CHAPTER 5

Slits and Their Receptors

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Abstract

Slit was identified in Drosophila embryo as a gene involved in the patterning of larval cuticle. It was later shown that Slit is synthesized in the fly central nervous system by midline glia cells. Slit homologues have since been found in C. elegans and many vertebrate species, from amphibians, fishes, birds to mammals. A single slit was isolated in invertebrates, whereas there are three slit genes (slit1-slit3) in mammals, that have around 60% homology. All encode large ECM glycoproteins of about 200 kDa (Fig. 1A), comprising, from their N terminus to their C terminus, a long stretch of four leucine rich repeats (LRR) connected by disulphide bonds, seven to nine EGF repeats, a domain, named ALPS (Agrin, Perlecan, Laminin, Slit) or laminin G-like module (see ref. 17), and a cystein knot (Fig. 1A). Alternative spliced transcripts have been reported for Drosophila Slit2, human Slit2 and Slit3, and Slit1. Moreover, two Slit1 isoforms exist in zebrafish as a consequence of gene duplication. Last, in mammals, two Slit2 isoforms can be purified from brain extracts, a long 200 kDa one and a shorter 150 kDa form (Slit2-N) that was shown to result from the proteolytic processing of full-length Slit2. Human Slit1 and Slit3 and Drosophila Slit are also cleaved by an unknown protease in a large N-terminal fragment and a shorter C-terminal fragment, suggesting conserved mechanisms for Slit cleavage across species. Moreover, Slit fragments have different cell association characteristics in cell culture suggesting that they may also have different extents of diffusion, different binding properties, and, hence, different functional activities in vivo. This conclusion is supported by in vitro data showing that full-length Slit2 functions as an antagonist of Slit2-N in the DRG branching assay, and that Slit2-N, not full-length Slit2, causes collapse of OB growth cones. In addition, Slit1-N and full-length Slit1 can induce branching of cortical neurons (see below), but only full-length Slit1 repels cortical axons.

Structure-function analysis in vertebrates and Drosophila demonstrated that the LRRs of Slits are required and sufficient to mediate their repulsive activities in neurons. More recent detailed structure function analysis of the LRR domains of Drosophila Slit, revealed that the active site of Slit (at least regarding its pro-angiogenic activity) is located on the second of the fourth LRR (LRR2), which is highly conserved between Slits. Slit can also dimerize through the LRR4 domain and the cystein knot. However, a Slit1 spliced-variant that lacks the cysteine knot and does not dimerize is still able to repel OB axons.

Introduction

The first roundabout gene, robo, was identified in Drosophila during a comprehensive screen for genes regulating midline crossing in the CNS. If SAX-3 is the unique robo ortholog in

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C.elegans, three robo genes have been found in Drosophila,\textsuperscript{5,29,30} zebrafish,\textsuperscript{31,32} chick,\textsuperscript{9} (Chedotal unpublished data) and mammals.\textsuperscript{7,12,33} Robo proteins belong to the immunoglobulin (Ig) superfamily and have five Ig-like domains followed by three fibronectin type III (FNIII) repeats, a transmembrane portion and a long intracellular tail containing up to four conserved cytoplasmic motifs, CC0-CC3, with no obvious catalytic domains (Fig. 1B). The first two Ig domains are the most highly conserved portion and are also found in another protein called Robo4 or magic roundabout that is only expressed by endothelial cells and plays a role in angiogenesis.\textsuperscript{34,35} However, Robo4 lacks the last three Ig domains, some FNIII domains and the CC1 and CC3 motifs found in other Robo proteins. Moreover, its capacity to bind Slits is still debated.\textsuperscript{36,37}
CC0 has no known function but is a site of tyrosine phosphorylation. CC1 also contains tyrosine residues that can be phosphorylated and was shown to bind to the P3 domain of the netrin-1 receptor, deleted in colorectal cancer (DCC; see below).

CC2 is a proline-rich sequence that matches the consensus binding site for Drosophila Enabled (Ena; see below), CC3 is also a polyproline stretch. Drosophila Robo2 and Robo3 lack the CC2 and CC3 domains and the second half of CC2 is also not conserved in mouse and zebrafish Robo3. Furthermore, mouse Robo3/Rig-1 (Rig-1 for retinoblastoma inhibiting gene 1P3) lacks the CC1 motif but zebrafish Robo3 has it. In addition, some spliced variants of mouse Robo3/Rig-1, including a secreted form, may exist. Last, Robo 1 can be cleaved in transfected cells.

