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REVIEW —*Current Perspective*—

Functions of Semaphorins in Axon Guidance and Neuronal Regeneration

Yoshio Goshima[1,2], Yukio Sasaki[1], Takashi Nakayama[3], Takaaki Ito[4] and Toru Kimura[5]

Departments of [1]Pharmacology, [3]Biochemistry and [4]Pathology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan

[2]CREST, Japan Science and Technology Corporation (JST)

[5]Sumitomo Pharmaceuticals Research Center, 3-1-98, Kasugade-Naka, Konohana, Osaka 554-0022, Japan

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ABSTRACT—The semaphorin family comprises secreted and transmembrane signaling proteins that function in the nervous, immune, respiratory and cardiovascular systems. Sema3A, a secreted type of semaphorin, is now recognized as the most potent repulsive molecule inhibiting or repelling neurite outgrowth. The biological actions of Sema3A are mediated via neuropilin (Npn)-1, a receptor or one of the components of a receptor complex for Sema3A. Although the molecular mechanisms of Sema3A-Npn-1 signaling are largely unknown, a pertussis toxin-sensitive trimeric G protein(s), Rac-1, collapsin response mediator protein (CRMP), cyclic nucleotides and tyrosine kinase(s) have been implicated as essential and/or modulatory components of these processes. As repulsive molecules could be impediments to axon outgrowth, determining how these repulsive molecules exert their actions has the potential of uncovering new therapeutic approaches to injury and/or degeneration of neuronal tissues.

Keywords: Semaphorin, Neuropilin, Axon guidance, Growth cone, Neuroregeneration

Injury and/or degeneration of neuronal cells, especially those of the CNS, presents a challenging problem for neuroscientists as well as clinicians. Regeneration of adult CNS neurons is likely to follow principles of axonal guidance similar to those used during development. Axon pathfinding and target recognition rely on the ability of the growth cone (the leading edge of the extending axon) to sample its environment and integrate multiple guidance cues in order to affect steering events. Recent studies have revealed the importance of positive, or attractive, and negative interactions in mediating axon guidance, and some of these cues have been characterized at the molecular level (1). The inability of injured axons in the adult mammalian CNS to regenerate thus might due in large part to the "repulsive guidance molecules" that repel and inhibit neurite outgrowth.

The semaphorins are a large family of secreted and membrane-associated proteins. Semaphorins appear to affect axon steering, fasciculation, branching and synapse formation through their action as chemorepellents. In addition, it has been implicated that semaphorins play important roles in the immune system and may affect organogenesis (2, 3), vascularization and angiogenesis (4), and it may also affect the progression of certain cancers (5). To date, the semaphorin family includes at least 19 different members in the worm- and lower vertebrates and at least 3 different members in the invertebrates (Fig. 1). All semaphorins contain an extracellular domain of about 500 amino acids termed a semaphorin (sema) domain and a class-specific C terminus that may contain additional sequence motifs. Semaphorins also differ with respect to membrane anchorage (secreted, transmembrane, and glycosylphosphatidylinositol-linked). These overall structural characteristics, in combination with phylogenetic tree analyses, allow at present for the designation of eight subclasses into which all known semaphorins can be assigned (6). Sema3A is one of the most potent growth cone collapsing factors, and as little as 10 pM Sema3A can alter growth cone morphology. The growth cone collapses as these processes shrink toward the center of the growth cone. The collapsed growth cone is then temporarily paralyzed, failing to extend or retract lamellipodia and filopodia and failing to advance (7). Recently, we found a novel action of Sema3A to facilitate antero- and retrograde

