ-secretase complex) mutant mice, spinal motor neurons (MNs) ectopically cross the floor plate due to inappropriate netrin1 attraction (Bai et al., 2011). This netrin1 response is obtained through the accumulation of Dcc stubs in the absence of γ-secretase cleavages (Fig. 4B2). Dcc stubs might function by preferentially binding to full-length Dcc receptors in MNs through an unknown mechanism, thus shielding them against interacting with the Robo1 receptor, which can inhibit netrin1 responsiveness. The role of γ-secretase in spinal commissural neurons, which also employ netrin1-Dcc and slit-Robo1 as some of the major pathways to guide their axons across the floor plate, awaits further investigation. Spinal commissures suffer severe disorganization at the floor plate in *Ps1* KO embryos, but this phenotype has not been directly tied to the cleavage of Dcc or any other guidance molecules (Bai et al., 2011). However, a strikingly different signaling strategy operates in *Drosophila* commissural interneurons, where the Dcc homologue Fra is cleaved by γ-secretase to release its ICD as an active signaling molecule (Neuhaus-Follini & Bashaw, 2015). The Fra ICD can translocate to the nucleus to transcriptionally activate the expression of its target gene *commissureless*, which is required cell-autonomously in commissural neurons to facilitate commissural axon midline crossing (Neuhaus-Follini & Bashaw, 2015; Yang, Garbe, & Bashaw, 2009). Given that the P3 motif, which houses both the nuclear export signal and the transcriptional activation domain of Fra, is well conserved from invertebrates to vertebrates, it is tempting to speculate that similar transcriptional function might exist for Dcc as well. Indeed, *in vitro* evidence suggests that both Dcc and neogenin ICDs can localize to the nucleus, and in the case of neogenin, a role in regulating transcription has also been reported (Goldschneider, Rama, Guix, & Mehlen, 2008; Taniguchi, Kim, & Sisodia, 2003). Lastly, γ-secretase processing of neogenin is required for its function across different species, including the regulation of (1) neural tube elongation in zebrafish (Brown et al., 2019), (2) RGC axon targeting in the chick optic tectum (Banerjee et al., 2016), (3) cortical neuron migration and optic nerve regeneration in mouse (van Erp et al., 2015), and (4) transcriptional regulation of gene expression in human cancer cell lines (Goldschneider et al., 2008), attesting to the significance and the

universality of regulated proteolysis for the proper signaling propagation of guidance receptors both during development and in disease pathogenesis.

In addition to Dcc and neogenin, the DSCAM ICD generated by γ-secretase cleavage can travel to the nucleus to influence the transcription of many genes involved in axon guidance and other aspects of neural circuit formation (Sachse et al., 2019). Forced expression of DSCAM ICD inhibits neurite outgrowth in primary mouse cortical neurons, yet it remains to be seen whether this inhibited outgrowth has physiological consequences. One perplexing aspect of such membrane-to-nucleus pathways is how proteolysis of a guidance receptor might interfere with downstream signaling outputs that only the full-length receptor can activate. One possibility involves activating the full-length receptor and the γ-secretase cleavage of the receptor with different ligands. Since netrins are not required in the transcriptional activation of *commissureless* (Yang et al., 2009), *Drosophila* commissural neurons might separate the function of full-length Fra and Fra ICD by activating γ-secretase cleavage with an alternative ligand, in a spatially and temporally regulated manner. Physical segregation of full-length receptors and receptor ICDs is another possible mechanism through which neurons could regulate guidance receptor functions. Future studies should address how receptor ICDs are retrogradely transported from the growth cone, where ligand stimulation is presumably detected, to the nucleus.

To further our understanding of the functional significance of proteolytic processing of axon guidance receptors, substantial future efforts should be directed toward uncovering the regulatory mechanisms that modulate the activity of the proteases themselves. ADAM10, ADAM17, BACE1 and γ-secretase are all broadly expressed in the developing nervous system and often target the same membrane receptors as proteolytic substrates to produce distinct amino and carboxy termini with variable functions. One strategy to ensure the specificity of proteases in regulating the precise cleavage of their substrates would be to spatially segregate the proteases into distinct subdomains on the plasma membrane or into different cellular compartments (Lichtenthaler et al., 2018). However, spatial segregation is unlikely to be the only means to regulate protease activity, since ADAM10 and BACE1, ADAM10 and γ-secretase, and BACE1 and γ-secretase can each interact and form proteolytically active multi-protease complexes in the developing mouse brain, suggesting physical separation might not hold true for all signaling pathways (Chen, Kim, et al., 2015; Wang, Wang, & Pei, 2018). It is interesting to note that γ-secretase could inhibit ADAM10 but enhance BACE1 processing of APP (Chen, Kim, et al., 2015). ADAM10, on the other hand,

might facilitate BACE1 cleavage of CHL1 (Wang et al., 2018). Although the physiological significance of these regulatory interactions has yet come to light, these observations imply the existence of crosstalk within a functionally interconnected protease network.

Alternatively, neurons can also employ context-specific modulators to regulate the functions of proteases. It is well-established that interacting with different members of the tetraspanin C8 subgroup can differentially influence the trafficking, activity and substrate specificity of ADAM10, whereas those properties of ADAM17 are regulated by the two inactive rhomboid proteases iRhom1 and iRhom2 (Hsia et al., 2019; Lambrecht et al., 2018; Li et al., 2015; Vincent, 2016). Despite their high expression levels in the nervous system, little information is available on the involvement of tetraspanins and iRhoms in regulating axon guidance receptor function. Additionally, tissue inhibitor of matrix metalloproteinase-3 (TIMP3) inhibits ADAM17 activity and can induce neurite outgrowth in the hippocampus both in primary neurons and *in vivo*. However, whether the growth-promoting effect of TIMP3 is dependent upon modulating ADAM17 function has not been established (Gibb et al., 2015). Secreted Frizzled Related Proteins (Sfrps), which share similarities with TIMPs in their C-terminal netrin-related motif, can bind to and suppress ADAM10 cleavages of Notch and APP (Esteve et al., 2011). In *Sfrp*-null mouse embryos, RGC axon guidance and fasciculation defects are observed in both the developing retina and optic nerve (Marcos et al., 2015). Enhanced processing of N-cadherin and L1, two cell adhesion molecules that are also known ADAM10 substrates, correlates with the genetic ablation of *Sfrp*s or the overexpression of ADAM10, yet causal relationships between specific ADAM10 substrates and RGC axon growth and guidance have not been established *in vivo*. The Robo receptors are attractive candidates, since inactivation of the slit-Robo pathway phenocopies the various defects that are present in *Sfrp* mutants (Marcos et al., 2015). Finally, ADAM10 might not be uniquely responsible for the axonal abnormalities observed in *Sfrp* mutants as it remains possible that Sfrps can also target and modulate ADAM17 function (Esteve et al., 2011; Marcos et al., 2015). Given the large number of existing physiological substrates for any of these proteases, global KOs of their essential modulators will likely result in altered proteolytic processing of several proteins and thus give rise to exceedingly complicated phenotypes. Future studies with conditional KO models for each of these proteases and their modulators, allowing close inspection of individual axon guidance events, will provide us with more insights into the many molecular mechanisms regulating proteolytic processing of guidance receptors.