In *Drosophila*, genetic and biochemical evidence demonstrated that Slit is a ligand of the Robo1-Robo3 receptors. Likewise, mammalian Slits can bind to all Robo receptors with comparable affinity. Slit cleavage fragments appear to have different cell association characteristics, with the smaller C-terminal fragment being more diffusible and the larger N-terminal and full length fragments being more tightly cell-associated. In addition, the C-fragment does not bind to Robo. More recent studies have shown that in *Drosophila* all three Robo receptors compete for a single active binding site in the second LRR of Slit and that neither the FNIII domains nor Robo dimerization are required for Slit binding. The major Robo1-3 binding site of Slit is in the second of the four LRRs, is evolutionary conserved and has a similar affinity for all Robos. However, Slit affinity is higher when all LRRs are present, probably due to its dimerization. On the receptor side, several results suggest that the first two Ig domains of Robos are required for Slit binding. First, the genetic deletion of Igl and Ig2 results in abnormal lung development. Second, antibodies against Robo Igl inhibit tumor growth in mice and neurite outgrowth in vitro. Third, Robo1 Igl-2 are important for Slit binding and function in vitro.

Several studies suggest that Slit can bind to other proteins than Robo, in particular heparan sulfate glycosaminoglycans that are negatively charged carbohydrates found on the cell surface. Slit1 and Slit2 were shown to bind to heparin column and to Glypican-1, a glycosyl phosphatidyl inositol (GPI)-anchored heparan sulfate proteoglycan known to interact with positively charged molecules. Biochemical data suggest that Slit binds to glypican-1 through its C-terminus. Moreover, heparinase III treatment reduces Slit2 activity and binding to Robo1. In *Drosophila*, expression of the transmembrane heparan sulfate proteoglycan syndecan in target cells appears to be required for Slit signaling. There is also genetic evidence in mouse supporting interaction between Slit and heparan sulfates in vivo. Heparan sulfates could help stabilizing the Robo/Slit complex or function as coreceptors presenting Slits to Robos or to alternative receptors.

**Robo Partners**

The analysis of Frazzled-Robo chimeric proteins in *Drosophila*, first revealed that the cytoplasmic domain of Robo is required to control the lateral positioning of post-crossing axons. Genetic and biochemical studies have then led to the identification of a number of transmembrane and cytoplasmic proteins that may participate or modulate Slit signaling through Robo receptors (Fig. 2). However, only a few of these proteins have been shown to directly participate to Robo signaling upon binding Robo CC domains.

**Abelson Tyrosine Kinase**

In *Drosophila*, Robo was found to be a substrate for the cytoplasmic tyrosine kinase Abelson (Abl). Abl is able to phosphorylate Robo’s CC0 and CC1 leading to Robo inactivation. In the *Drosophila* visual system, Abl was also found to interact with Robo2 and Robo3. An Abl substrate, the actin binding protein Enabled (Ena), is also involved in Robo repulsion in *Drosophila* and *C. elegans*. Ena was shown to bind Robo’s CC2 motif, therefore participating to Robo signaling. However, other studies showed that Abl rather than inactivating Robo, could...
Figure 2. A central role for Abelson tyrosine kinase in Robo repulsion. Abl can inactivate Robo signaling through phosphorylation of CC0 and CC1. Other data suggest that Abl is recruited following Robo activation. Abl interacts with multiple effectors such as Enabled (Ena), capulet (CAP) and Orbit/MAST/CLASP that control cytoskeletal dynamics. Slit binding to Robo was also shown to inactivate the cell adhesion molecule N-cadherin. Abl also mediates interaction between Robo and N-cadherin, most likely through an unknown partner. Robo can also bind to the netrin-1 receptor DCC upon slit binding. This leads to the inhibition of netrin-1 attractive activity.

promote repulsion downstream of Robo. The adenyl cyclase associated proteins (CAP) regulate actin polymerization and bind to the SH3 domain of Abl. Interestingly, axon guidance defects at the midline were observed in the Drosophila CAP homolog capulet (capt), or in capt-slit or capt-robo-robo2 transheterozygotes. In this system, Abl and Capt are recruited by Robo activation and inhibit actin polymerization, therefore acting positively in the Slit pathway. Thus, Abl may play both positive and negative roles in Slit signaling. Slit binding to Robo was also shown to inactivate the cell adhesion molecule N-cadherin that mediates homophilic binding. Interestingly, Abl is required for Robo binding to N-cadherin. Robo is
thought to inhibit N-cadherin function by interfering with its cytoplasmic domain and by inducing a decrease in N-cadherin-mediated adhesion. Slit binding to Robo increases the phosphorylation of β-catenin and thus its ability to bind to N-cadherin. This induces the binding of Robo to N-cadherin resulting in its inhibition.55

