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THE FUNCTION OF SEMAPHORINS DURING NERVOUS SYSTEM DEVELOPMENT

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1. ABSTRACT

The wiring of the nervous system is established through a progressive refinement of the choices made by a growing axon. The growth cone is a highly motile structure at the tip of the axon that integrates the multitude of signals present in its environment and translates these signals into structural changes of the cytoskeleton that determine the rate and direction of extension. Four families of guidance cues were identified that provide directional information to growing axons: the ephrins, the netrins, the slit proteins, and the semaphorins. The semaphorins represent the largest family of guidance cues identified so far that can be divided into 8 classes based on the degree of sequence similarity between their semaphorin domains and the presence of class specific carboxy-terminal domains (CTD). Collectively, the *in vitro* data indicate that semaphorins function mainly as chemorepellents that direct axons away from tissues marked by their expression, but can also act as chemoattractants in some cases. Genetic analysis of the semaphorins and their receptor showed that these proteins play an important role in the development and function of different tissues other than the nervous system. The effects of the different semaphorins are mediated by a variety of receptor complexes that include members of the neuropilin and plexin protein families as well as other membrane proteins belonging to the immunoglobulin superfamily. Plexins directly and indirectly interact with members of the Rho-like GTPases, the kinases Fes, Fyn, and Cdk5, the oxidoreductase MICAL, lipoxygenase, and the CRMP proteins. The signal transduction cascades that include these signalling proteins and link semaphorin receptors to the cytoskeleton are still incompletely understood.

2. INTRODUCTION

The wiring of the nervous system is established through a progressive refinement of the choices made by a growing axon (1-3). The first step of this program begins when neurons extend an axon that navigates through the developing embryo to its synaptic target. During this phase, the growth cone at the tip of the axon determines the direction of neurite growth. The growth cone is a highly motile structure that is responsible for sensing and integrating the multitude of signals present in its environment and translates these signals into structural changes that determine the rate and direction of extension (1). Guidance cues that provide directional information are especially important for the navigation of growth cones. A variety of *in vitro* and *in vivo* approaches in both invertebrate and vertebrate model systems led to the identification of at least four different families of guidance cues: the membrane-bound ephrins, the secreted netrin and slit proteins, and the semaphorins that include both membrane-bound and secreted members. These four families share three common features. Firstly, the proteins are bi-functional molecules that can act as attractive or repulsive signals depending on the composition of receptors and signalling cascades present in the cells. Secondly, different guidance cues control axonal outgrowth in a cooperative fashion. For example, the commissural axons of the vertebrate spinal cord are guided by the coordinated action of members of the netrin family, acting as chemoattractant, and members of slit and semaphorin families acting as chemorepellents. Finally, the guidance signals are evolutionarily conserved. Members of all four protein families are found from nematodes to humans and in many cases have similar roles in the guidance of axons.

3. SEMAPHORINS AND THEIR ROLE DURING NERVOUS SYSTEM DEVELOPMENT

3.1. The semaphorin family

The semaphorins represent the largest family of guidance cues identified so far. Semaphorin 3A (Sema3A, originally called collapsin), the first molecularly

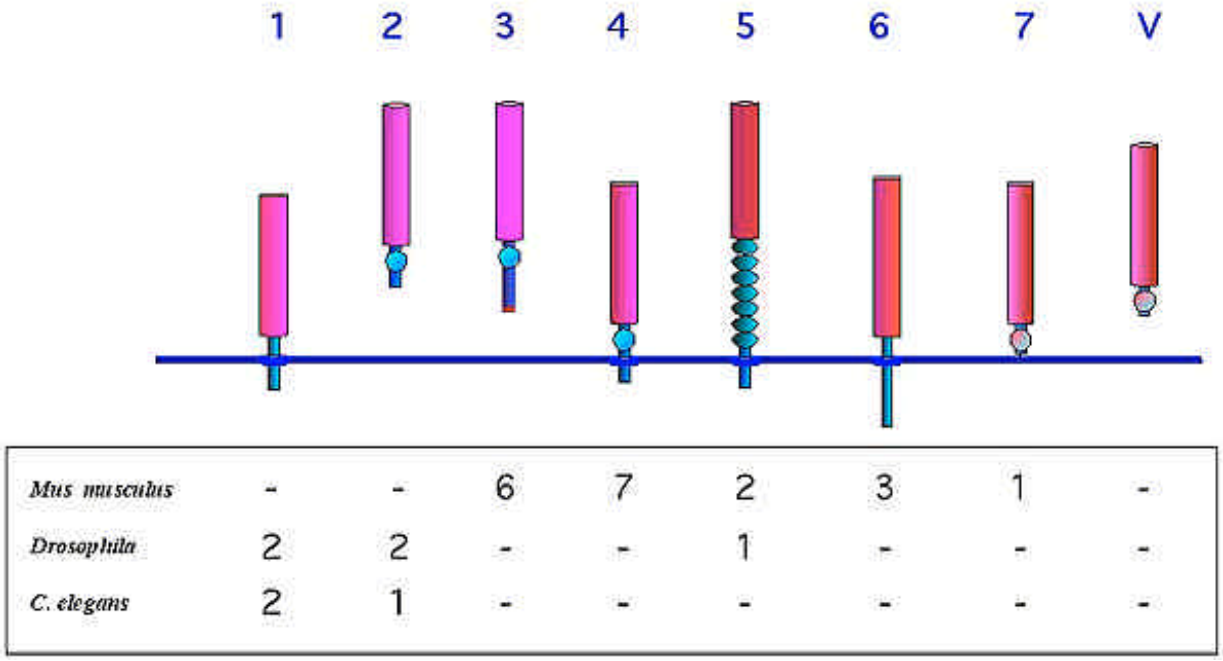

Figure 1. The semaphorin protein family. The different semaphorin classes containing family members found in invertebrates (1, 2, 5) and vertebrates (3-7) are indicated together with the number of members identified for each class in Caenorhabditis elegans (CE), Drosophila melanogaster (DM) and Mus musculus (MM). The distinction between the different classes is based on the degree of sequence similarity between their semaphorin domains and the presence of class specific carboxy-terminal domains. Red cylinders represent semaphorin domains, blue spheres immunoglobulin domain, ovals thrombospondin type 1 repeats, red rectangles the basic domain, and blue rectangles transmembrane domains

characterized chemorepellent, was identified as an activity in membrane preparations from embryonic or adult chick brain that induced the collapse of sensory growth cones (4-6). The growth cone collapse assay was instrumental in the subsequent biochemical isolation of Sema3A and the cloning of the corresponding cDNA. Sema3A displayed significant homology to Fasciclin IV (later renamed Sema-1a), a gene identified in a screen for glycoproteins that are expressed along specific axonal pathways in the central nervous system (CNS) of the grasshopper (7, 8). Sema3A/Collapsin and Sema-1a/Fasciclin IV were the first members of a large family of proteins that all share a conserved domain, the semaphorin (sema) domain (8-10) at their N-terminal end that consists of approximately 500 amino acids containing 14 highly conserved cysteine residues and one potential N-glycosylation site.