3. Downstream signaling of axon guidance receptors

Thus far, we have discussed how axon guidance receptors are regulated, now we move on to how guidance receptors signal to the underlying growth cone cytoskeleton to control specific guidance decisions. Axon guidance receptors primarily induce growth cone turning by impinging on the axonal cytoskeleton to induce movement of the growth cone in a directional manner. A fundamental question is how these guidance receptors achieve specificity in signaling pathways in order to generate distinct functional outputs. Diversity in signaling can be achieved through different downstream targets. However, in many cases, a common set of effectors is recruited in response to a number of different guidance cues. In such instances, differences in signaling can be elicited via regulation of the spatiotemporal activation of an effector molecule. In previous years, much of the effort to elucidate signaling cascades downstream of guidance receptors has focused on RhoGTPases which play pivotal roles in axon growth and guidance. This work has been extensively covered in previous reviews and will not be discussed here (Niftullayev & Lamarche-Vane, 2019). In this section, we discuss recent insights into novel roles of certain actin and microtubule effectors in axon guidance, with a special emphasis on how their spatiotemporal activation can contribute to signaling specificity.

4. Actin binding proteins

The actin cytoskeleton is the primary driving force for growth cone guidance and exploration. When actin polymerization is inhibited using cytochalasin, neurons lose their ability to respond to guidance cues (Bentley & Toroian-Raymond, 1986). Actin remodeling drives the formation of lamellipodia and filopodia that typically underlie growth cone motility and protrusion. It seems reasonable then to infer that actin polymerization is associated with attractive guidance cues while actin depolymerizing agents are recruited by repulsive guidance cues. The reality, however, is more complicated. Many repulsive guidance receptors recruit downstream effectors that enhance actin polymerization, and recent studies (discussed below) highlight the nuanced and complex nature of these actin rearrangements and their importance in repulsive signaling. Aside from propelling the growth cone forward, actin effectors can also influence several other actin-dependent processes including membrane trafficking and endocytosis, that play important roles in regulating receptor signaling. Finally, actin effectors are also likely to act in a context-

dependent manner, influenced by the repertoire of other signaling proteins present, and the cellular compartments within which they are activated. In this section, we highlight recent work describing the role of the Wave regulatory complex and Ena/VASP proteins, two major actin effector families, in axon guidance.

4.1 The WASP family of nucleation promoting factors

The actin-nucleating Arp2/3 complex is one of the most well-studied actin binding protein complexes. Upon activation, the Arp2/3 complex nucleates branched actin filaments and is important for the lamellipodial actin network although its role in filopodia formation remains controversial (Yang & Svitkina, 2011). The Arp2/3 complex is activated by nucleation promoting factors (NPFs) like the Wiskott-Aldrich Syndrome protein (WASP) family of NPFs, which consists of five subfamilies in mammals: (i) WASP and N-WASP (ii) the three isoforms of SCAR/WAVE (iii) WHAMM (iv) WASH and (v) JMY. For details on the cellular functions of the WASP family of NPFs, we refer the reader to reviews that explore this topic (Alekhina, Burstein, & Billadeau, 2017; Tyler, Allwood, & Ayscough, 2016). Here, we will highlight the recent work on the SCAR/WAVE subfamily in axon guidance.

The WAVE regulatory complex (WRC) consists of five different proteins: CYFIP/Sra1, Nap1/Kette, Abi, HSPC300/Brick1 and WAVE/SCAR. WAVE contains a VCA region that can bind to Arp2/3 and induce branched actin polymerization. In *Drosophila* and *C. elegans*, SCAR/WAVE has been implicated in axon guidance and targeting (Shakir et al., 2008; Stephan, Gohl, Fleige, Klambt, & Bogdan, 2011; Xu & Quinn, 2012) however the recruitment and activation of the WRC in response to guidance cues is still poorly understood. Recent work identified a unique binding site for the WRC known as WRC-interacting receptor sequence (WIRS; (Chen et al., 2014). The WIRS motif is present in various transmembrane proteins, including several axon guidance receptors. A number of studies have since followed up on the importance of this binding site in various cellular processes. In *Drosophila*, the WIRS motif present in neuroligins was found to be essential for the recruitment of the WRC to postsynaptic membranes at neuromuscular junctions to maintain normal synapse formation and synaptic transmission (Xing et al., 2018). In *C. elegans*, the synaptic cell adhesion protein SYG-1 requires its WIRS motif to interact with the WRC for proper axonal arborization and synapse assembly (Chia, Chen, Li, Rosen, & Shen, 2014). The WIRS-WRC interaction is also important

for neogenin to regulate junctional stability in human epithelial cell lines (Lee et al., 2016). These WIRS-WRC interactions can restrict WRC-mediated actin assembly to desired subcellular locations and allow for high spatial and temporal specificity in directing actin polymerization. Since there are WIRS motifs in several axon guidance receptors including Robos and Dcc, it will be interesting to determine if they are functional and if their interaction with the WRC is regulated by the binding of their respective ligands. The WIRS motif has a conserved threonine or serine residue that is essential for its interaction with the WRC, and phosphorylation of various WIRS sites has been previously documented (Hornbeck et al., 2012). Further investigation is necessary to determine if phosphorylation can regulate the WIRS-WRC interaction. Another interesting question is whether in addition to recruiting the WRC, these WIRS-containing receptors can also influence its activity. The cytoplasmic tails of different protocadherin receptors were found to have differential effects on the activity of the WRC in a pyrene-actin polymerization assay (Chen et al., 2014). However, whether this phenomenon has physiological significance is still unclear. Altogether, identification of the WIRS motif has thus provided a direct link between axon guidance receptors and WRC-mediated actin dynamics, and is an important contribution to our understanding of WRC recruitment.