Abl function downstream of Robo may involve microtubule associated proteins (MAP) in addition to actin binding proteins.56 The MAP Orbit/MAST, ortholog of the vertebrate cytoplasmic linker protein (CLIP)-associated proteins (CLASP) are microtubule-associated plus end tracking proteins that seem to reduce microtubule stability. Genetic evidence supports a role for Orbit/MAST downstream of Abl in the Slit repellent pathway.

Although all these studies suggest that Abl plays a pivotal role in mediating Robo signaling in Drosophila, it remains to determine if Abl function is conserved in vertebrates.

**Rho Family of Small GTPases**

Rho family of small GTP-binding proteins (Rho GTPases) are major modulators of the actin cytoskeleton and play a central role in axonal growth and cell migration.57 Rho GTPases are activated upon GTP binding and inactive when bound to GDP. The switch from their active to their inactive state is controlled by two families of proteins: the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). GEFs activate Rho GTPases while GAPs inactivate them by inducing GTP hydrolysis.57

Many studies have shown that Rho GTPases play an important role in the modulation of Slit function (Fig. 3). First, activation of RhoA in Drosophila causes axons to cross the midline.58 Second, in this system, Rac1 inactivation or Cdc42 activation can overcome the effect of constitutively active Robo suggesting that Cdc42 and Rac1 function downstream of Robo.59 Likewise, in vertebrate neurons, a constitutively active Cdc42 blocks the repulsive effect of Slit.60 Biochemical studies have shown that the SH3-SH2 adaptor protein Dock, can bind directly to Robo and that this interaction requires the SH3 domain of Dock and the CC2 and CC3 motifs of Robo.61-62 Dock is known to interact with key regulators of the actin cytoskeleton such as p21-activated protein kinase (Pak), a serine-threonine kinase which in turn can interact with RhoGTPases such as Rac1 and Cdc42. Slit binding to Robo increases the level of Dock-Robo association, recruits Pak and stimulates Rac (in particular Rac1). Thus Slit regulates the assembly of a multiprotein complex composed of Dock, Pak and Rac that couples Robo receptor activation to the regulation of the actin cytoskeleton.

Robo also controls the activity of Rho GTPases through a family of Slit/Robo specific GAPs, SrGAP1-3, that were identified using yeast two hybrid and Robo1 CC3 domain as a bait. SrGAPs consist of a RhoGAP domain, a SH3 domain and a Fes/CIP4 (FCH) homology domain. SrGAP1 and srGAP3 bind to the CC3 domain of Robo1 through their SH3 domain. Slit2 increases Robo1 binding to srGAP1 and its activity and this regulation requires CC3. In turn, SrGAP1 inactivates Cdc42 and activates RhoA but not Rac1.60 Contrary to srGAP1, srGAP3 is mainly a repressor of Rac1.63 Recently, it was also shown that in *Drosophila*, Slit/Robo signaling could also control Rac activity upon binding the GAP Vilse/CrossGAP, that is conserved in vertebrates.64,65 Genetic evidence showed that CrossGAP may be involved in Robo-dependent axonal repulsion and tracheal cell migration and that it specifically inactivates Rac. Moreover, CrossGAP WW domains can directly bind to the CC2 domain of Robo and thus may not function downstream of other Robo receptors that lack the CC2 domain.64 Moreover, the two proteins can be coimmunoprecipitated from brain extracts.65 Although it inactivates Rac, Vilse seems to have a positive role in Robo repulsion.64