More than 30 semaphorins have been identified to date that can be divided into 8 classes based on the degree of sequence similarity between their semaphorin domains and the presence of class specific carboxy-terminal domains (CTD) (11, 12) (figure 1). Classes 1 and 2 are found only in invertebrates, while classes 3 to 7 contain the vertebrate family members, and class V members are encoded by viral genomes. The class 5 is the only that includes both vertebrate and invertebrate members (13-15). The semaphorin family contains both secreted (class 2 and 3) and membrane-bound proteins that are anchored in the plasma membrane by a transmembrane domain (classes 1, 4, 5, and 6), or a GPI-anchor (class 7) (figure 1). The CTD of most semaphorins is composed of an immunoglobulin-like domain (with a C2-type Ig homology) (classes 2-4 and 7) followed by a unique carboxy-terminal end. The CTD of the class 5 semaphorins contains 7 type I thrombospondin (TSP) repeats that are also found in extracellular matrix proteins, such as Thrombospondin 1 and -2 (13).

Just a few members of this large protein family have been characterized in detail. The growth cone collapse assay (5) and co-cultures of explanted tissue and cells producing recombinant semaphorins (9, 10) have been essential to study the function of these proteins and the molecular mechanism underlying their signalling (figure 2). The structural requirements for their function as guidance signals are best understood for the class 3 semaphorins. The presence of a sema domain and dimerization are required for their activity. The sema domain of Sema3A is sufficient to induce the collapse of growth cones only when produced in dimer form (16, 17). The specific effects of class 3 semaphorins on different types of axons are determined by a relatively short stretch of amino acids within the sema domain (18). Class 3 semaphorins are synthesized as largely inactive pro-proteins that require the proteolytic processing by the pro-protein convertase furin to become fully active. Several highly conserved clusters of basic amino acid residues are located in the sema domain and the C-terminus of the protein that contain consensus recognition sites for furin-like proteases. The proteolytic processing results in the generation of several isoforms that differ in their repulsive activity depending on the

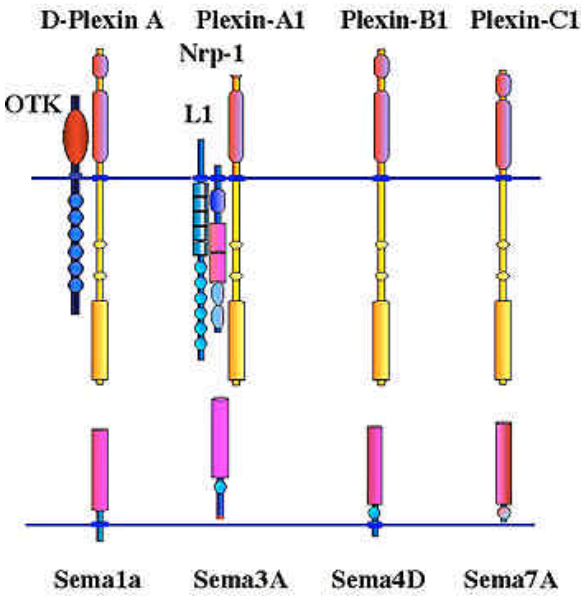

Figure 2. Sema3A induces the collapse of COS-7 cells and growth cones. Coexpression of Plexin-A1 and Nrp-1 in COS-7 cells allows the reconstitution of a functional Sema3A receptor. Addition of Sema3A results in the collapse of these cells within 10 minutes. This response resembles the collapse of sensory growth cones induced by sema3A and serves as a model system to study this receptor

combination of cleaved sites (19). As the relative amount of the isoforms changes during mouse embryogenesis, the proteolytic processing of class 3 semaphorins may be important for the modulation of their chemorepellent activity during development.

3.2. Semaphorin receptors

An expression screen using Sema3A as a probe led to the identification of Neuropilin-1 (Nrp-1) as an essential component of the Sema3A receptor (20) (table 1, figure 3). Nrp-1 was originally identified as an antigen specifically expressed in the optic tectum of Xenopus tadpoles (21). In addition to its function as a semaphorin receptor, it can also mediate heterophilic cell adhesion (22, 23). Incidentally, in the same screen also another subunit of the Sema3A receptor, Plexin-A1, was isolated (see below) (24, 25). Nrp-1 and the closely related protein Nrp-2 bind all tested class 3 semaphorins, but differ in their affinity for individual members (Rohm and Püschel, unpublished results) (26, 27). Sema3A preferentially binds Nrp-1, while Nrp-2 acts as a receptor for Sema3F and Nrp-1/Nrp-2 heterodimers for Sema3C. The effects of blocking antibodies confirm that Nrp-1 is required for the effects of Sema3A and Nrp-2 for those of Sema3F while inhibition of either Nrp -1 or Nrp -2 blocks repulsion by Sema3C (28, 29). The different class 3 semaphorins have specific effects on Nrp-1 and -2 expressing neurons. Sema3A strongly repels Nrp-1-positive neurites, such as sensory or sympathetic axons, while Sema3C or -3F affect only sympathetic axons, which also express Nrp-2 (9, 10, 29-31). In addition, Takahashi *et al*. (32) reported that Sema3B and -3C act as agonists for receptors containing Nrp-2, whereas they behave as competitive antagonists for Sema3A on receptors containing Nrp-1. Therefore, the specific effects of secreted class 3 semaphorins result from differences in their ability to activate neuropilin containing receptors. However, the molecular determinants for their agonistic or antagonistic effects remain to be identified.

Neuropilins differ not only in their specificity but also their affinity from semaphorin receptors present on neurons. The affinity of Nrp-1 for Sema3A (dissociation constant (K_D) = 0.33 - 1.15 nM) (20, 28) is at least one order of magnitude lower than that determined for neuronal Sema3A-binding sites (0.03 nM) or the EC_{50} for the collapse of sympathetic growth cones (0.05 nM) by Sema3A (33, 34).

Several results indicated that the neuropilins are not sufficient to form functional and specific receptors for class 3 semaphorins. Embryonic day (E8) chick retinal ganglion neurons do not express Nrp-1 and do not respond to Sema3A (22, 30), but become susceptible to the repulsive effects of Sema3A upon expression of NRP-1 from viral vectors(35). The cytoplasmic domain of Nrp -1 is dispensable for its ability to confer Sema3A-sensitivity to retinal axons. Replacement of the cytoplasmic and transmembrane domains of Nrp -1 by a heterologous sequence or a GPI-anchor did not impair its ability to confer Sema3A-sensitivity suggesting that additional receptor subunit(s), present in E8 chick retinal ganglion neurons, are responsible for activating downstream signal transduction cascades. Thus, additional components are required not only to form high affinity binding sites but also signaling-competent receptors specific for a single class 3 semaphorin.

