

Signaling of Secreted Semaphorins in Growth Cone Steering

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Introduction

Secreted semaphorins [class 3 semaphorins (3A-3F) in vertebrates and class 2 in invertebrates] play essential roles in the establishment of neuronal circuitry by mediating axon steering and fasciculation during development of the nervous system.¹⁻³ Semaphorin 3A (Sema3A) was the first secreted form of semaphorins purified from adult chick brains and characterized as a chemorepellant with the ability to induce rapid collapse or repulsion of dorsal root ganglion (DRG) growth cones in vitro and to repel populations of neurons in vivo.⁴⁻⁸ In the grasshopper, a graded distribution of Sema-2a has been shown to be essential in guiding the tibial (Ti1) pioneer neurons in the developing limb.⁹ Like other families of guidance cues, such as netrins and ephrins, semaphorins function not only as repellents but also as attractants to neuronal growth cones, depending on the composition of receptors and signaling cascades presented in the cells. Sema3C, for example, can act as a chemoattractant to embryonic cortical axons.¹⁰ Sema3B has recently been shown to attract and repel commissural axons in vitro and is critical for the positioning of anterior commissural projection.¹¹ In a slice overlay assay, Sema3A was shown to attract the dendritic growth cones of cortical neurons,¹² while repelling the axonal growth cones of the same neurons.¹³ The molecular mechanisms in mediating and modulating growth cone steering responses to class 3 semaphorins have been best characterized within the semaphorin family both in vitro and in vivo, and are the main focus of this chapter. Interested readers can consult other chapters of the book and several other comprehensive reviews on semaphorins and their signaling.^{3,14-16}

In Vitro Neuronal Growth Cone Steering Assays

Since in vitro assays have been indispensable in determining the function and molecular mechanisms of class 3 semaphorin signaling in growth cone steering, we will first briefly introduce and describe some of these in vitro assays. For all growth cone steering assays, a gradient of the semaphorin protein is produced, either by being released from source cells (natural semaphorin producing cells or cell lines transfected with semaphorin expression constructs), or from a micropipette loaded with purified recombinant protein.

Growth Cone Turning Assay^{17,18}

This assay has been extensively used with *Xenopus* spinal neurons, retinal ganglion cells and mammalian neurons to determine signaling mechanisms of various diffusible guidance cues, including class 3 secreted semaphorins. A microscopic gradient is established by controlled

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pulsatile ejection of solutions containing semaphorins from a micropipette and the growth cone responses within the gradient can be quantified by the degree of the turning angle with respect to the original direction of neurite extension. The *in vitro* gradient is very stable and reliable, thus allowing quantitative analysis of the steering decision of individual neuronal growth cones in response to a defined gradient of guidance cues. In addition, pharmacological and genetic manipulations as well as high resolution imaging of cellular events (e.g., Ca^{2+} imaging) are relatively easy to be achieved in this system.

Collagen Gel Repulsion Assay^{19,20}

In this assay, neuronal tissue explants and aggregates of COS cells expressing class 3 secreted semaphorins are embedded in three-dimensional collagen gels for coculture of a few days. Axonal outgrowth is then visualized by fixation and immunostaining with antibodies to neurofilament. The chemotropic activity of the protein of interest is quantified by the axon outgrowth ratio P/D, where P is the extent of axonal outgrowth on the side proximal to the COS cell aggregate, and D is the extent of axonal outgrowth distal to the cell aggregate. A P/D ratio below one, therefore, indicates chemorepulsion.

Slice Overlay Assay^{12,21}

In this assay, dissociated cells isolated from the developing nervous system are labeled and cultured over neural tissue slices from various developmental stages and regions. This system can potentially provide individual neuronal growth cones with an environment that better mimics the endogenous milieu. The axonal or dendritic orientation of dissociated cells in response to guidance cues provided by the slice is then analyzed by the morphology of neurons.

Receptor Complex in Mediating Growth Cone Turning Responses to Class 3 Semaphorins

The chemotropic effects of class 3 semaphorins on growth cone steering are mediated by a functional receptor complex comprised of neuropilins (Neuropilin-1 and Neuropilin-2) as the ligand-binding component²²⁻²⁶ and Plexin-As (A1-A4) as the signal transducing component (Fig. 1).²⁷⁻²⁹ The neuropilin/plexin receptor complex appears to have different binding specificity for class 3 semaphorins and exhibits mostly complementary and distinct temporal and spatial expression profiles in developing neurons of both the central (CNS) and the peripheral nervous systems (PNS), which may explain the neuronal subtype specificity of chemotropic effects of class 3 semaphorins.