**The Netrin Receptor DCC**

Deleted in colorectal cancer (DCC) is a transmembrane receptor for the secreted protein netrin-1 (see Chapter by Moore et al). Robo1 can bind DCC and this results in the inhibition of netrin-1 attraction.60 This silencing activity requires the binding of the CC1 domain of Robo 1 and to the P3 domain of DCC. The *C. elegans* homolog of DCC, UNC-40, can also
Figure 3. Rho GTPases in Slit/Robo signaling. Rho family of small GTP-binding proteins (Rho GTPases) are activated upon GTP binding and inactive when bound to GDP. Rho GTPases play an important role in the modulation of Slit function. The SH3-SH2 adaptator protein Dock, can bind directly to Robo. Dock interacts with key regulators of the actin cytoskeleton such as p21-activated protein kinase (Pak), which in turn can interact with RhoGTPases such as Rac1 and Cdc42. Slit binding to Robo increases the level of Dock-Robo association, recruits Pak and stimulates Rac. Robo also controls the activity of Rho GTPases through a family of Slit/Robo specific GAPs. SrGAP1 inactivates Cdc42 and activates RhoA but not Rac1. Slit/Robo signaling could also control Rac activity upon binding the GAP Vilse/CrossGAP. CrossGAP WW domains can directly bind to the CC2 domain of Robo. Although it inactivates Rac, Vilse seems to have a positive role in Robo repulsion.

bind SAX-3. Interestingly, Slit also binds netrin-1, but the functional consequence of this interaction is unknown.

Other Modulators of Slit/Robo Function

ECM molecules, specially laminin-1 have been shown to influence the response of retinal axons to netrin-1. Thus, exposure of Xenopus retinal axons to laminin converts their response to netrin-1 from attraction to repulsion, apparently by lowering cAMP levels in the growth
cones.\textsuperscript{67} The level of cyclic nucleotides in the growth cone in part determines the action of many guidance cues.\textsuperscript{68} It was found that Slit2-N growth-promoting action could be converted into an inhibition by lowering cGMP levels. Along this line, the activation of CXCR4 by the chemokine SDF-1 reduces the repulsive activity of Slit2 on retinal axons indirectly, by stimulating PKA\textsuperscript{69} and increasing cAMP levels. Other second messengers such as calcium may also participate to Slit signaling.\textsuperscript{70} Interestingly, as for netrin-1, a laminin-1 peptide was able to convert Slit2-N activity and this may also involve integrins.\textsuperscript{71} Accordingly, there is genetic evidence in flies for a regulation of Slit action by integrins.\textsuperscript{62}

Last, there is also genetic evidence suggesting that receptor-linked tyrosine phosphatases,\textsuperscript{72} Calmodulin and the Ras/Rho GEF Son of Sevenless (SOS) critical for Ras activation\textsuperscript{73} may participate to Slit/Robo signaling. Both Sos and CaM signaling pathways are required to prevent certain axons from crossing the midline. However, the link with the transduction of Robo signaling is unclear and these proteins may just function in parallel pathways.

**Molecular Control of Slit and Robo Expression**

**Transcriptional Regulation**

In *Drosophila*, Slit function is controlled by the BTB transcription factor Lola.\textsuperscript{74} In addition, several transcription factors were shown to control Slit expression in fly embryo\textsuperscript{75} such as the PAS bHLH single minded.\textsuperscript{76} Slit promoter region also contains binding sites for the SOX HMG domain protein Fish-Hook and the POU domain protein Drifter.\textsuperscript{75} Likewise, in the chick retina optic layer, the Lrx homeobox gene family member, Lrx4, negatively controls Slit1 expression.\textsuperscript{77} Last, Islet-2, a LIM/homeodomain-type transcription factor of the Islet-1 family was also proposed to control in zebrafish sensory neurons the expression of some factors important for Slit signaling.\textsuperscript{78} One of these factors may be the semaphorin receptor plexin-A4.\textsuperscript{79}

Similarly, Robos could be subject to transcriptional regulation.\textsuperscript{74,80} For instance, in fly embryo, Robo expression in the mesoderm is likely to be controlled by homeotic genes such as homothorax\textsuperscript{81} and there is a box binding site in the robo2 gene.

**Post Transcriptional and Post-Translational Regulation Commissureless**

In *Drosophila* and rodents, Robo expression is regionally restricted\textsuperscript{29,41,82} to longitudinal axons and absent from commissures. In fly, this localization of Robo to the post-crossing segment of commissural axons is controlled by the transmembrane protein commissureless (Comm).\textsuperscript{82,83}

Comm was initially proposed to be expressed and required at the midline for appropriate midline crossing and to triggers Robo internalization.\textsuperscript{84} However, more recent studies have shown that Comm is expressed by commissural axons and acts autonomously in commissural neurons.\textsuperscript{85} Moreover, there is no need for Comm at the midline for restoring midline crossing in *comm* mutants.\textsuperscript{86} Comm protein appears to be prevented from reaching the contralateral portion of commissural axons and accumulates at the midline. In turn, Comm prevents the delivery of Robo at the growth cone, by recruiting it to late endosomes.\textsuperscript{86} It is still unclear why Robo is present on the post-crossing segment.