The identification of VESPR (Plexin-C1) and Drosophila Plexin-A as receptors for the vaccinia virus encoded semaphorin A39R (SemaVa) and Drosophila Sema-1a, respectively, suggested that the Plexins are candidates for the missing signal-transducing subunit (36, 37). The plexins are a family of large integral membrane proteins with a highly conserved cytoplasmic domain. In C. elegans and Drosophila two plexins (Plexin-A and -B) were identified (37, 38), while the mammalian genome contains nine plexin genes that fall into four distinct groups (types A - D; Table 1) (38, 39). At their amino-terminus they contain a sema domain, which shows a moderate degree of sequence identity to the corresponding domain of semaphorins. As this homology includes 14 conserved cysteine residues it is likely that the semaphorin domains of plexins and semaphorins adopt similar structures (37). Together with the receptor protein tyrosine kinases Met, Ron, and Sea, plexins and semaphorins form a superfamily of semaphorin domain-containing proteins (38, 40, 41). In addition, their extracellular domains are characterized by two or three Met-related sequence (MRS) repeats also found in many other proteins (38, 42). In vertebrates, plexins are widely expressed in the developing central and peripheral nervous system including hippocampal, cortical, sensory, and sympathetic neurons (39, 43). mRNAs of all four A-type Plexins can be detected in dorsal root ganglia

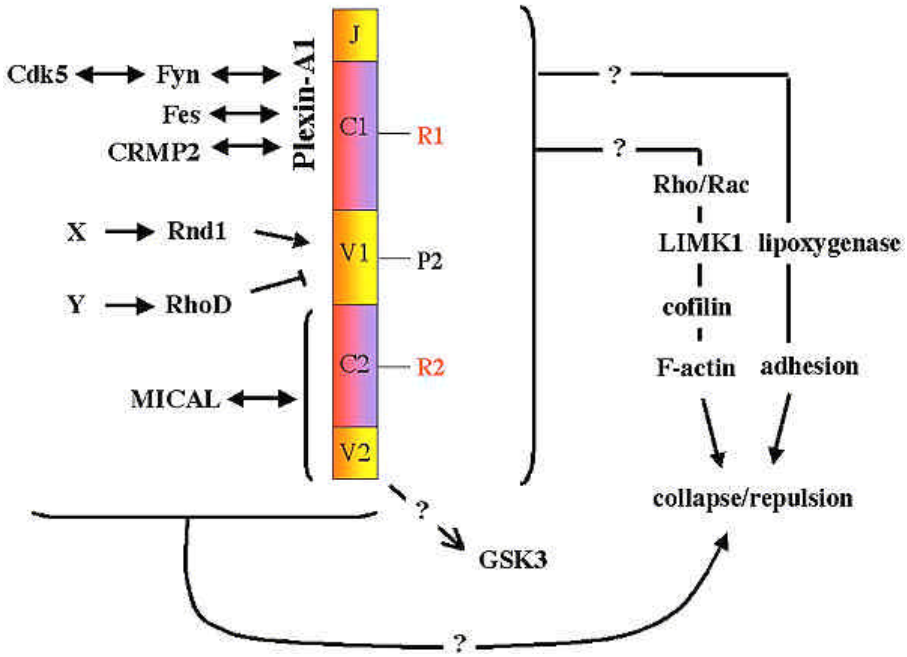

Figure 3. Semaphorin receptors. The different identified semaphorin receptors are shown together with their respective ligands. Red and yellow cylinders represent sema domains (red: semaphorin, yellow: plexin), blue spheres immunoglobulin domain, ovals thromobspondin type 1 repeats, red rectangles the basic domain and blue rectangles transmembrane domains

where Plexin-A1 shows the lowest and Plexin-A3 and -A4 the highest expression levels.

As in Drosophila (37), genetic analysis and biochemical assays showed that C. elegans Plexin-A (plx-1) acts as the receptor for the two transmembrane semaphorins Sema-1a and Sema-1b (44). As C. elegans Sema-2a does not bind Plexin-A, it is likely that Plexin-B is involved in mediating the effects of the class 2 semaphorins in invertebrates (44). The genomes of Drosophila and C. elegans do not contain recognizable neuropilin homologs, suggesting that invertebrate plexins may not require additional receptor subunits for ligand binding or that other proteins may perform the function of neuropilins.

Binding assays with transfected cells demonstrated that Sema4D binds Plexin-B1 while Plexin-C1 is a receptor for Sema7A (38). The class 3 semaphorins, however, do not bind directly to plexins but require the neuropilins for their interaction with the A-type plexins. Independent of the presence of ligands, several plexins (Plexin-A1, -A2, -A3, and -B1) are able to form complexes with both Nrp-1 or Nrp-2 (38, 45, 46). The formation of a complex containing both an A-type plexin and a neuropilin is essential for mediating the repulsive effects of Sema3A (38, 45, 46). Deletion of the conserved cytoplasmic domain of Plexin-A1 or -A2 results in a dominant-negative receptor that can suppress repulsion by Sema3A in Xenopus motor neurons and mouse sensory neurons (38, 46). Co-expression of NRP-1 and Plexin-A1 in COS-7 cells allows the reconstitution of a functional Sema3A receptor in a heterologous system (45) (figure 2). These results demonstrate that the Sema3A receptor consists of Nrp-1 as the ligand binding subunit and an A-type plexin as the signal-transducing subunit. It remains to be shown that Plexins are also required for mediating the attractive effects of class 3 semaphorins (47, 48) (see 3.6.).

Analysis of the Plxna3 knock-out confirmed its involvement in mediating class 3 semaphorin signalling (39). Sympathetic neurons explanted from mutant animals are completely unresponsive to Sema3F and only partially sensitive to Sema3A. Thus Plexin-A3 is an essential component of the Sema3F receptor while the effects of Sema3A are mediated by at least one additional protein, most likely another A-type plexins. This conclusion is supported by the observation that the same nerves that are severely defasciculated in the Sema3a and Nrp-1 knock-out animals show a much milder defasciculation phenotype in Plxna3 mutants. Homozygous Plxna3 mutant mice were viable and fertile and showed only minor defects in peripheral innervation. The ophtalmic branch of the trigeminal nerve was defasciculated in E10.5 to E12.5 mice. In addition, defects in hippocampal projections were observed.