SCAR/WAVE proteins can also function in endocytosis of surface receptors. The WASP subfamily of Arp2/3 NPFs is traditionally accepted as the NPF responsible for regulating internalization events at the plasma membrane (Benesch et al., 2005; Merrifield, Qualmann, Kessels, & Almers, 2004; Tyler et al., 2016). However, in invertebrates like *Drosophila* and *Caenorhabditis*, SCAR/WAVE is a key regulator of endocytic events (Bai & Grant, 2015; Fricke et al., 2009; Giuliani et al., 2009; Patel & Soto, 2013; Shivas & Skop, 2012). New work suggests that this finding can be extended to vertebrate systems as well. In mouse hippocampal neurons, knocking down components of the WRC, but not N-WASP, impairs BDNF-mediated internalization of TrkB, suggesting that the WRC is the NPF responsible for BDNF-induced endocytosis of TrkB (Xu, Fu, Zhu, & Liu, 2016). However, TrkB does not contain a WIRS motif and there is no evidence for a direct interaction between TrkB and the WRC. Retrolinkin, an endosomal vesicle protein important for BDNF-TrkB trafficking (Fu et al., 2011), interacts with CYFIP, and knockdown of retrolinkin in hippocampal neurons shows a decrease in colocalization between WAVE1 and BDNF-activated TrkB, suggesting that retrolinkin functions in the recruitment of the WRC to TrkB. However, since no physical interaction

between retrolinkin and TrkB has been reported, it remains unclear how the WRC is recruited to and activated by TrkB. The WRC-induced endocytosis of TrkB appears to be clathrin-independent, consistent with its clathrin-independent role in endocytosis of the interleukin-2 receptor (IL-2R; (Basquin et al., 2015). IL-2R directly interacts with the WRC via a WIRS motif present in its cytoplasmic tail and mutations in the WIRS motif disrupt this interaction and inhibit IL-2R endocytosis. Interestingly, IL-2R endocytosis also requires subsequent activation of N-WASP, providing a unique example of two Arp2/3 activators regulating different steps of endocytosis initiation in a temporally coordinated manner.

Taken together, these findings offer significant advances in understanding the mechanisms underlying recruitment and activation of the WRC, yet how these events are actively regulated in response to specific guidance cues remains largely unknown. Future studies should be directed toward uncovering if and how receptor-WRC interactions are modulated upon ligand binding. Additionally, while the different WASP subfamilies generally have distinct physiological functions, in some cases, they can act cooperatively to regulate a single actin-driven process. Thus, cooperativity between different WASP subfamilies may represent an additional mechanism to diversify individual receptor signaling pathways.

4.2 Ena/VASP proteins

The Ena/VASP family consists of actin-regulatory proteins that associate with the barbed ends of actin filaments and antagonize filament capping, thereby resulting in the generation of long, unbranched F-actin filaments. *Drosophila* and *C. elegans* each have a single Ena/VASP ortholog while mammals have three: Mena, VASP and EVL (Drees & Gertler, 2008). Ena/VASP family members are concentrated at the leading edge of lamellipodia and the tips of filopodia. They are thus perfectly poised to function as immediate modifiers of the actin cytoskeleton in response to guidance cues (Fig. 5). Ena acts in attractive signaling downstream of Dcc and netrin1, functioning to increase filopodia protrusion and elongation (Gitai, Yu, Lundquist, Tessier-Lavigne, & Bargmann, 2003; Lebrand et al., 2004; Yu, Hao, Lim, Tessier-Lavigne, & Bargmann, 2002). A number of studies have demonstrated the requirement for Ena in slit-Robo dependent repulsive signaling as well (Bashaw, Kidd, Murray, Pawson, & Goodman, 2000; Yu et al., 2002) and the *Drosophila* Robo receptor has been shown to directly interact with Ena (Bashaw et al., 2000).

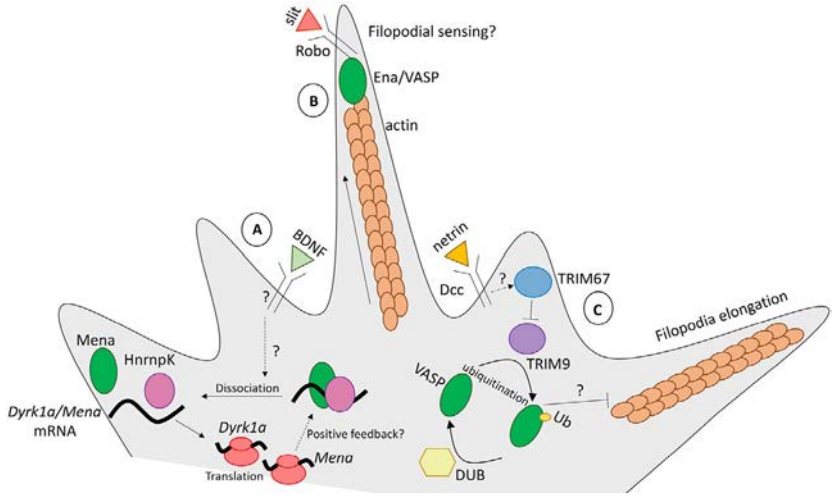

Fig. 5 Different roles of Ena/VASP proteins in axon guidance signaling. (A) BDNF stimulation induces dissociation of the complex of Mena, HnrnpK and *Dyrk1a* mRNA resulting in disinhibition of translation of *Dyrk1a*. Mena can also regulate translation of its own mRNA raising the potential for a positive feedback loop. (B) Robo recruits Ena/VASP proteins in response to slit stimulation to facilitate an initial extension of filopodia toward slit followed by retraction away from it possibly contributing to filopodial sensing of the environment. (C) TRIM9-mediated ubiquitination of VASP inhibits its function in filopodial formation although the mechanistic details are unclear. Netrin stimulation results in TRIM67-mediated inhibition of TRIM9 and activation of de-ubiquitinating (DUB) enzyme that removes the ubiquitin moiety from VASP resulting in its activation and promoting the growth of filopodia. Ub, ubiquitin; DUB, de-ubiquitinating enzyme.