Comm is a predicted transmembrane protein of 370 amino acids with no known domains. Structure-function analysis revealed that the N-terminal and transmembrane domains of Comm are required to downregulate Robo.\textsuperscript{87} The intracellular portion of Comm is also essential for its function and contains an endosomal sorting domain that is required, together with the membrane proximal region of *comm* (108-131) to relocalize Robo in transfected cells.\textsuperscript{86} It also includes a binding site for the ubiquitine ligase dNedd4 and interaction with Nedd4 is required for Comm to localize within vesicles in transfected S2 cells.\textsuperscript{88} In yeast two hybrid, Nedd4 was shown to bind Robo.\textsuperscript{88} However, more recent studies have shown that comm ubiquitination is not required for its function and that Nedd4 does not influence midline guidance in vivo.\textsuperscript{86} Robo2 and Robo3 expression can also be negatively regulated by Comm when the protein is overexpressed but this probably does not occur in vivo.\textsuperscript{45,36} In normal
condition, the restricted expression of Robo2 and Robo3 expression may be controlled by other Comm proteins, but may also be transcriptionally regulated.5

Despite its major role in Drosophila, so far no commissureless homolog has been found in vertebrates suggesting the existence of additional regulatory mechanisms and modulators one of which could be Robo3/RigI.

In the mouse spinal cord, commissural axons become responsive to midline repellents, including Slit2, after crossing.89 Moreover, in mouse spinal cord, Robo1 and Robo2 expression is upregulated after crossing.90 This suggests that the expression and function of vertebrate Robo is also precisely controlled at the midline. Surprisingly, this regulation seems to involve the receptor Robo3/RigI. Rig1 expression overlaps with Robo1 in dorsal spinal cord41 and is downregulated in post-crossing axons and neurons.51,91 In addition, axons from RigI knockout exhibit a premature response to Slit. In the spinal cord and hindbrain,91 Rig1 seems to function as an inhibitor of Slit signaling in precrossing axons. Accordingly, there is a significant rescue of midline crossing by commissural axons in rig1/slit2 and rig1/robo1 double mutants and rig1/slit1/slit2 triple mutants.31,90 Rig1 exact function is unknown. It may sequester Slit, or interfere with Robo1 signaling, but there is still no evidence for direct Robo/Rig1 interaction.41

Other Regulators

As mentioned above, all Slits, and possibly some Robo receptors can be proteolytically processed into shorter fragments. The enzymes regulating the cleavage of these proteins are unknown, although there is some evidence92 for a role of the metalloprotease of the ADAM family kuzbanian in Drosophila. There is also some data supporting a posttranscriptional modulation of Slit function by the Arf6-GEF, Schizo, through a regulation of endocytosis or membrane dynamics.93

Multiple Functions for Slit/Robo in the Nervous System

Slits play a major role in axon guidance in many systems and animal species. In most cases Slits act as repellents but there is some evidence that they may act positively on some axons.71,77

Midline Crossing

Slit and Robo are primarily known for their function in regulating midline crossing in the nervous system. In Drosophila robo mutants, many axons abnormally cross the CNS midline and some multiple times.29 In Drosophila, Robo also controls midline crossing in the olfactory system.94 In the CNS of slit mutant, axons converge to the midline and remains there. Slit was later shown to be a repellent for noncrossing axons and for commissural axons once they have crossed the midline. Biochemical and genetic studies showed that Slit is produced by midline glia cells and that its binding to Robo triggers axonal repulsion. The different midline phenotype between robo and slit mutants suggested that additional Slit receptors may be present on commissural axons. Accordingly, Robo2 was shown to act redundantly with Robo to control midline crossing in Drosophila.45 However, each receptor has a unique role and their function in controlling midline crossing is only partially redundant. In contrast, Robo3 does not seem to play a role in midline crossing in fly.5

Interestingly, this essential function of Slit/Robo at the CNS midline is evolutionary conserved from C. elegans to humans.33 In all these species, Slits are expressed at or near the midline, such as the floor plate and septum in vertebrates, or are expressed around decussating axons, canalizing them as they approach the midline. Thus, in vertebrates, Slit/Robo were shown to govern midline crossing by retinal axons95,96 commissural axons in the spinal cord,41,90 olfactory bulb axons,37 cortical axons,98,99 precerebellar axons.41 They were also shown to control midline crossing by migrating neurons in the hindbrain.91