At present, it is not clear if all A-type plexins can function as the signal transducing subunit of a class 3 semaphorin receptor. Experiments in COS-7 cells suggest that Plexin-A1 and -A2 are able to act as Sema3A receptors while Plexin-A3 is not (45). In contrast, inactivation of the mouse Plexin-A3 gene convincingly shows that Plexin-A3 can transduce repulsive signals and contribute to Sema3A and Sema3F signaling *in vivo* (39). The inability of Plexin-A3 to mediate cell collapse in response to Sema3A in COS-7 cells may result from inefficient post-translational

Table 1. Composition of semaphorin receptors. Known components and associated proteins of the various semaphorins are listed

Class	Plexin	Co-receptor	Other Receptors	Reference
1	Plexin-A	OTK		44, 54
2	Plexin-B ?			
3	A-type Plexins	Nrp-1, Nrp-2		20, 28, 29, 38, 45, 46
4	Plexin-B1	Met	CD72, Tim-2	53, 142, 143
5	?			
6	?			
7	Plexin-C1			38
8	Plexin-C1			36

processing or trafficking of Plexin-A3. Indeed, we observed that Plexin-A3 is retained to a large extent in intracellular compartments in COS-7 cells (Rohm and Püschel, unpublished results). In addition to their role in signal transduction, complex formation with Plexins also changes the ligand-binding properties of neuropilins. Neuropilin/plexin complexes display an increased specificity for secreted semaphorins (45, 46). A Nrp-1/Plexin-A1 complex prefers Sema3A over Sema3C or Sema3F, while Nrp -2/Plexin-A2 preferentially binds Sema3F. The biochemical basis for the increased specificity remains, however, controversial. While Takahashi *et al.* (45) reported an increase in the affinity of Nrp -1/Plexin-A1 for Sema3A, Rohm *et al.* (46) demonstrated an increase in the number of Sema3A binding sites detectable on transfected 293T cells when Nrp -1 and Plexin-A1 were co-expressed in comparison to cells expressing only Nrp -1 (45, 46). In contrast, the number of binding sites for Sema3C was reduced. The reason for this discrepancy is presently unclear, but may result from differences between the assay systems.

The amino-terminal semaphorin domain of Plexin-A1 associates with the carboxy-terminal half of the plexin ectodomain and thereby keeps Plexin-A1 in the inactive state (49). Consequently, deletion of the semaphorin domain or the complete ectodomain of Plexin-A1 results in the formation of a receptor that is constitutively active, both in a heterologous cell system (COS-7 cells) and in neurons. The semaphorin domain and the C-terminal half of the Plexin-A1 ectodomain interact independently with Nrp -1. In Nrp -1 the binding site for the semaphorin domain of Plexin-A1 does not overlap with that for Sema3A. These results suggest that prior to ligand binding the intramolecular interaction between the subdomains of Plexin-A1 results in a self-inhibition, which is released upon binding of Sema3A to Nrp -1.

One additional component of the mammalian Sema3A receptor is the cell adhesion molecule L1-CAM (50). Sensory axons explanted from L1-CAM knockout mice are insensitive to the repulsive effects of Sema3A. Furthermore, addition of a soluble dimeric L1-CAM ectodomain converts the repulsion of wild-type cortical and sensory axons into an attraction by Sema3A (51). It is unknown if L1-CAM is directly involved in signal transduction by the Sema3A receptor. Recent results show that a trans-interaction of L1-CAM and Nrp-1 triggers production of NO and activation of guanylyl cyclase that induces the reversion of Sema3A-mediated repulsion to attraction in cortical neurons (see 3.3.) (51, 52). Pharmacological inhibition of either soluble guanylyl cyclase or neuronal NO synthetase prevents this reversion.

The molecular composition of other semaphorin receptor complexes is less well characterized than that of the Sema3A receptor. Several results indicate that, at least in some cases, the other plexins also associate with additional subunits. In addition to its collapsing effects on hippocampal neurons, Sema4D triggers invasive growth of epithelial cells *in vitro* that is similar to the effects of Scatter factor/hepatocyte growth factor (HGF) through its activation of the receptor tyrosine kinase Met (53). The Sema4D receptor Plexin-B1 associates with Met and binding of Sema4D to Plexin-B1 stimulates the tyrosine kinase activity of Met that phosphorylates both receptors.

Another membrane protein that associates with plexins is Off-track (OTK) that forms a complex with Drosophila Plexin-A and genetically acts downstream of Sema-1a (54). OTK can also be co-immunoprecipitated with vertebrate A- and B-type plexins after heterologous expression. Off-track belongs to the immunoglobulin superfamily and contains a cytoplasmic domain with sequence similarity to tyrosine kinases. OTK probably is not an active kinase due to the absence of several key residues in the catalytic domain. It can mediate homophilic adhesion, which results in tyrosin phosphorylation of its intracellular domain.

3.3. Signal transduction by plexins

Incubation of sensory growth cones with Sema3A results in their collapse within 10 - 20 minutes. At least two signaling pathways are involved in this response (figure 4). One involves the small GTPase Rac and leads to the depolymerization of actin filaments (55). A second leads to the loss of substrate adhesion and requires 12/15-lipoxygenase (56). However, the identity of the molecules that link Rac or 12/15-lipoxygenase to the semaphorin receptor remain unknown. Recent work identified several proteins that interact directly or indirexty with the cytoplasmic domain of plexins and are required for the effects of Sema3A. These are the GTPases Rnd1 and RhoD, the tyrosin kinases Fes, Fyn, and Cdk5, and the CRMP2/CRAM complex (figure 4).

Work from many labs demonstrated that Rho-like GTPases are central regulators of cytoskeletal dynamics and control the organization of actin filaments and microtubules (57). Activation of Rho induces neurite retraction while active Rac promotes extension (58-62). Therefore, it is surprising that the Rho-like GTPase Rac1 is involved in mediating actin depolymerization during Sema3A-induced growth cone collapse (63-65). In contrast to the retraction of neurites formed by neuronal cell lines and the effects of ephrins that are both mediated by Rho