Recent work to resolve the paradoxical role of Ena/VASP proteins in repulsive signaling has uncovered a novel response to slit in dorsal root ganglion axons (McConnell et al., 2016). When DRG axons encounter slit, they initially extend long filopodia toward the source of slit before retracting. This filopodial extension requires the activity of Ena/VASP proteins and is necessary for subsequent slit-induced retraction. Disrupting the Ena/VASP interaction site on Robo abolishes the extension of filopodia and the subsequent repulsion. In mouse embryos deficient for Ena/VASP proteins, DRG axons fail to bifurcate along the rostro-caudal axis and instead aberrantly extend into the dorsal midline similar to the phenotype seen in embryos lacking Robo or slit (McConnell et al., 2016). Together with the *in vitro* data, these observations strongly suggest that Ena/VASP proteins and Robo function together to guide DRG axons. slit-induced filopodia are likely to function in sensing the environment surrounding a growth cone, thereby facilitating a better resolution of guidance gradients (Fig. 5B).

Guidance cues can achieve rapid localized cytoskeletal changes by using molecular switches that allow for immediate regulation of cytoskeletal effector proteins. One such switch involves the rapid, reversible, non-degradative ubiquitination of VASP (Menon et al., 2015) Fig. 5C). The ubiquitination of VASP is mediated by TRIM9, a member of the tripartite motif (TRIM) family of E3 ubiquitin ligases. TRIM9 along with the invertebrate orthologs, MADD-2 and Asap. has previously been implicated in netrin-Dcc signaling (Hao et al., 2010; Morikawa, Kanamori, Yasunaga, & Emoto, 2011; Winkle et al., 2014). However, while *Trim9* loss of function phenocopies *netrin* and *Dcc* mutants in invertebrates (Hao et al., 2010; Morikawa et al., 2011), conditional *Trim9* knockout mice show thickening of the corpus callosum, a phenotype opposite to *Dcc* and *netrin* mutants. This suggests that TRIM9 function might have diverged in mammals (Menon et al., 2015). Mechanistically, TRIM9-mediated ubiquitination of VASP in cortical neurons, has no effect on VASP stability but results in relocalization of VASP away from filopodial tips (Fig. 5C). Although it is unclear how ubiquitination affects VASP localization, it appears that ubiquitination of VASP affects the rate of its dissociation from filopodial tips, which in turn reduces filopodial stability. Consistent with this idea, *Trim9* mutant growth cones show increased filopodial number and length. Upon netrin stimulation, VASP ubiquitination is reduced. This reduction is lost in the presence of a deubiquitinating enzyme (DUB) inhibitor, suggesting that VASP is de-ubiquitinated downstream of netrin signaling (Menon et al., 2015).

More recently, TRIM67 was found to competitively inhibit TRIM9-dependent ubiquitination of VASP (Boyer, McCormick, Menon, Urbina, & Gupton, 2020) Fig. 5C. Cortical neurons lacking TRIM67 show increased ubiquitination of VASP, presumably mediated by TRIM9. *In vivo* analysis of *Trim67* conditional mutant mice shows thinning of the corpus callosum similar to *netrin* and *Dcc* mutants, although the defects in *netrin and Dcc* are much more severe with complete agenesis of the corpus callosum. This might suggest compensatory mechanisms *in vivo*, or perhaps can be partly attributed to the use of whole animal knockouts for *netrin* and *Dcc*. Importantly, the ligase domain of TRIM67 appears to be important for the reduction in VASP ubiquitination. Is TRIM67 ubiquitinating TRIM9 to inhibit TRIM9 activity or is it acting indirectly via other substrates? Despite the contrasting phenotypes of *Trim9* and *Trim67* mutants *in vivo*, both *Trim67* and *Trim9* mutant cortical neurons have an increased growth cone area and number of basal filopodia, *in vitro*. Perhaps this reflects a continuing need for TRIM9 and TRIM67 downstream of netrin signaling to allow for repeated cycling of VASP between ubiquitinated and de-ubiquitinated states for

successive rounds of filopodial attachment and detachment. This could represent a model similar to the cycling of RhoGTPases where dominant negative and constitutively active forms of RhoGTPases often show the same phenotype. This balance between TRIM9 and TRIM67 function may fine-tune VASP activity to an optimal level permitting filopodial sensing of the environment.

In addition to its canonical role as a cytoskeletal regulator, Mena, the mammalian ortholog of Ena, can function to regulate local translation both at the basal level and in a cue-dependent manner (Vidaki et al., 2017) Fig. 5A. Mena can bind to its target mRNAs, including the kinase *Dyrk1a*, through its association with the RNA-binding protein (RBP) hnrnpK. In neurons deficient in Ena/VASP proteins, both basal and BDNF-elicited local translation of *Dyrk1a* is impaired (Fig. 5A). Mena acts downstream of many axon guidance receptors that are known to regulate local translation, and thus can serve as a link to the translational machinery. This dual function of Mena places it in a pivotal position to coordinate the crosstalk between cytoskeletal reorganization and local protein synthesis downstream of guidance cues.

These recent studies are paving the way for understanding how a core set of actin-binding proteins can function downstream of both attractive and repulsive guidance receptors. The work on slit-induced filopodial extension in DRG axons, lends credence to the idea that actin polymerization events are essential for repulsive signaling and are more nuanced and complex than previously thought. Further, the discovery of novel functions of actin-binding proteins, namely the regulation of local translation by Mena, can substantially advance our understanding of how these proteins function in several different guidance pathways yet produce distinctive signaling outputs.