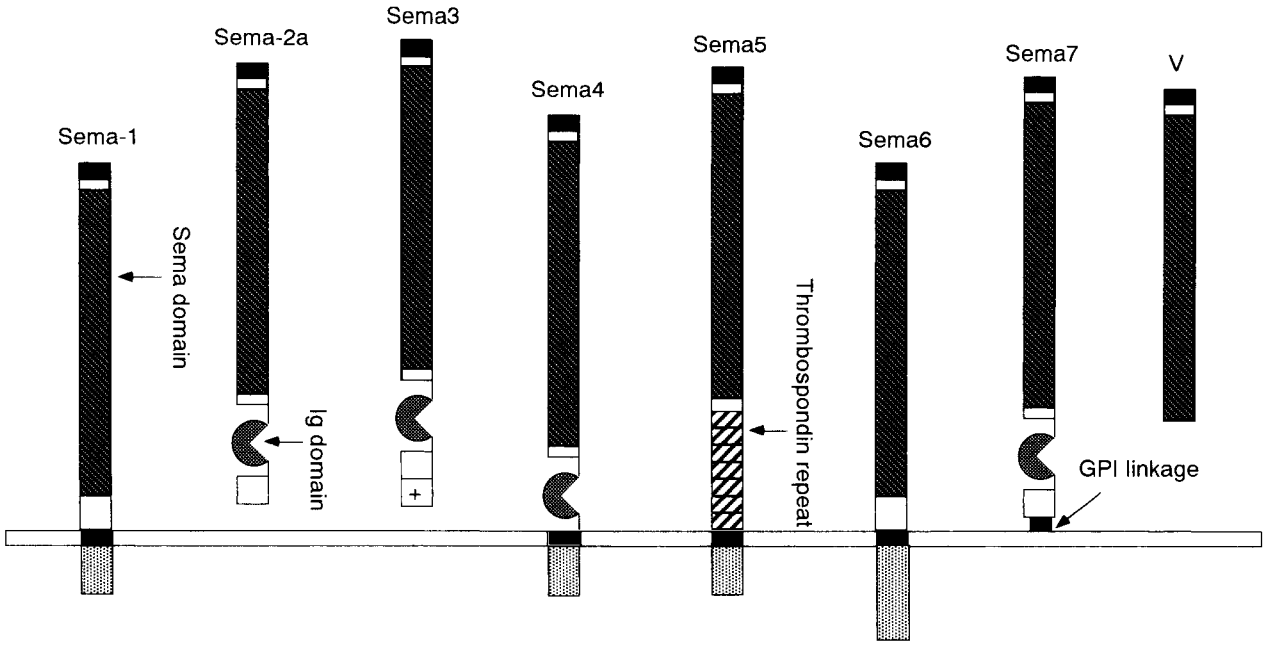

Fig. 1. The semaphorin family.

axoplasmic transport of organelles (8, 9). Neuropilin-1 (Npn-1) has been shown to be a receptor or one of the components of a receptor complex, for Sema3A, but the molecular mechanisms by which Sema3A exerts its effects via Npn-1 remain to be determined (10 – 14). In this review, we give an overview of the functions of semaphorin in special reference to possible signal-transduction mechanisms.

Structures and functions of type 3 semaphorins and their receptors

The functions of most semaphorins and their receptors are largely unknown. To date within the semaphorin family, transmembrane and secreted semaphorins belonging to classes 1, 2 and 3 have been best characterized functionally at the cellular level. In fact, several class 3 semaphorins bind to and require Npns, a small family of transmembrane proteins, for transducing a repulsive guidance signal (10, 12, 13). Npn is a type I membrane protein that was initially identified in the *Xenopus* tadpole nervous system by hybridoma techniques and then in the chick, mouse, rat and human (10). Because another Npn-related molecule has been identified, the original neuropilin is now referred to as Npn-1 and the new Npn, as Npn-2. Npn-1 and-2, however, have very short cytoplasmic domains that contain no obvious signaling motifs that are dispensable for repulsive semaphorin guidance (14). This strongly suggests that Npns serve an essential role in assembling a receptor complex that includes a yet unknown transmembrane signaling component. Indeed, Npn-1 also serves as a receptor component for an isoform of vascular endothelial growth factor ($VEGF_{165}$), augmenting the functional response to $VEGF_{165}$ by the receptor tyrosine kinase KDR/Flk-1 (4). At present, there are no clear predictions about the nature of the transmembrane signaling component that interacts with a class 3 semaphorin/Npn complex to initiate growth cone-steering responses (See Appendix). Sema3A-induced growth cone collapse requires the two segments of the ectodomain of Npn-1, the CUB domain and the juxtamembrane portion, or MAM domain, while the transmembrane segment and cytoplasmic tail of Npn-1 are not required for biologic activity (14). One attractive model describing the receptor for secreted semaphorins therefore includes a Npn and an additional component(s) of a receptor complex that cooperates to mediate semaphorin-sensitive growth cone collapse (Fig. 2).

At the beginning, Sema3A has been found to be able to function in vitro as a collapsing factor for sensory growth cones (7). It repels NGF-responsive sensory afferents in a collagen-gel matrix but has much lesser effect on neurotrophin-3-responsive sensory afferents (15). Sema3A is expressed at high levels in the ventral spinal cord when it produces a chemorepellent selective for NGF-responsive afferents. These findings suggest that Sema3A plays a role in generating the distinct projection patterns of these sensory afferents. Sema3A can also act as a selective chemorepellent for several populations of spinal and cranial sensory and motor axons. Target disruption of the

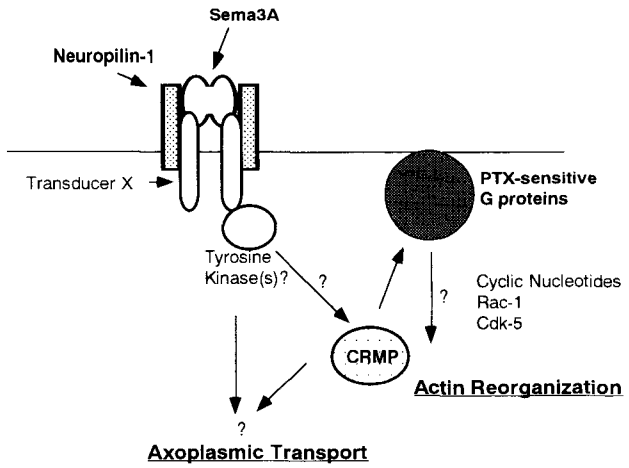

Fig. 2. A model for signal transduction mechanisms of Sema3A.