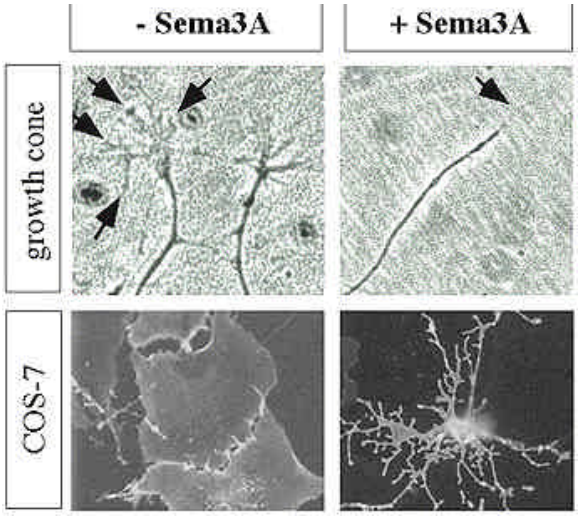

Figure 4. Signaling by A-type Plexins. A schematic representation of the intracellular domain of Plexin-A1 is shown. The two blocks of homology to Ras GAPs are labeled as C1 (conserved region 1) and C2. These are flanked by the juxtamembrane region (J), and two less well conserved regions V1 (variable region 1) and V2. V1 contains sequences essential for the interaction with GTPases in Plexin-A1 and -B1. P2 indictes the position of a mutation in three amino acid residues that abolishes GTPase binding (83, 86). R1 and R2 correspond to the arginine residues that are essential for the function of Plexin-A1 as Sema3A receptor subunit (82). Rnd1 and RhoD act upstream of Plexin-A1 and regulate its activity by their antagonistic effects. The activity of these GTPases is regulated by the unknown factors X and Y. Plexin-A1 induces actin depolymerization through its influence on the balance of Rho and Rac activity and the loss of integrin-mediated substrate adhesion by activation of 12/15-lipoxygenase. The molecular links that connect Rac and Rho to active PlexinA1 are still unknown. LIM Kinase I regulates cofilin, an actin-depolymerizing factor. Other proteins involved in signalling by A-type plexins are the kinases Fes, Fyn, and Cdk5, the collapsin response mediate protein CRMP2, and MICAL

(61, 62), Sema3A-induced growth cone collapse is inhibited by dominant-negative RacN17 (63-65). Downstream of Rac, phosphorylation of cofilin, a regulator of actin polymerization, by LIM Kinase 1 is essential for Sema3A induced growth cone collapse (66). Most of the signaling events, however, that translate the binding of Sema3A to its receptor into changes in the balance of Rho and Rac activity (59) and structural changes of the cytoskeleton remain to be elucidated.

The second pathway required to induce growth cone collapse involves the production of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) and the loss of integrin-mediated substrate adhesion (56). Sema3A stimulates the synthesis of 12(S)-HETE, which is is able to induce growth cone collapse. Treatment with a lipoxygenase inhibitor prevents both the loss of adhesion and growth cone collapse, but not the loss of F-actin from sensory growth cones.

In addition to Rho-like GTPases and lipoxygenase, several cytosolic proteins were implicated in mediating the effects of Sema3A. One of the proteins shown to interact with the cytoplasmic domain of A-type plexins is the tyrosine kinase Fes (67). Upon binding of Sema3A, Plexin-A1 recruits Fes and stimulates its kinase activity. Interaction of Nrp-1 and Plexin-A1 prevents the recruitment of Fes by Plexin-A1 in the absence of the ligand. Active Fes phosphorylates Plexin-A1 and another component of the signaling cascade, the collapsin response mediator protein 2/CRMP-associated molecule (CRMP2/CRAM) heterotetramers that form a complex with Fes (67, 68). The phosphorylation of plexins and/or CRMPs is essential for signal transduction, as expression of a kinase-deficient Fes mutant inhibits growth cone collapse by Sema3A.

CRMP-2 belongs to a family of 5 related proteins in vertebrates (CRMP-1 to -4, and CRAM/CRMP-5), which has an unknown biochemical function and shows sequence similarity to dihydropyrimidinase, the C. elegans protein Unc-33 and bacterial enzymes (69-71). CRMP proteins are phosphoproteins that are phosphorylated by Rho-kinase (ROK) and Fes and form homo- and heterotetramers (67, 72, 73). Several observations support a role of CRMP proteins in the development of axonal projections. Mutations in Unc-33 result in defects of axon growth (74). Neutralization of CRMP-2 blocks the ability of Sema3A to induce the collapse of sympathetic growth cones (70). CRMP-2 also binds tubulin dimers and stimulates microtubule assembly (75).

In addition to Fes, the kinases Fyn and Cdk5 are also involved in mediating the effects of Sema3A (76). Plexin-A1 and -A2 interact with the Src family kinase (SFK) Fyn and indirectly through Fyn with Cdk5. Activated Fyn phosphorylates Cdk5 and Plexin-A2 on tyrosine residues. Sema3A induces the phosphorylation and activation of Cdk5 by Fyn, which is essential for the collapse of sensory growth cones by Sema3A. Pharmacological inhibition of either Fyn or Cdk5 blocks the collapse of sensory growth cones by Sema3A. Axons from explanted E17 $Fyn^{-/-}$ and E12 $Cdk5^{-/-}$ dorsal root ganglia show an attenuated response to Sema3A. This effect is more pronounced in $Cdk5^{-/-}$ than in $Fyn^{-/-}$ neurons. As E12 sensory axons from $Fyn^{-/-}$ mice are not affected by Sema3A (76) other SFKs like Src may also be involved in Sema3A-induced signaling events and compensate for the loss of Fyn at earlier stages. A role for Fyn in the function of Sema3A is also supported by their genetic interaction as doubly heterozygous $Sema3a^{+/-}/Fyn^{+/-}$ and $Sema3a^{+/-}/Cdk5^{+/-}$ mice show defects in the orientation of cortical dendrites.

A yeast two-hybrid screen with the cytoplasmic domain of Plexin-A identified Drosophila MICAL as a binding partner (77). A N-terminal domain of 500 amino acids in Drosophila MICAL and its three vertebrate homologs displays sequence similarity to flavoprotein monoxygenases and binds FAD. A loss-of-function mutant of MICAL shows axon guidance defects similar to those seen in PlexA or Sema-1a mutants. Mutation of the

consensus FAD binding motif disrupts the ability of of MICAL transgenes to rescue the phenotype of MICAL mutants. Human MICAL-1 interacts with Plexin-A3 and -A4 suggesting that this interaction is conserved (77). Flavoprotein monooxygenase inhibitors neutralize the repulsive effects of Sema3A on sensory axons *in vitro*. These data suggest that MICAL is an essential component of the signaling cascade mediating the repulsive effects of several semaphorins and that it putative oxidoreductase activity is required for its function. However, the inhibitor used for these experiments is not specific for flavoprotein monooxygenases, but inhibits also other enzymes such as metalloproteinases (77). It remains to be shown directly that MICALs posseses oxidoreductase activity.

One additional component involved in the response of neurons to Sema3A is the Ser/Thr kinase GSK-3α/β (78). Sema3A activates GSK-3α/β by stimulating its dephosphorylation. Pharmacological inhibition of GSK-3α/β activity prevents Sema3A-induced growth cone collapse, but activation of GSK-3α/β by wortmannin is not sufficient to induce it.

The response of axons to guidance signals such as netrin, slit or semaphorins can be modulated by cyclic nucleotides (52, 79). In the axon turning assay with Xenopus spinal neurons increasing the intracellular cGMP concentration converts the repulsion by Sema3A into an attraction. Elevation of cGMP levels is also able to block the collapse of sensory growth cones be Sema3A. The asymmetric distribution of soluble guanyl cyclase may be responsible for the opposite effects of Sema3A on the apical dendrites and axons extended by cortical neurons (see 3.6.) (48).