5. Microtubule associated proteins

It is widely accepted that the actin cytoskeleton is instrumental in driving growth cone motility in response to axon guidance cues. However, there is a rapidly growing body of evidence to suggest that microtubules (MTs) play more than just a passive role in axonal navigation and extension. The idea of MTs steering growth cones stems from one of the early observations that localized application of Taxol, a MT-stabilizing drug, induces attraction toward the site of application, while Nocodazole, a MT-destabilizer, induces repulsion away from it (Buck & Zheng, 2002). MTs contribute to axonal

growth and guidance by advancing and stabilizing filopodia and by directing the asymmetric delivery of intracellular vesicles which contain new membrane components and receptor proteins necessary for membrane protrusion. Additionally, MTs are constantly switching between phases of growth and catastrophe, and this dynamic instability can help growth cones explore and navigate their surrounding environments. Here, we focus on recent work identifying the microtubule-associated proteins (MAPs) required for regulating MT dynamics to influence growth cone steering.

MTs are the stiffest of the cytoskeletal structures (Fletcher & Mullins, 2010). They are composed of α- and β-tubulin heterodimers that polymerize in a head-to-tail arrangement to give rise to protofilaments that in turn assemble to form polarized tubes. TUBB3, a neuronal isoform of β-tubulin, interacts with netrin receptors and plays a role in netrin-induced attraction and repulsion (Huang et al., 2018; Qu et al., 2013; Shao, Yang, Huang, Alarmanazi, & Liu, 2017). TUBB3 can interact with Dcc and this interaction is enhanced in the presence of netrin (Qu et al., 2013). *TUBB3* knockdown affects netrin-induced neurite outgrowth and attraction both *in vitro* and *in vivo* in chick spinal cords. Indeed, certain disease-associated missense mutations in TUBB3 that partially disrupt its interaction with Dcc, fail to rescue the netrin-dependent outgrowth and branching defects induced by *TUBB3* knockdown in cultured cortical neurons (Huang et al., 2018). Electroporation of these TUBB3 variants in spinal cords of chick embryos inhibits netrin-induced attraction of axons in an open-book turning assay. Because Dcc is absent in avians, it is likely that TUBB3 functions downstream of neogenin, a functional substitute for Dcc in chick; however, this requires further study. In mammals, *TUBB3* mutants show defects in formation of the corpus callosum and anterior commissure, phenotypes similar to that seen in *Dcc* and *netrin* mutants, further suggesting a role for TUBB3 in Dcc signaling (Fazeli et al., 1997; Serafini et al., 1996; Tischfield et al., 2010). Future studies should determine the interaction sites on Dcc that are responsible for binding to TUBB3, permitting manipulations that would specifically disrupt TUBB3 function in Dcc signaling rather than globally depleting TUBB3. Unc5 can also interact with TUBB3, although in contrast to Dcc, netrin stimulation reduces this interaction (Shao et al., 2017). The current model suggests that netrin stimulation causes TUBB3 to dissociate from Unc5 on the proximal side, allowing for collapse and repulsion away from netrin.

In addition to interacting with MTs directly, guidance receptors can also recruit MAPs that can bind to MTs and regulate MT dynamics. Several of

these MAPs can bind to actin in addition to microtubules and can thus serve as points of crosstalk between the two cytoskeletal networks. Most MT bundles are sequestered in the central domain of the growth cone presumably restricted by actin retrograde flow, but a few extend further out into the periphery. These pioneer MTs can undergo crosslinking with peripherally located F-actin bundles that help guide them into filopodia. Subsequent microtubule capture and stabilization can facilitate directed growth. MT-actin interactions are crucial for achieving proper axon guidance (Coles & Bradke, 2015) and much effort has been directed toward identifying MAPs which have actin and MT crosslinking abilities. For example, XMAP215 and navigator1 (Nav1), two previously known MAPs, were recently shown to also directly interact with F-actin and regulate MT-actin interactions (Sanchez-Huertas et al., 2020; Slater et al., 2019). XMAP215 and Nav1 join the expanding repertoire of crosslinker proteins that includes cytoplasmic linker associated proteins (CLASPs), adenomatous poliposis coli (APC), spectraplakins and others. Although removal of several of these proteins has been shown to impair responsiveness to guidance cues *in vitro*, mechanistic insight into their recruitment and activation downstream of specific guidance receptors is relatively lacking.

MT studies are complicated by the fact that MAPs act in tip-stabilizing complexes, where problems of redundancy between complex components limit the extent of knowledge that can be gained from individual knockout studies. Further, MAPs can have roles independent of MT binding, as was recently reported for MAP6 in Sema3E signaling (Deloulme et al., 2015) and many MAPs can bind to actin as well as MTs, which complicates the interpretations from MAP knockout studies. Finally, some MAPs, like CLASPs, can both promote and inhibit MT elongation (Bearce, Erdogan, & Lowery, 2015) highlighting a complex regulation that may buffer against unwanted polymerization events. As such, reductionist approaches studying single MAPs in isolation are inherently limited. Additionally, with advancements in the fields of optogenetics and live-imaging microscopy, investigators can study the impact of localized MAP manipulations as individual growth cones are visualized in living tissue.

6. Spatiotemporal signaling

It is easy to imagine signal diversification when effectors can perform distinct functions downstream of different receptors, although many effectors have just a single known function. In such instances, differences in signaling

can be achieved by regulating the spatiotemporal activation of these effectors. When and where in a cell, these effectors are active, can influence both the duration of their signaling and the protein assemblies with which they interact, resulting in differential signaling outputs (Fehrenbacher, Bar-Sagi, & Philips, 2009; Sugiyama, Fairn, & Antonescu, 2019). In this section we discuss spatial and temporal activation of downstream effectors of axon guidance, and novel tools for studying this local signaling.

6.1 Endosomal signaling

Endosomes play pivotal roles in axon pathfinding by regulating surface levels of axon guidance receptors (Fig. 6). Endocytosis is a well-documented mechanism for attenuating receptor signaling by decreasing the number of surface receptors and targeting them for degradation. In recent years however, several novel functions for endosomes have emerged, from serving as hotspots for local translation to functioning as platforms for diverse signaling cascades. Here, we focus on the role that endosomal signaling plays in axon guidance.