In the vertebrate visual system both ipsilaterally and contralaterally projecting axons respond to Slits and in their absence, pathfinding errors are observed prior to crossing. In this system, Slit expressing cells surround retinal axons, channeling the axons before and after the chiasm up to the diencephalon.96,100 The same occurs in the neocortex where Slit2 expression
in the glial wedge and induseum griseum prevent callosal axons from entering the septum. \(^98,99\) In vertebrates, the function of the three \textit{slit} genes, that are often totally or partially coexpressed \(^101\) appears largely redundant. Thus, axonal tracts are only slightly perturbed in mice deficient for a single \textit{slit} gene and sometimes for two \textit{slit} genes. \(^90,95,97,98\) This redundancy may explain why some major commissures such as the anterior commissure and the hippocampal commissure are normal in mice deficient for both Slit1 and Slit2. Accordingly, it is only in the spinal cord of triple Slit1/2/3 knockouts \(^90\) that many commissural axons stay at the midline and recross it, a phenotype reminiscent of the \textit{Drosophila slit} mutant. The organization of the brain of \textit{slit1/slit2/slit3} triple knockouts will have to be fully studied to determine if Slit/Robo controls the development of all commissural tracts in vertebrates.

**Projection Map Formation**

In many systems, in particular those conveying sensory informations, axonal projections are topographically ordered in the target territory. Slit and Robo seem to play an important role in regulating axonal targeting in vertebrates and invertebrates. Thus, in the \textit{Drosophila} visual system, Slit and Robo control the segregation of lamina cells (that express Slit) and lobula cells (that express all Robo receptors) by preventing cell mixing. \(^102\) Likewise, in the visual system of zebrafish, Robo2 (\textit{Astray}) in addition to control axon guidance at the chiasm regulates pathfinding within the tectum. \(^96,100\)

In the \textit{Drosophila} olfactory system, distinct subtypes of olfactory axons express various combinations of Robo receptors and Robo controls axonal positioning in the olfactory lobes. \(^94\) In rodents, the projection from the vomeronasal organ (VNO) to the accessory olfactory bulb (AOB) is topographically organized. Neurons in the apical part of the VNO send axons to glomeruli in the anterior half of the AOB and VNO neurons in the basal part project to the posterior AOB. All VNO axons were shown to express \textit{robo1} mRNA during development, while \textit{robo2} is present only in basal ones. \(^101,103\) Slit1 and Slit3 are also expressed in the VNO (preferentially in the apical part) and the anterior AOB and VNO axons are repelled by Slit in collagen gel. \(^101,103,104\) The important role of Slit1 in VNO axon targeting was recently confirmed in vivo using \textit{slit1}-deficient mice. \(^104\) In zebrafish, Robo2 controls the development of olfactory projections from to the olfactory bulb, in particular the establishment of the glomerular map. \(^105\)

**Branching**

In vertebrate, Slit2 was originally purified as a factor able to stimulate the formation of axon collateral branches by NGF-responsive neurons of the dorsal root ganglia (DRG). \(^21\) It was also shown that only the N-terminal fragment of Slit2, but not the full length protein is capable of stimulating DRG elongation and branching. \(^21,24\) Moreover, full-length Slit2 can antagonize the effect of Slit2-N. \(^24\) Slit2 also controls the branching/arborization of central trigeminal sensory axons in the brainstem of rodents \(^106\) and in zebrafish. \(^78\) In this later case, the branching activity of Slit2 is modulated by the semaphorin receptor plexin-A4. \(^79\) Last, although DRG express Robo2, \(^21\) and trigeminal axons express both Robo1 and Robo2, the axonal receptor mediating Slit branching activity is unknown.

Interestingly, Slit/Robo not only influence axonal branching but also dendritic branching. First, in Drosophila, the directionality of dendritic outgrowth at the midline is controlled cell-autonomously by Robo. \(^107\) In \textit{robo} mutant, dendrites of motor neurons grow abnormally toward the midline while no phenotype was observed in \textit{robo2} and \textit{robo3} mutants. Likewise, Slit1 has a dual activity on rat cortical neurons, \(^108\) as it repels their axons but induces dendritic growth and branching. This effect appears to involve Robo signaling.