Incubation of axons formed by Xenopus retinal ganglion cells with Sema3A induces a marked increase in translation in the growth cones (80). Translation inhibitors block the repulsion and growth cone collapse induced by Sema3A. A similar observation has been made for the attraction of Xenopus spinal neurons by netrin-1 and implicated in the resensitization of growth cones after exposure to netrin-1 (81). Therefore, it is possible that protein translation is not required directly for the response to Sema3A, but for the resensitization of growth cones and their ability to maintain their sensitivity to the repulsive signal Sema3A (81).

3.4. The role of GTPases for the signal transduction by plexins

The cytoplasmic domain of the plexins does not contain any homology to the catalytic domains of other well characterized receptors, but shows sequence similarities to GTPase activating proteins (GAPs) specific for Ras-like GTPases (figure 4) (82). GTPases act as molecular switches that regulate multiple cellular processes by activating downstream effectors when in the GTP-bound form. GAPs stimulate their intrinsic GTPase activity and terminate signaling by GTPases. The GAP homology is split into two blocks separated by a sequence of variable size in different plexins that is less well conserved between different family members (figure 4). The GAP homologies include two arginine residues that correspond to the catalytic residues found in rasGAPs. These arginines are essential also for the function of plexins as semaphorin receptors and mutation of either amino acid residue suffices to completely block the ability of Plexin-A1 to induce the collapse of COS-7 cells (82). However, so far there is no direct evidence that plexin may indeed have GAP activity.

Their homology to GAPs suggested that Plexins might regulate the activity of or interact with small monomeric GTPases of the Ras or Rho families. Indeed, several groups reported the binding of GTPases to plexins (82-86). While Rac1 interacts with Plexin-B1, Plexin-A1 binds Rnd1 and RhoD, and Drosophila Plexin-B forms complexes with Rho and Rac. Rnd1 and RhoD have antagonistic effects on the activity of Plexin-A1 and probably are involved in the initiation or modulation but not the execution of cytoskeletal collapse by Plexin-A1 (86). They appear to act upstream of Plexin-A1 to regulate its activity as a Sema3A receptor. Whereas the interaction between Rnd1 and Plexin-A1 triggers signaling by Plexin-A1 and results in cytoskeletal collapse in the absence of any ligand, RhoD has the opposite effect and blocks Plexin-A1 activity (figure 4). Activation of Plexin-A1 by Rnd1 may be a prerequisite for its ability to induce cell or growth cone collapse upon Sema3A binding. The regulation of Plexin-A1 activity by Rnd1 and RhoD does not require the presence of Nrp-1. The role of Nrp-1 in the receptor complex, thus, may be restricted largely to ligand-binding.

Sema4D specifically binds to Plexin-B1 and increases its interaction with Rac (87). Sequences between the two GAP homologies (V1 in figure 4) are essential for Rac binding that is abolished by mutation of three residues in V1 (83). Co-expression of active Rac and Plexin-B1 enhances Sema4D/Plexin-B1 binding, mainly by increasing the cell surface expression of Plexin-B1 in additon to a slight increase in receptor affinity (87). An intact Rac binding site is essential for the efficient expression of Plexin-B1 on the surface of transfected cells. Plexin-B1 and PAK compete for binding to active Rac *in vitro* and in transfected cells suggesting that Plexin-B1 may inhibit PAK activation by sequestering active Rac (87). Activation of a CD2-Plexin-B1 chimera in 3T3 cells by clustering with anti-CD2 antibodies results in the formation of stress fibers, a phenotype typical for active Rho (84). This phenotype was completely blocked by inhibition of either Rac or Rho. However, the Rac binding site in Plexin-B1 was not required, suggesting that the direct binding of Rac to this plexin is dispensible for this effects. Similar to mammalian Plexin-B1 Drosophila Plexin-B binds active Rac. In addition, a different region of PlexB that is not conserved in mammalian plexins interacts with Rho (85). A genetic analysis suggests that Drosophila PlexB suppresses Rac and enhances Rho activity. All B-type plexins contain a C-terminal PDZ-domain binding motif, which mediates the interaction with the Rho guanine nucleotide exchange factors (GEFs) PDZ-RhoGEF and LARG (88-91). Binding of Plexin-B1 by Sema4D activates these GEFs and leads to an increase in active Rho. The interaction with PDZ-RhoGEF is required for the induction of growth cone collapse after treatment of hippocampal neurons with Sema4D.

3.5. Class 1 and 2 semaphorins

In C. elegans one secreted (Sema-2a encoded by mab-20) and two membrane-bound semaphorins (Sema-1a and Sema-1b, encoded by smp-1 and -2) were described (92, 93). All three semaphorins are predominantly required during epidermal morphogenesis for modulating transient contacts between cells to prevent the formation or maintenance of inappropriate cell contacts (92). The adult male tail contains nine male-specific genital sensilla (ray 1 to 9) that develop from nine precursor cell clusters. In smp-1 or smp-2 mutants the most prominent phenotype is the frequent displacement of sensory ray 1 to a more anterior position. This defect may result from a failure to terminate the association with epidermal cells of the tail. In mab-20 mutants defects in the hypodermal enclosure of the embryo and frequent fusions of sensory rays were observed (93). Although all three mutants also display additional axon guidance defects, the major function of semaphorins in C. elegans appears to be the regulation of cell shape changes and cell association (92).

The Drosophila genome contains 5 semaphorin genes encoding three membrane-bound (Sema-1a, Sema-1b and Sema-5c) and two secreted proteins (Sema-2a and Sema-2b) (8, 14, 15, 94, 95). The integral membrane protein Sema-1a is present on most axons of the central nervous system, but not in the peripheral sensory neurons. One function of Sema-1a is to mediate motor axon defasciculation as an axonally localized repellent (95). In embryos deficient for the Sema-1a gene specific motor axons stall and fail to defasciculate at specific choice points.