Endosomes can serve as major signaling hubs where continued ligand-receptor association leads to sustained signaling from the endosomal compartment, constituting a population known as "signaling endosomes." Signaling endosomes were traditionally viewed as long-distance, retrograde messengers that transmit information from the nerve terminals to the soma, an important component of neurotrophin signaling (reviewed in Barford, Deppmann, & Winckler, 2017). Now, the definition of signaling endosomes has expanded to include those that signal locally within the growth cone. Some receptors initiate different signaling cascades at the plasma membrane and in endosomes, thus requiring internalization to recruit specific effector molecules. There are several instances of guidance cue-induced signaling that is specifically initiated in endosomes. slit-induced endocytosis of the Robo receptor is required for the recruitment of its downstream effector SOS, a GEF for Rac1 activation (Chance & Bashaw, 2015) Fig. 6A. Disrupting endocytosis of Robo results in a failure to recruit SOS to Robo-induced cellular processes. Endosomal-specific Rac1 activation is also induced by the TrkA receptor, which is internalized upon NGF stimulation (Harrington et al., 2011). Activated Rac1 subsequently recruits cofilin which presumably severs actin filaments to enable the retrograde transport of these TrkA-NGF signaling endosomes to the soma to support neuronal survival (Fig. 6A). Finally, RhoA, another member of the RhoGTPase family is also activated on early endosomes upon NogoAΔ20

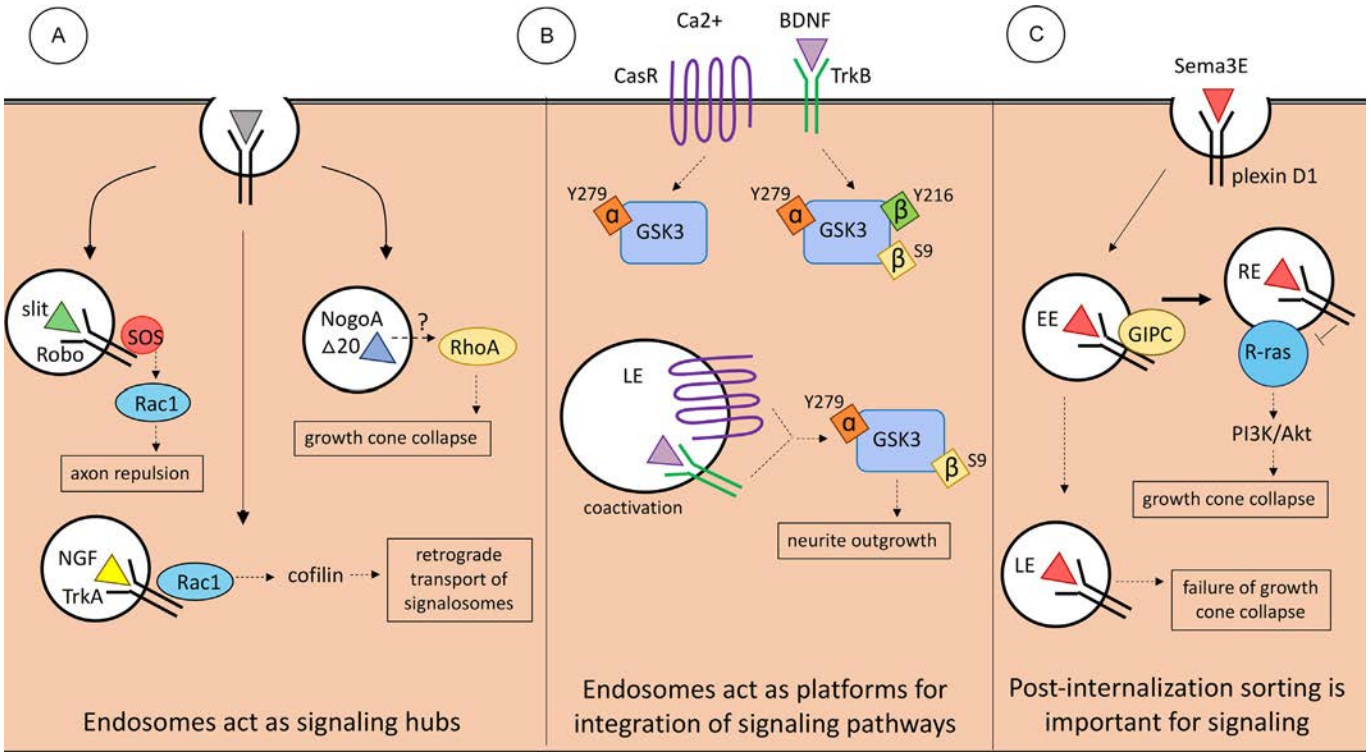

Fig. 6 See figure legend on opposite page.

stimulation (Joset, Dodd, Halegoua, & Schwab, 2010). The growth inhibitory fragment NogoAΔ20 is internalized via the pinocytic chaperone, pincher-dependent macroendocytosis, and generates signaling endosomes that are retrogradely transported to negatively regulate neuronal growth programs in the cell body. These signaling endosomes can also act locally to mediate growth cone collapse in response to NogoAΔ20 (Fig. 6A). Disrupting endocytosis of NogoAΔ20 abolishes RhoA activation and impairs NogoAΔ20-dependent growth cone collapse (Joset et al., 2010). Previously, endocytosis was thought to function primarily in repulsion and growth cone collapse, however a recent study has demonstrated a role for endocytosis in Shh-mediated attractive signaling. Shh induces endocytosis of its receptor, Boc, via an endocytic adapter, Numb, and this internalization is required for Shh-dependent axon turning (Ferent et al., 2019). One demonstrated role for Shh-mediated endocytosis is to facilitate Ptch1 internalization, permitting disinhibition of smoothened (Smo). However, it is tempting to speculate that Shh-induced endocytosis might function as more than just a means for Smo disinhibition, by actively regulating local signaling; perhaps even serving as a site for integration with the Shh-induced DOCK/ELMO/Rac1 pathway (Makihara et al., 2018).