The growth cones of cultured embryonic *Xenopus* spinal neurons or retinal ganglion cells exhibit robust repulsive turning responses to a gradient of Sema3A in a chemotropic growth cone turning assay.^{18,30} Similar repulsive responses were observed in a collagen gel repulsion assay using mammalian sensory neurons.³¹ Neuropilin-1 and Plexin-A1 mediate Sema3A-induced repulsion in these neurons, since application of a function-blocking antiserum against the extracellular domain of Neuropilin-1 or overexpression of a truncated form of Plexin-A1 lacking the highly conserved cytoplasmic domain completely abolished the growth cone repulsion.^{18,29,31} In addition, dendritic growth cones also require Neuropilin-1 function for Sema3A-induced guidance responses. Experiments using function-blocking antibody in a slice overlay assay showed that Neuropilin-1 serves as a Sema3A receptor in mediating both the chemoattractive guidance of cortical apical dendrites towards the pial surface and the chemorepulsive guidance of cortical axons toward the white matter in response to a Sema3A gradient.^{12,13}

Similar to Sema3A, other members of the class 3 semaphorin family (Sema3B, 3C, 3D, 3E, 3F) also exhibit chemotropic guidance activities *in vitro* but differ in their binding specificities for neuropilins. For example, Sema3B, 3C and 3F appear to preferentially bind to the Neuropilin-2 homodimer or Neuropilin-1/2 heterodimer, whereas Sema3A preferentially binds to Neuropilin-1 homodimers.²⁴⁻²⁶ It has been shown that sympathetic axons coexpress