**Longitudinal Tract Formation**

In \textit{Drosophila}, Robo, Robo2 and Robo3 are expressed in overlapping domains within longitudinal tracts of the CNS, and this combination of Robo receptors is thought to control the lateral position of longitudinal axons. Thus, genetic alterations of the Robo code displace
longitudinal axons along the mediolateral axis. However, it is not known if these changes involves Slit signaling. As Robos are immunoglobulins and able to mediate homophilic and heterophilic binding, it is possible that the control of lateral positioning by Robo involves Robo-Robo interactions and selective axonal fasciculation. In mouse, there is also some data supporting a differential expression of Robo receptors by longitudinal axons that project at distinct ventro-dorsal position in the spinal cord.41

**Control of Cell Differentiation**

In fly, serotoninergic neurons are bilaterally organized and must cross the midline to achieve their differentiation. Robo2 and Robo3 were shown to regulate the expression of the serotonin transporter (SerT) as many serotoninergic neurons fail to express SerT in robo2 and robo3 mutants. Moreover, Robo2 and Robo3 are required for eagle expression, a transcription factor controlling serotoninergic differentiation.109 Interestingly, SerT activity is normal in slit mutants suggesting that Robo2/3 function in serotoninergic differentiation is Slit independent. In Drosophila, Slit also promotes the terminal asymmetric division of ganglion mother cells by regulating the asymmetric distribution of Inscuteable and by downregulating the expression of POU genes.110 In vertebrates,111 Robo1 may also control cell differentiation as its overexpression in Xenopus leads to ectopic neuronal differentiation. Last, during kidney development in mouse, slit2 and robo2 inactivation leads to supernumerary ureteric buds, possibly through posttranscriptional effect on other developmental genes.12

**Cell Migration**

Another important function for Slits and Robos is the control of cell migration in the nervous system (both neurons and glia) and in several other tissues. As for axons, Slits were found to be important regulators of the behavior of migrating cells at the midline. But in contrast with axons, migrating cells can either be attracted or repelled by Slits. In Drosophila, longitudinal glia is generated from glioblasts that migrate ventrally to contact pioneer neurons at a distance from the midline. These cells express Robo1 and in robo mutant, glial cells migrate over the midline113 suggesting that Slit is repulsive. Likewise, Muscle precursors in Drosophila embryos114 fail to migrate away from the midline in slit mutant. In this system also, Slit produced by midline glia acts as a repellent. However, at later stages Slit expressed at muscle attachment sites attracts muscle precursors that express both Robo and Robo2. Interestingly, Comm also cooperates with Robo and Robo2 to control muscle precursors migration.115 The mechanism responsible for the switch from repulsion to attraction is still unknown but may involve signaling through different Robo receptors as suggested in other systems. Hence, Robo2 was proposed to mediate the long-range attraction of tracheal cell into the CNS115 while Robo may mediate a repulsive action of Slit on tracheal cells. This different activity of the two receptors may rely on differences in their cytoplasmic domains. In C. elegans, Slit was also shown to be a positive regulator of neuronal migration C.elegans along the anterior posterior axis.6

In vertebrates, Slits and Robo participate to the migration of many neurons in the CNS and PNS but so far there is only evidence for a repulsive activity. Moreover, whereas Slit and Robo were shown to guide tangentially migrating neurons, they do not seem to participate to radial migration. During development and throughout adulthood, several types of olfactory bulb (OB) interneurons (the granule cells and the tufted cells) are generated from progenitors located in the so-called subventricular zone (SVZ) that surrounds the lateral ventricles116 and migrate to the OB via the rostral migratory stream (RMS). The rostral migration of SVZ-derived neuroblasts was shown to involve chemorepellents secreted by the septum.117-119 Biochemical and in vitro studies have since demonstrated that Slit1 and Slit2 are mediating this repulsive activity.15 Accordingly, some SVZ-derived neuroblasts showed abnormal migration pattern in slit1 deficient-mice.119 However, those cells were also shown to express Slit1119 that may act cell autonomously. Migrating OB neuroblasts express robo2 and robo3 mRNAs101 and srGAP1.60 Although dominant-negative srGAP1 blocks Slit repulsion of SVZ cells,60 the contribution of Robo signaling in this system is largely unknown. Elegant in vitro assay also showed that Slit
repels migrating svz cells without blocking their migration. Thus, Slit may just control the
directionality of the migration without affecting cell motility, although this issue is still
controversial. In chick embryo, Slit2 repels the migration of trunkal but not vagal neural
crest cells that take different migratory pathways during development. As observed in the
SVZ system, Slit2 appears to enhance cell motility: neural crest cells migrate further in the
presence of soluble Slit2.