Signaling endosomes are perfectly poised to serve as platforms for crosstalk between signaling pathways (Fig. 6B). Different guidance receptors internalized into a common vesicle can elicit signaling cascades that interact to generate an entirely novel output (Markworth et al., 2019). For example, CasR and TrkB can interact in a ligand-independent manner, and upon coactivation in chick sensory neurons, CasR and TrkB colocalize and co-traffic in Rab7-positive late endosomes. Both CasR and TrkB have a common downstream

Fig. 6 Endosomal signaling in axon guidance. (A) Some axon guidance receptors recruit effector molecules specifically at endosomal membranes and generate a local signaling output which is distinct from that occurring at the plasma membrane. Examples include Robo and SOS, TrkA and Rac1, and NogoA△20 and RhoA. (B) Endosomes can serve as platforms for the integration of signaling pathways. Independently, CasR and TrkB show different phosphorylation profiles of their common downstream target, GSK3. Upon coactivation, CasR and TrkB colocalize in late endosomes where CasR alters the GSK3 phosphorylation target sites of TrkB. (C) Post-internalization sorting into different endosomal populations can contribute to diversity in signaling as different endosomal pools have unique repertoires of effector molecules localized to them. Upon Sema3E binding, plexin D1 is internalized and further sorted into recycling endosomes by GIPC where it inactivates the R-ras population primarily expressed on the membranes of recycling endosomes. Missorting of plexin D1 into late endosomes fails to elicit inactivation of R-ras.

effector, GSK-3, which can be phosphorylated at multiple residues. When independently activated, CasR and TrkB show different phosphorylation profiles of GSK-3 and coactivation of CasR and TrkB results in a non-additive effect of GSK-3 phosphorylation, suggesting that CasR changes the GSK-3 phosphorylation target sites of TrkB (Fig. 6B). The authors hypothesized that GSK-3 cycles between active and inactive states. Supporting this idea, phosphorylation of tau, a downstream target of GSK-3, shows a cycling behavior when CasR and TrkB are coactivated but not when CasR is activated alone (Markworth et al., 2019). Since phosphorylation of tau can affect its ability to promote MT assembly (Lindwall & Cole, 1984), cycling between tau dephosphorylation and phosphorylation can potentially induce successive rounds of microtubule assembly and disassembly to regulate neurite outgrowth. It is unclear whether TrkB activation alone is also capable of eliciting the cycling behavior of phosphorylated tau and what that might mean for the biological significance of this integration of signaling. It remains to be seen whether CasR and TrkB interact directly to regulate crosstalk, and if late endosomes are actively contributing to the integration of their signaling or if they are simply acting as a platform for the localization of these receptors.

Once internalized, receptors can be sorted into recycling endosomes to be returned to multiple locations on the cell surface. This allows cells to fine tune the distribution of receptors and the extent of signaling. Post-internalization sorting is an important regulatory step in several axon guidance pathways and is vital for proper spatiotemporal signaling (Fig. 6C). Adaptor proteins are critical regulators of post-internalization sorting. Recently, an adaptor protein, HD-PTP, was found to be indispensable for ephrin-mediated growth cone collapse. HD-PTP, an accessory protein for the ESCRT complex, regulates post-endocytic sorting of EphB2 complexes by protecting them from lysosomal degradation and facilitating their recycling back to the plasma membrane (Lahaie et al., 2019). In some cases, recycling endosomes do not function solely to replenish surface receptors, but actively contribute to signaling cascades. For instance, active R-ras is detected primarily on the membranes of recycling endosomes. Plexin D1 is internalized in response to Sema3E and requires sorting by the adaptor protein GIPC, into recycling endosomes where it inactivates R-ras (Fig. 6C). Disrupting the interaction between plexin D1 and GIPC results in missorting of plexin D1 from recycling endosomes to late endosomes and loss of R-ras inactivation, along with a failure of Sema3E-induced growth cone collapse (Burk et al., 2017). A very interesting role for recycling endosomes in co-adaptation of ephrinA/EphA signaling was recently demonstrated in chick RGCs (Fiederling et al., 2017). Upon prolonged exposure to guidance signals, axons become less

sensitive to those signals. For continued navigation, axons must adapt over time in order to overcome the decreased sensitivity to guidance signals. In this study, the authors use computational modeling and an *in vitro* "gap assay" to demonstrate that axons adapt to both forward and reverse ephrinA/EphA signals and this co-adaptation is based on vesicular trafficking. Upon internalization of ephrinAs and EphAs, enhanced cis signaling in recycling endosomes desensitizes growth cones to trans signals while resensitization is achieved by recycling of ephrinAs and EphAs back to the growth cone surface (Fiederling et al., 2017). These studies highlight the importance of post-endocytic sorting in axon guidance and lend credence to the idea that different endosomal populations have unique signaling profiles that can further contribute to signal diversification.

6.2 Tools for studying spatiotemporal signaling

Specific neuronal connections are established through the concerted action of several guidance receptor pathways that signal through common effector molecules whose dynamics are subject to rigorous spatial and temporal regulation. Thus, to dissect the molecular mechanisms involved in guidance receptor signaling, it is imperative to study the spatiotemporal signaling of these effectors together with their regulatory mechanisms. Unfortunately, the commonly used methods of studying signal transduction traditionally involve cell lysis or fixation, offering only limited spatial and temporal resolution. To overcome these challenges, many novel tools have been developed in the last decade to help visualize and manipulate these signaling molecules with greater precision. Here we provide a brief overview of these novel tools and refer readers to several excellent reviews for further information (Ross, Mehta, & Zhang, 2016; Rost, Schneider-Warme, Schmitz, & Hegemann, 2017).

Advancements in optical imaging have enabled the precise observation of signaling molecules at subcellular resolution and with high temporal specificity. Improvements in the design and sensitivity of genetically encoded fluorescent biosensors have made them powerful tools for monitoring effector activity in living cells. The most common biosensors are the fluorescence resonance energy transfer (FRET)-based biosensors, which are engineered by sandwiching the sensing unit between two fluorescent proteins that act as a FRET pair (Marx, 2017). FRET biosensors offer an advantage over single fluorescent protein biosensors because they are ratiometric, making them more reliable and robust. When targeted to specific subcellular compartments, these biosensors can provide valuable information on the functional compartmentalization of signaling in neurons. However, many fluorescent

biosensors are limited by their reliance on visible light which penetrates poorly through tissues. Far-red and near-infrared sensors, which can penetrate deeper into tissues, may be more suitable for *in vivo* imaging (Chernov, Redchuk, Omelina, & Verkhusha, 2017).