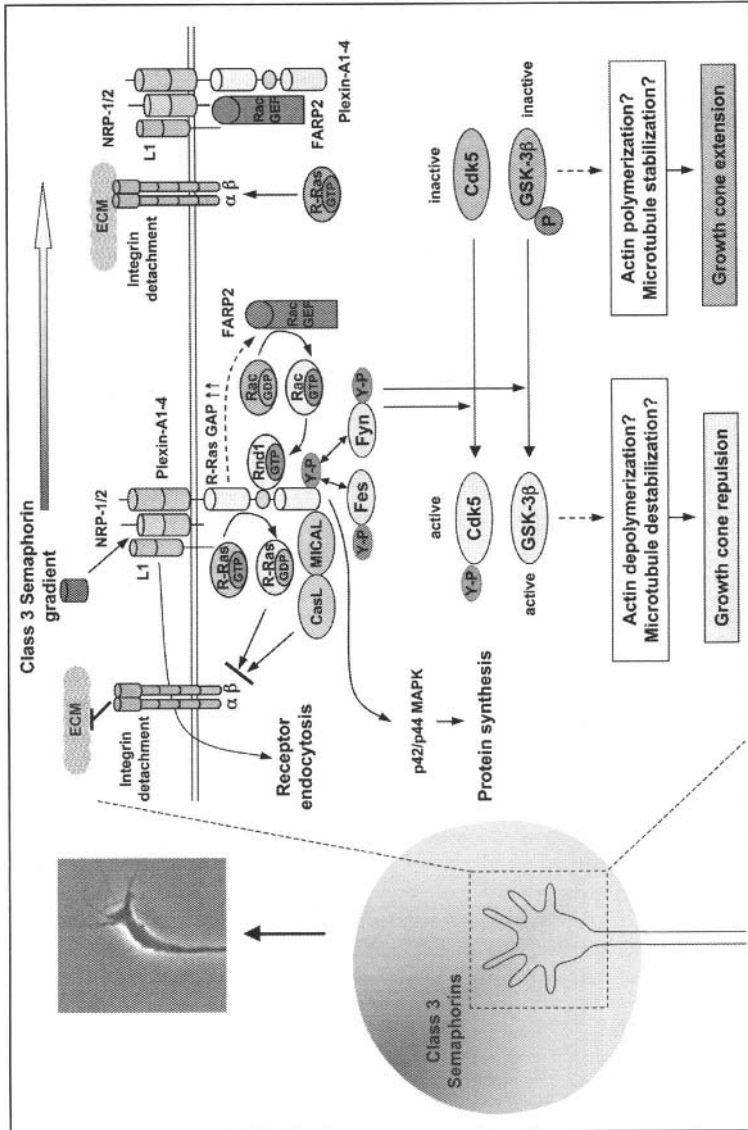


Figure 1. Components of class 3 secreted semaphorin signaling for growth cone steering. In neuronal growth cones, class 3 secreted semaphorins bind to a receptor complex with Plexin-As (A1-4) and Neuropilins (Neuropilin-1 or Neuropilin-2) to propagate a number of signaling pathways. Sema3A binding to Neuropilin-1 triggers the dissociation of RacGEF, leading to the activation of plexin-A1 downstream signaling events, such as activation of R-Ras GAP of plexin-A1 and subsequent down regulation of R-Ras, which in turn limits integrin-mediated attachment. The activated semaphorin receptor complex also stimulates the activity of protein kinases Fes, Fyn, Cdk5 and GSK-3β and these activations may induce biased cytoskeletal reorganization, such as actin polymerization and microtubule assembly, thereby leading to neuronal growth cone repulsion.

Neuropilin-1 and Neuropilin-2, whereas sensory axons express only Neuropilin-1, therefore, sensory axons are sensitive to Sema3A, whereas sympathetic axons show responsiveness to all three Sema3s.^{23,24} Interestingly, Sema3B and Sema3C were shown to block Sema3A binding to Neuropilin-1 and abolish the repellent actions of Sema3A on sensory neurons by competing the binding to Neuropilin-1.²⁵

In addition to Plexin-A1, other Plexin-A family members are involved in transducing the signals of class 3 semaphorins, but with less specificity. Overexpression of a dominant negative form of Plexin-A2 in DRG sensory neurons renders their axons insensitive to Sema3A,³¹ suggesting that Plexin-A2 also partially contributes to the receptor signaling of Sema3s. In a more recent study using the collagen gel repulsion assay, analysis of Plexin-A3, Plexin-A4 and Plexin-A3/-A4 double knockout mice showed that while Plexin-A3 and -A4 together mediate the responses to class 3 semaphorins in both sensory and sympathetic neurons, Sema3A repulsive signaling is mediated principally by Plexin-A4 via Neuropilin-1 and Sema3F repulsive signaling is mediated principally by Plexin-A3 via Neuropilin-2.^{32,33}

The specificity of receptor binding and repulsive effects of different class 3 semaphorins seems to be conserved in both PNS and CNS. Differential expression patterns of class 3 semaphorins^{34,35} and their receptors (neuropilins^{24,35,36} and plexins^{32,37}) in the developing hippocampus and afferent connections have also been implicated in the specificity of the chemorepulsive actions of different class 3 semaphorins.^{32,34-36} Notably, hippocampal axons explanted from the embryonic dentate gyrus (DG), CA1 and CA3 regions express both Neuropilin-1 and Neuropilin-2 and are repelled by both Sema3A and Sema3F in a collagen gel repulsion assay.³⁴ Moreover, function-blocking antibodies against Neuropilin-1 block the repulsive effect of Sema3A but not Sema3F,³⁴ while hippocampal axons from Neuropilin-2 knockout mice (*Nrp2*^{-/-}) lose their responsiveness to Sema3F but not Sema3A.³⁸ Analysis of Plexin-A3 knockout mice with collagen gel repulsion assays showed that Plexin-A3 also contributes to chemorepulsive effects of Sema3A and Sema3F on hippocampal axons.³²

Intracellular Mediators for Class 3 Semaphorin-Induced Growth Cone Turning

While the intracellular pathways, from the receptor activation to changes of cytoskeleton proteins that result in growth cone steering in response to class 3 semaphorins, are still not fully understood, several molecules and mechanisms have been identified to be involved in the cytoplasmic signaling of this family of guidance cues over the past decade.

Protein Kinases

After the initial observation of tyrosine phosphorylation of plexins *in vitro*,²⁹ several cytoplasmic kinases have been implicated in semaphorin-plexin-mediated growth cone responses (Fig. 1). For example, tyrosine kinase Fes,³⁹ threonine-serine kinase cyclin-dependent kinase 5 (Cdk5)⁴⁰ and glycogen synthase kinase-3 (GSK-3)⁴¹ have been shown to mediate sema3A-induced growth cone collapse. However, the functional roles of these molecules in growth cone steering have not been fully explored. A correlative study using Sema3A and *fyn* knockout mice also suggests a role of Fyn, a Src family nonreceptor tyrosine kinase, in mediating the guidance of apical dendrites of large pyramidal neurons to sema3A.⁴⁰ In cortical slices prepared from null mutants, some pyramidal neurons exhibit an atypical morphology of dendritic orientation in both *fyn*^{-/-} and Sema3A^{-/-} cortices using Golgi impregnation analysis. This study, however, did not provide direct evidence for the role of Fyn in dendritic guidance in response to Sema3A.