In the telencephalon, Slit1 repels in vitro the migration of GABAergic interneurons from
the ganglionic eminence. However, the phenotypic analysis of slit1/slit2 deficient mice
revealed that they are not necessary for tangential migration of GABAergic interneurons to
the cortex in vivo. On the other hand, this study revealed that Slits influence the migration
of cholinergic neurons of the basal magnocellular complex.

In the hindbrain, Slit and Robo participate to the migration of rhombic lip derivatives both
in chick and rodents. Although Slits primarily act as repellents for rhombic lip-derived
cells, they were also proposed to antagonize the attractive activity of floor plate-derived
netrin-1.

Last, Slits and Robo also influence the migration of other vertebrate cells; either negatively
as shown for leukocytes, but sometimes positively as shown for endothelial cells. In this
later case, Slit attractive activity involves Robo1 signaling.

A Role for Slit and Robo in Neurological Disorders?

There is relatively little direct evidence so far indicating that Slit and Robo may be involved
in pathological processes except in cancers. However, several patients suffering from a rare
genital syndrome named Horizontal gaze palsy with progressive scoliosis and hindbrain
dysplasia (HGPPS) were recently shown to bear mutations in the ROB03 gene. In these pa-
tients, the pyramidal tract and the dorsal column- medial lemniscus are uncrossed. Moreover,
there is a reduced pontine nucleus and abducens nuclei. As shown in robo3 knockout mice the
pontine nucleus defect is probably caused by an abnormal migration during development. It
is still unknown if the other human brain defects are also present in mice lacking Robo3 and as
those die at birth, behavioral analysis are not possible.

One of the Slit/Robo GAP, SrGAP3 has a putative role in idiopathic mental retardation as
it is mutated in patients with X chromosome-linked MR. However, the cellular basis for these
defects and the normal function of SrGAP3 in the CNS are unknown.

The incapacity of adult axons to regenerate in the CNS of mammals is known to rely for a
large extent on the existence of inhibitors of axonal growth expressed either in the glial scar or
in myelin. Several studies have shown that repulsive axon guidance molecules may be respon-
sible for some of the inhibition. Slit an Robo expression after injury has not been extensively
studied so far. However, in a model of cryoinjury, Slit2 was found to be expressed in reactive
astrocytes together with glypican-1. In addition, adult DRG neurons also express mRNAs
for Robo2 and Slit1 but their expression does not change after sciatic nerve transection or
dorsal column lesion in the spinal cord. Thus, it is still unclear if Slit and Robo play any role in
preventing axonal regeneration.

Perspectives

There is mounting evidence that Slits regulate a large range of biological functions, from
axon guidance, neuronal migration, immune response to cell differentiation most likely
through Robo signaling. However there are still many open questions.

First, could Robo functions independently of Slit in particular through Robo/Robo interaction
and what are the signaling pathways involved. Thus, in the Drosophila PNS, Robo2 ex-
pressed on visceral mesoderm binds Slit and present it to Robo expressing chordotonal sensory
neurons. This may also involve Robo-Robo2 direct interaction. In vitro experiments also showed
that the growth of retinal and olfactory Robo expressing axons is stimulated on Robo expressing
cells, suggesting that Robo might work as cell-adhesion molecule to regulate outgrowth. In
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Drosophila, Robo and Robo2 can dimerize in vitro\(^{40}\) and ectopic expression of low level of Robo2 causes a Robo-like phenotype suggesting that Robo2 could interfere with Robo function.\(^5\)

The function of Slits and Robos to in the normal adult brain and in pathological condition also remains to be clarified. Many data support a role for these molecules in tumorigenesis, in particular in gliomas\(^{128}\) but this needs to be further demonstrated. As all Slits and Robos are expressed in adult neurons it is likely that they modulate synaptic transmission as shown recently for other secreted axon guidance molecules of the semaphorin family.\(^{135}\) Many answers to these questions should come from the analysis of mouse deficient for one or several of these proteins.

References

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84. Georgiou M, Tear G. Commissureless is required both in commissural neurones and midline cells for axon guidance across the midline. Development 2002; 129(12):2947-2956.


87. Georgiou M, Tear G. The N-terminal and transmembrane domains of Commissureless are necessary for its function and trafficking within neurons. Mech Dev 2003; 120(9):1009-1019.


129. Filbin MT. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 2003; 4(9):703-713.