With the advent of super-resolution (SR) microscopy, live imaging at nanoscale resolution is now possible. However, most SR techniques use very high intensity light which can cause photobleaching, phototoxicity and other adverse consequences, prohibiting long-term imaging of dynamic cellular events. To this end, super-resolution based on reversibly photoswitchable fluorescent proteins (RSFPs) is proving to be extremely useful. Reversible saturable optical fluorescence transition (RESOLFT) microscopy uses RSFPs that can undergo repeated cycles of photoactivation from a dark state to a fluorescent state, and enables live imaging for long periods with negligible photodamage (Kwon et al., 2015). Bioluminescence offers another strategy to avoid the problem of phototoxicity and many bioluminescence resonance energy transfer (BRET) sensors are being developed. While most luminescent proteins are dimmer, compromising resolution, Nano-lantern and other color variants enable high-speed multicolor luminescence in living cells (Takai et al., 2015). Other efforts are being directed at physically magnifying the sample itself in a new form of super-resolution microscopy known as expansion microscopy (Chen, Tillberg, & Boyden, 2015). This method uses a swellable polymer that expands such that labeled biomolecules within the sample can be separated to distances that enable their resolution with traditional microscopy.

In addition to being able to detect the spatiotemporal activation of effector molecules, it is imperative to develop tools that can precisely manipulate effector activity in time and space. Optical manipulation of specific effector activities allows for increasingly finer control in live cell studies. This can be achieved through the use of naturally occurring light-activated proteins like photoactivatable adenylyl cyclases (PACs) which can be optically activated to regulate cAMP levels in a spatiotemporal manner. Optical manipulation can also be achieved through the optical caging of proteins, wherein the target protein or its activity is blocked by a photolabile group that enables light-induced activation with high resolution. Alternatively, light-induced dimerization (LID) can be used to facilitate spatiotemporal activation of signaling by regulating protein interactions and effector recruitment. In LID, one component of the dimer is targeted to a specific subcellular compartment while the other component is expressed as a cytosolic protein. The dimerization induced by light facilitates rapid and precise recruitment of

the cytosolic protein to the subcellular compartment. LID can also be used to achieve homo-oligomerization of proteins thereby allowing for finer control over receptor activation. This has been demonstrated for optoTrk receptors, which can recapitulate BDNF-TrkB signaling in response to light in cultured hippocampal neurons (Chang et al., 2014). Lastly, magnetic nanoparticles (MNPs) are another rapidly developing tool for manipulating single molecules with high spatiotemporal precision (Nimpf & Keays, 2017). MNPs can be used to control the spatial organization of cell surface receptors and induce signal transduction as reported for Notch receptors (Seo et al., 2016). However, technical considerations remain with regard to the delivery of these MNPs within cells and current research is geared toward enabling genetic encoding of MNPs.

There have been tremendous advancements in the fields of optogenetics, magnetogenetics and nanoscale microscopy. The availability of these novel tools for visualizing and manipulating effector molecules with high spatiotemporal precision, offers many exciting avenues of investigation. An ongoing effort is the design and optimization of these tools for various different effectors involved in guidance receptor signaling, and the improvement of existing technologies to make them more amenable to *in vivo* imaging.

7. Concluding remarks

In this chapter, we have sought to highlight recent advances and new concepts emerging from the investigation of axon guidance receptor regulation and signaling. When conserved families of guidance cues and receptors were first described, their functions were grouped into the two major categories of attraction and repulsion. They were further ascribed either short-range or long-range functions, or both. This proved to be a useful framework for thinking about axon guidance mechanisms, but not surprisingly this categorization represented an over-simplification of the diverse actions of these signaling proteins. In addition, while first described for their actions in the developing nervous system, it is now appreciated that these conserved families of signaling proteins regulate diverse aspects of neuronal morphogenesis, homeostasis and function.

Investigations into the mechanisms of receptor regulation over the past ten years have begun to highlight the myriad of ways in which interactions between receptors and coreceptors, as well as interactions between receptors for distinct guidance cues serve to diversify and modulate signaling outputs.

This has challenged the notion that guidance pathways only act in isolation to mediate discrete steps of the axon guidance process, and that it is only the net balance between attraction and repulsion that determines guidance outcomes. Instead, these pathways are often integrated and converge to yield distinct and sometimes synergistic effects on axonal responses. This new understanding of the complex cross talk between signaling pathways has been dramatically accelerated by the burgeoning body of structural work that sheds light on how receptor-ligand and receptor-receptor interactions control receptor activation. In addition to new roles for interactions between distinct guidance pathways, significant progress has been made in understanding how proteolytic processing of axon guidance receptors can regulate receptor availability and downstream signaling. One particularly interesting emerging theme is that receptors often undergo processive proteolysis to generate intracellular domains which can signal in new ways, including by acting as direct regulators of transcription. This suggests the intriguing possibility that axon guidance pathways used for building neuronal circuits could be repurposed to control other neuronal properties by directly regulating gene expression.

As we have seen for mechanisms of receptor regulation, the study of the complex mechanisms of signaling downstream of axon guidance receptor activation has also been a fertile area of investigation. One of the most challenging aspects of understanding axon guidance receptor signaling has been the finding that frequently the same signaling molecules can act in both axon attraction and axon repulsion. Higher resolution imaging and a finer focus on the spatial regulation of signaling events has begun to shed light on this paradox. For example, recent studies of Ena/VASP proteins point to the importance of local signaling within filopodia to allow growth cones to sample gradients of extracellular cues and reveal that an important aspect of the response to repellant cues is to first extend filopodia. As new tools become available to image the activity of signaling molecules in sub-domains of the growth cone, we expect that a clearer delineation of the roles of proteins that act in both attraction and repulsion will emerge. New studies of actin regulatory proteins such as Ena/VASP have also highlighted how these proteins can play distinct roles in the growth cone depending on how upstream pathways impinge upon them, and where within the growth cone they are activated. For instance, Ena/VASP can regulate filopodial extension in response to netrin or regulate local protein translation in response to BDNF. A clear challenge for the future will be to achieve an understanding of how these distinct activities are regulated spatially and temporally to confer distinct signaling outcomes.

Finally, it is important to recognize that without exception, conserved families of axon guidance proteins play broad roles in the development and function of multiple organ systems outside of the nervous system, and disruption of their signaling pathways is implicated in many human diseases. The broad and diverse roles of axon guidance signaling pathways creates an even stronger imperative to dissect their mechanisms of action. Thus, future studies of the regulation and function of axon guidance signaling pathways will not only increase our understanding of the development and function of the nervous system, they will also offer broad insights into development and disease.

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