MICAL

The MICAL (molecule interacting with Cas ligand) family of cytosolic, multidomain, flavoprotein monooxygenases has recently been identified as a binding partner to plexins and is involved in semaphorin-plexin-mediated axon guidance.⁴² Interestingly, the monooxygenase

enzyme activity seems to be required for growth cone turning responses to Sema3A and 3F. In a collagen gel repulsion assay, a flavoprotein monooxygenase inhibitor neutralizes Sema3A- and Sema3F-mediated repulsion of both DRG and superior cervical ganglion (SCG) axons, respectively.^{42,43} These results, together with a recent report that 12/15-lipoxygenase is required for Sema3A-mediated axonal collapse,⁴⁴ suggest an important role of oxidation on the regulation of inhibitory effects of class 3 semaphorins on neuronal growth cones.

Rho Family of GTPases

Direct growth cone turning is mediated by biased cytoskeletal reorganization localized within a growth cone. Small GTPases of the Rho family (which include Rac, Rho and Cdc42) provide an important link between semaphorin receptor signaling and cytoskeletal dynamics in neurons.⁴⁵ Although many of the Rho GTPases, such as Rnd, Rac and Rho, have been shown to mediate semaphorin signaling in other biological events, their roles in mediating growth cone turning has not been firmly established. Their regulators, including activator GEFs (guanine exchange factors) and inactivator GAPs (GTPase activating proteins), however, have been shown to be involved in Sema3A-induced growth cone turning. Recently, a FERM domain-containing Rac-GEF protein, FARP2, has been identified to serve as a physical link between Sema3A binding and Rac activation.^{46,47} Sema3A-induced repulsion of axons of DRG neurons was completely abolished when these neurons were infected with adenovirus encoding short hairpin RNA (shRNA) against FARP2 or mutant forms of FARP2.⁴⁷ Interestingly, the intracellular domain of plexins contains two highly conserved regions that share a high degree of homology to the GAP domain as well as containing two arginine residues that are essential for the catalytic activity, thus plexin itself may function as a GAP. Indeed, several lines of evidence demonstrated that Plexin-A1 and Plexin-B1 are GAPs for the Ras-family GTPase R-Ras and the activation of GAP activity of plexins leads to inactivation of R-Ras, resulting in detachment of cells from the extracellular matrix.^{48,49} Together with the observation that stimulatory β 1 integrin antibodies significantly block Sema3A-mediated growth cone repulsion,⁴⁷ these studies implicate the importance of membrane adhesion for Sema3A signaling and growth cone migration.

New Protein Synthesis

Local protein synthesis in the axon has been implicated in acute growth cone responses to several families of guidance cues, including Sema3A. Sema3A treatment resulted in a marked increase in protein synthesis in isolated growth cones of *Xenopus* retinal ganglion cells within minutes.⁵⁰ In addition, both Sema3A-induced growth cone collapse and repulsive turning were blocked by protein synthesis inhibitors. p42/p44 MAP kinase (MAPK) activated by Sema3A may be upstream to Sema3A-induced protein synthesis and subsequent chemotropic activity of growth cones.⁵¹ Recently, it was shown that Sema3A induces intra-axonal translation of RhoA mRNA, and this local translation of RhoA is necessary and sufficient for Sema3A-mediated growth cone collapse.⁵² β -actin is another potential candidate protein that is translated in response to guidance cues, since β -actin mRNA is transported down to growth cones⁵³ and disruption of β -actin mRNA and protein localization to the growth cone leads to impaired growth cone motility.⁵⁴

Microdomain Signaling

Lipid rafts are plasma membrane microdomains enriched with cholesterol and glycosphingolipids, which provide an ordered lipid environment for localized trafficking and signaling.⁵⁵ A recent study showed that lipid rafts also mediate inhibitory effects of Sema3A on growth cones in both *Xenopus* spinal neurons.⁵⁶ Disruption of lipid rafts by membrane cholesterol depletion effectively blocks Sema3A-induced repulsion and extension of growth cones in *Xenopus* spinal neuron cultures.⁵⁶ In addition, a brief exposure to Sema3A increases the association of Neuropilin-1 with lipid rafts, implying asymmetric receptor-raft association and localized signaling in the growth cone during guidance responses. Activation of MAPK following