Sema-2a is expressed by developing muscles and acts as a target-derived chemorepellent for motor axons. Ectopic expression of Sema-2a in muscles prevents specific neurons (RP3 and DC1) from forming a normal synaptic arborization (96). Loss-of-function mutants in Sema-2a result in the formation of promiscuous contacts with neighbouring inappropriate muscles in addition to the normal synaptic sites (94). These data suggest that one function of Sema-2a is to act as a selective target-derived signal that prevents the formation of ectopic synaptic terminals. Netrin-B acts as a short-range targeting signal for the RP3 growth cone. Removal of one or two copies of Sema-2a in the NetB mutant background suppresses the NetB phenotype indicating that the targeting defect caused by the decreased attraction in the absence of Netrin-B can be compensated for by lowering the repulsion mediated by Sema-2a (94). Thus, the correct establishment of axon terminals by the RP3 growth cone is determined by the balance of attractive and repulsive signals. A function of Sema-2a as a chemorepulsive guidance signal was also shown in the grasshopper embryo, where it regulates axonal fasciculation, as well as determines the direction of axonal growth of the Ti pioneer axon (97). When both Sema-2a and Sema-1a are neutralized by blocking antibodies, the incidence and severity of guidance defects is increased. These two semaphorins appear to provide functionally distinct guidance information in the developing grasshopper limb bud. When exogenously supplied Sema-1a is homogenously distributed it causes perturbation in the navigation of axons that suggest an attractive and/or permissive function (98, 99). Thus, Sema-1a can have both attractive and repulsive effects on different classes of sensory neurons in the grasshopper embryo (100).

Recent results indicate that Sema-1a is also involved in the formation of synapses in the giant fiber system of Drosophila where it is required both pre- and postsynaptically. Gain- and loss-of-function phenotypes suggest a bi-directional signalling by Sema-1 at the synapse, where it may act both as a receptor and as a ligand (101).

3.6. Vertebrate semaphorins (Classes 3 to 7)

Most of the vertebrate semaphorins show complex and dynamic expression pattern both in the embryonic and adult peripheral and central nervous system. However, only the function of the secreted class 3 semaphorins has been investigated in some detail. The analysis of the membrane-bound semaphorins is hampered by the absence of suitable *in vitro* assays. A combination of *in vitro* and *in vivo* approaches has demonstrated the involvement of semaphorins in the guidance of axons and dendrites, axonal fasciculation and neuronal migration in vertebrates. *In vitro*, the secreted class 3 semaphorins have strong repulsive effects on a large variety of axons. In addition to its effects on sensory growth cones, Sema3A acts as a repulsive or growth cone-collapsing signal for many central and peripheral neurons. These include the axons from sympathetic, sensory, and motor neurons (17, 19, 33, 102-105), as well as axons formed by olfactory, pontocerebellar, cortical and hippocampal neurons (31, 33, 47, 106-108). While most types of neurons tested are sensitive to Sema3A, other class 3 semaphorins affect a much small number of cell types (19, 31, 108).

Semaphorins can also act as attractants, at least *in vitro*. Sema3C attracts axons from cortical explants (47) and Sema3B those from olfactory bulb axons (107). Sema3A can also differentially affect the dendritic and axonal projections of the same neuron. In the cortex, Sema3a is expressed in the cortical plate and attracts the apical dendrites of pyramidal neurons, while it repels their axons (48, 109). In Sema3a$^{-/-}$ mice, a large number of apical dendrites extended by layer V, but not by layer III pyramidal neurons, fail to orient correctly towards the pial surface (48, 76). The intracellular level of cGMP is one of the factors that determine the response of neurons to Sema3A (see 3.3.). *In vitro*, elevation of the cytosolic cGMP concentration converts repulsion induced by Sema3A to attraction, while a decrease in cGMP enhances the repulsive activity of Sema3A (52). The soluble guanylate cyclase is asymmetrically localized in pyramidal neurons with higher levels in dendrites than in axons, which may explain the opposing effects of Sema3A on dendrites and axons (48).

Collectively, the *in vitro* data indicate that semaphorins function mainly as chemorepellents that direct axons away from tissues marked by their expression. The phenotype of Sema3a deficient animals was, however,

surprisingly modest compared to the strong *in vitro* effects of this protein (110). For example, *in vitro* assays and the expression pattern in embryonic spinal cord suggested a role in determining the lamina-specific termination of sensory afferents in the spinal cord (9, 10, 111). In the spinal cord of Sema3a $^{-/-}$ mice, a subclass of sensory neurons was reported to extend some axons ventrally into a region that they avoid in wild type mice (112). However, this finding was not confirmed in an independently generated mutant (113). It is not clear, if these conflicting results are due to differences in the genetic background or the methods used for their analysis. Furthermore, other pathways in the CNS, which on the basis of *in vitro* experiments and the Sema3a expression pattern might be expected to be perturbed in the Sema3a mutants, were largely unaffected in mutant animals (110, 113). Several peripheral axons (like the vagal, accessory, trigeminal, facial and glossophoringeal nerves) that are repelled by Sema3A *in vitro* are highly defasciculated in the knockout mouse. However, these axons project normally to their appropriate targets (113). These results suggest that Sema3A does not act as a chemorepulsive guidance signal for sensory axons, but promotes axonal fasciculation in a process called surround repulsion (114). A clearer case for Sema3A as a guidance signal has emerged for the projection of sensory axons from the olfactory epithelium to the olfactory bulb. Nrp-1^{+} axons normally avoid the Sema3A expressing ventral olfactory bulb, but are misrouted in Sema3a$^{-/-}$ mice and are no longer restricted to defined region of the olfactory bulb. These aberrant axon trajectories lead to the formation of glomeruli (the termination sites of olfactory axons) in atypical positions (115).

The overall modest effects of inactivating the Sema3a gene may be explained by the model that axons are guided by multiple cues that have potentially redundant functions. In addition, misguided axons may be removed later in development as shown in Sema3a$^{-/-}$ mice (116). Thus, eliminating a single guidance signal may result in an increased error rate, but does not cause gross abnormalities in the structure of the nervous system. This is the case for the navigation of commissural axons at the midline of the spinal cord that requires the coordinated action of proteins from at least three different families of guidance cues (3). Commissural neurons located in the dorsal half of the spinal cord extend axons toward the ventral midline, and cross it just once. Subsequently, they turn rostrally and grow alongside the midline for a considerable distance, but never cross back or enter the ipsilateral ventral horn of the spinal cord. Commissural axons are attracted to the midline by the diffusible chemoattractant Netrin-1 that is secreted by the floor plate (117, 118). Crossing-back is prevented, because the axons acquire responsiveness to repulsive guidance molecule secreted by the floor. Candidates for this activities are Slit-2 and Sema3B, which both act as chemorepellents for post-crossing commissural axons and push them away from the midline plate (119, 120). This example demonstrates that axonal trajectories are regulated by multiple cues, with complementary and partially redundant functions. Nerve trajectories are determined by the integration of different repulsive and attractive signals in the growth cone. These can act in a complementary or hierarchical fashion, with the response to one gating the response of another. As seen in the analysis of class 3 semaphorins, the discrepancies between the *in vitro* and *in vivo* data also show that genetic approaches are essential for understanding the physiological role of these proteins.