Sema3A treatment appears to depend on the integrity of lipid rafts and is required for Sema3A-induced growth cone repulsion.⁵¹ These results support a role for lipid rafts in mediating growth cone guidance by providing a molecular platform for the localized assembly of ligand-receptor complex and their downstream effectors for cytoskeletal rearrangement and local protein synthesis, including Neuropilin-1, plexins, Src family kinases (SFKs), Rho-GTPases and MAPK.^{55,56}

Modulation of Growth Cone Turning Responses to Class 3 Semaphorins

Cyclic nucleotides are potent modulators of growth cone turning in response to a group of guidance factors. For class 3 secreted semaphorin-induced growth cone responses, elevation of cyclic GMP (cGMP) signaling pathways converts Sema3A-induced repulsion of *Xenopus* spinal neurons into attraction in a growth cone turning assay.¹⁸ Although cAMP analogs had no direct effect on Sema3A-induced repulsion, the cAMP antagonist Rp-cAMPs blocks the conversion of the turning response to Sema3A in the presence of 8-Br-cGMP, suggesting that there is some interaction between cAMP- and cGMP-dependent pathways.¹⁸ The modulatory role of intracellular cyclic nucleotides in Sema3A-mediated repulsion was further supported by a recent report using a collagen gel assay showing that chemokine stromal cell-derived factor 1 (SDF-1) reduces the responsiveness of growth cones to Sema3A by elevating cAMP levels.⁵⁷ Interestingly, the level of cytoplasmic cGMP seems to act as an endogenous regulator of Sema3A signaling because pharmacological and histological evidence suggests that asymmetric localization of soluble guanylyl cyclase to the developing apical dendrites of cortical neurons allows Sema3A to act as a chemoattractant.¹³

Manipulation of the extracellular Ca^{2+} concentration, blockade of TRPC1-mediated Ca^{2+} influx or inhibition of the activity of CaM kinase II-calcineurin by specific inhibitors does not seem to influence Sema3A-induced growth cone repulsion of *Xenopus* spinal neurons.^{18,58,59} Electrical activity stimulation and resultant Ca^{2+} influx, however, was shown to modulate Sema3A-induced growth cone guidance behaviors by enhancing the repulsive activity of Sema3A.⁶⁰ Since the enhanced repulsive effect by electrical stimulation is abolished either by the removal of extracellular Ca^{2+} or with the addition of Sp-cGMPs, a membrane-permeable analog of cGMP, and mimicked by Rp-cGMPs, a competitive analog of cGMP without electrical stimulation, it was proposed that electric stimulation may indirectly influence the growth cone responses by mechanisms involving cGMP pathways.⁶⁰ The molecular mechanisms of cross-talk among cAMP, cGMP and Ca^{2+} signaling are still elusive.

The functional cross-talk between cell adhesion protein L1 and Sema3A is implicated in repulsive responses to Sema3A.^{61,62} L1, a member of the immunoglobulin superfamily of cell adhesion molecule (Ig CAM), directly associates with Neuropilin-1 and L1-deficient cortical and DRG axons lose their responsiveness to Sema3A, thereby acting as an integral part of the Sema3A-Neuropilin-1 receptor complex. L1 may also serve as a modulator of repulsive Sema3A signaling in two ways. First, L1 mediates the receptor internalization and thereby changes the sensitivity of growth cones to semaphorins.⁶³ Second, L1 may regulate the growth cone responses to Sema3A by decreasing the cGMP level. Addition of soluble L1-Fc chimera converted the Sema3A-mediated repulsion of wild-type but not L1-deficient axons into attraction through activation of NO/cGMP pathway.^{61,62} On the other hand, blockade of soluble guanylate cyclase prevented the L1-Fc-induced switch in the Sema3A responses.

Summary

Despite a tremendous amount of progress in the identification and characterization of many new players as components of class 3 secreted semaphorin signaling in growth cone steering (Fig. 1), our understanding of the molecular mechanisms is far from complete. More questions remain to be answered: how are differential cytoskeletal changes within a growth cone achieved

in response to semaphorins? What are the target(s) of cyclic nucleotide modulation? How does a growth cone make a reliable decision in response to a shallow gradient? And finally, how does a growth cone maintain its sensitivity to a decreasing concentration of semaphorins when it is growing away from the source? With a high degree of interest in the field with the development of novel technologies in analyzing growth cone steering, we expect to see a much more complete picture of semaphoring signaling in the near future.

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