Semaphorins not only guide axonal extension, but may also play a role in neural crest cell migration (121) and in the sorting of telencephalon-derived interneurons migrating to the striatum or cortex (122). *In vitro*, it has been shown that explanted neural crest cells from trunk and hindbrain regions avoid a Sema3A containing substrate, suggesting a role of semaphorins in the control of cell migration. This hypothesis is supported by the observation that the sorting of telencephalon-derived interneurons migrating to the striatum or cortex is dependent at least in part of Sema3A (122). Interneurons expressing the Sema3A receptor Nrp-1 migrate to the cortex while those lacking neuropilins go to the striatum that express several class 3 semaphorins.

The function of most membrane-bound semaphorins is unknown. The phenotypic analysis of Sema6a deficient mice revealed defects that are less severe than expected on the basis of the *in vitro* data and expression pattern of this protein. *In vitro*, Sema6A repels sympathetic and sensory axons (123). It is expressed in the developing cranial nerves, optic tract, sensory axons, and several tracts in the brain including the fasciculus retroflexus, stria medullaris, the anterior commissure, and thalamocortical axons. In homozygous Sema6a mutant mice caudal thalamocortical axons project abnormally down towards the amygdala region instead.
A knock-out of the Sema4d gene results in effects in B-cell maturation, but no overt defects in the nervous system (124). Gain- and loss-of-function experiments in mice showed that Sema4D acts as a positive modulator in the immune system, specifically by acting on the B-cell response to T-cell-dependent antigens (124, 125).

3.7. Semaphorin function in the vascular system

Genetic analysis of the semaphorins and their receptor showed that these proteins play an important role in the development and function of different tissues, other than the nervous system. There is increasing evidence for an important role of semaphorins and neuropilins in cardiovascular development. Analysis of Sema3a knock-out mice revealed hypertrophy of the right ventricle and dilation of the right atrium of the heart (112). Sema3c knock-out mice die perinatally from congenital cardiovascular defects, due to the improper separation of the cardiac outflow tract and the interruption of the aortic arch (126). These abnormalities are the consequence of improper remodelling of the truncus arteriousus and branchial arch arteries and are probably caused by an impaired migration of cardiac neural crest cells (127). Since Sema3c it is expressed in the outflow tract along the pathway followed by migrating cardiac neural crest cells, Sema3C might act as a chemoattractant, facilitating entry of the cells into the outflow tract (127). Interestingly, interruption of the aortic arch and improper remodelling of

the truncus arteriousus has also been described for the Nrp1 knock-out mouse (128). This is consistent with the hypothesis that Nrp-1 functions as a receptor for Sema3C. Nrp-1 and -2 are able to bind the VEGF-A isoform $VEGF_{165}$, and the heparin binding form of the placenta growth factor (PlGF) and VEGF-B. In addition, Nrp-2 can bind $VEGF_{145}$ (129). Nrp-1 overexpression in transgenic mice leads to vascular abnormalities, resulting in the production of a large excess of vessels in the nervous system (130). In contrast, regression of neural vascularisation has been observed in Nrp-1 deficient mouse embryos. In addition, transposition of large vessels, insufficient development of vascular networks in the yolk sac and defects in heart and large vessel development were described in these mutants. While small lymphatic vessels and capillaries are absent or severely reduced in $Nrp2^{-/-}$ mice, no obvious defects were detected in the formation of arteries or veins (131, 132). However, Nrp1, -2 double mutants showed a completely avascular yolk sac, and died around E8.5, a phenotype resembling deficiency of the VEGFA or VEGFR-2 proteins. $Nrp1^{-/-}$ $Nrp2^{+/-}$ and $Nrp1^{+/-}$ $Nrp2^{-/-}$ mutants survive until E10. The formation of capillary networks and the branching of large vessels, are impaired in the yolk sack of these mutants. In addition, avascular regions were observed in the head (131). This indicates that neuropilins have an essential role in during both vasculogenesis and angiogenesis. It is still unclear, if these cardiovascular defects are due to an impairment of VEGF or semaphorin to interact with neuropilins. On the basis of *in vitro* evidence, the vascular phenotype of neuropilin-deficient mice has been interpreted as resulting from impairment of VEGF signalling. *In vitro*, efficient transduction of the $VEGF_{165}$-mediated angiogenic signals by VEGFR-2 requires the presence of Nrp-1 dimers that bind with high affinity $VEGF_{165}$ and enhance the VEGFR-2 mediated chemotactic activity of $VEGF_{165}$ (133). This effect of neuropilins is abolished in the presence of Sema3A. Thus it has been proposed that neuropilins might function simultaneously and independently as receptors in the VEGF and semaphorin pathways, and competition between the ligands for binding to the receptor could modulate the activity of the neuropilins containing receptors (129). However, *in vitro* data indicate that semaphorins might play a direct role in cardiovascular development, suggesting the existence of direct cross talk between these two signalling pathway. Sema3A can directly inhibit endothelial cell motility and capillary sprouting (134). It has also been shown that Sema3A can repel neural progenitor cells and that this effect depends upon the presence of VEGFR-1 (135). The phenotype of the Sema3A deficient mice and the similarity between the cardiovascular phenotype of the Sema3c and Nrp1 mutant mice is the first indication *in vivo* of a direct role of semaphorins in certain aspects of cardiovascular development.

4. PERSPECTIVE

Despite tremendous progress in understanding the function of semaphorins many questions remain to be addressed. So far only four out of twenty mammalian semaphorins have been analyzed by genetic approaches. All have surprisingly mild phenotypes, when compared to their strong *in vitro* effects. Especially the function of the membrane-bound members remains to be elucidated. A second open question concerns the signaling pathways mediating the various effects of semaphorins. A number of receptors habe been identified, but it is likely that more remain to be isolated. The various components of these receptors reveal the possibility of substantial cross-talk between semaphorin receptors and other signaling systems, but the extent and function of this cross-talk still has to be addressed. In addition, it remains to be investigated, whether semaphorins indeed form concentration gradients *in vivo* and how growth cones or migrating cells are able to recognize these gradient and translate them into structural changes. Significant progress has been made in identifying components of the signaling cascade, which is activated by semaphorin receptors. However, probably many more molecules remain to be isolated. A major challange will be to understand, how the different components contribute to the regulation of cytoskeletal and membrane dynamics that have to be coordinated for the response to guidance signals. Evidence from *in vitro* and *in vivo* approaches show that semaphorins perform essential functions in regulating cell-cell contacts in various systems and we are only beginning to understand their role in the development and function of the nervous system.

Semaphorins and their receptors are also widely expressed in the adult organism. Recent results show that expression of several semaphorins is induced after injury to the nervous system and that they may contribute to the inability of the mammalian central nervous system to regenerate (136). Their expresssion in glial scars may prevent the extension of regenerating axons and the ability of Sema3A to induce apoptosis may cause to the loss of damaged neurons (137-141). It remains to be shown, however, that neutralization of semaphorin activity can contribute to promoting neuronal regeneration.

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