Sema3A gene in mice shows that the patterned pathway and projection of peripheral nerves are severely disorganized in the homozygous Sema3A mutant embryos (2, 16). In the homozygous mutant embryos, the ophthalmic, maxillary, mandibular, facial, glossopharyngeal and vagal cranial nerves do not form thick bundles but sprout widely, sometimes over-shooting far beyond the growing front of the normal nerves. Deficiency of Sema3A also disrupts pathways and projections of the spinal nerves. The main spinal nerve trunks are defasciculated; several axons leave the main spinal nerve trunk at arbitrary points and then grow into the skin surface in an irregular manner. Some spinal axons cross the dorsal midline and project onto the opposite side of the embryos, which is not their correct target (2, 16). Npn-1-deficient mice have a similar PNS phenotype to that of the Sema3A mutant. These findings indicate that chemorepulsive signals that are mediated by the interaction between Sema3A and Npn-1 are major mechanisms in the guidance of peripheral axons during embryonic development. The functions of Sema3A and Npn-1 in the CNS, however, are still obscure. In the CNS, Sema3A is expressed at a low level and Sema3A-deficient mouse embryos show no apparent abnormalities in the axonal projections. The projection of CNS axon is probably regulated in combination with several guidance molecules, in addition to the semaphorins (10).

One of the most intriguing properties of semaphorins is that they can act as both repulsive and attractive cues. For cortical axons, Sema3C acts as an attractive guidance signal, while Sema3A acts as a repulsive signal (17). Npn-1 and Npn-2, receptors for Sema3A and Sema3C, respectively, are present on cortical fibers both in vitro and in vivo at the time when corticofugal projections are established. Moreover, Sema3A mRNA is detected in the ventricular zone of the neocortex, whereas Sema3C mRNA is restricted to the subventricular zone. As a consequence, cortical axons are considered to be attracted towards the intermediate zone by Sema3C, whereas Sema3A prevents the fibers from entering the subventricular zone and ventricular zone. The biphasic character of semaphorin is also exemplified by the finding that Sema3A-induced repulsion can be changed to attraction by an analogue of guanosine 3′,5′-monophosphate in *Xenopus* spinal neurons (18). This implies that the response of a growth cone to a particular guidance cue may depend critically on other coincident signals received by the neuron.

Plexin is a receptor for type 1 semaphorins

Recently, a family of transmembrane proteins distinct from Npns, the plexins, have been discovered, and this family includes members capable of serving as semaphorin receptors both in the immune system and in the nervous system (19). In the embryonic *Drosophila* CNS, both PlexA and PlexB are expressed primarily when both central and peripheral pathfinding events are taking place. PlexA mutants show specific defasciculation defects of some motor neurons. The defasciculation defects are identical to those seen in the Sema-1a mutant (19). This suggests, especially in light of the fact that Sema-1a is found on all motor axons, that PlexA and Sema-1a function in a common pathway required for axon defasciculation. This idea is supported by dominant genetic interactions that, while neither heterozygote alone shows defasiculation defects, removing one dose of both PlexA and Sema-1a results in defects similar to either single mutant alone. These and other genetic interactions between PlexA and Sema-1a suggest that PlexA functions downstream from Sema-1a as a receptor. Consistently, a Sema-1a alkaline phosphatase fusion protein can bind with a high affinity to membranes from PlexA expressing COS cells. These biochemical and genetic data provide evidence that PlexA is a functional Sema-1a receptor and show that axonally distributed transmembrane semaphorins and their receptors play an essential role in mediating fasiculation events required for the generation of neural networks. Since class 6 semaphorins have been shown to be structurally and phylogenetically the closest to the insect sempahorins (20, 21), an assessment of the binding interactions among all known plexins and class 6 semaphorins will begin to address whether or not these recently demonstrated functional plexin/semaphorins relationships can be extended to interactions between plexins and other semaphorin classes.

Cytoplasmic domains of transmembrane type semaphorins

Most of the transmembrane type semaphorins so far identified have been shown to have proline-rich region in their cytoplasmic domains, and these semaphorins have been suggested to have the ability to deliver a signal to

other cells via engagements of its counter-receptor. Sema4D (CD100) can be associated with a serine/threonine kinase, and the kinase pathway may participate in the biological effects of these semaphorins (22, 23). In the cytoplasmic domain of Sema4F, a phosphorylation site for cyclic nucleotide-dependent protein kinases is found (24). Sema6B may associate with a tyrosine kinase c-Src (23). Indeed, the cytoplasmic domain Sema6B contains several proline-rich potential SH3 domain binding sites, which bind specifically the SH3 domain of the c-Src. Recently, using a yeast two-hybrid system, a PDZ-containing neural protein has been identified as an Sema4C cytoplasmic domain-associated protein (25). It is unknown whether or not these components are actually involved in the signaling mechanisms of these semaphorins.

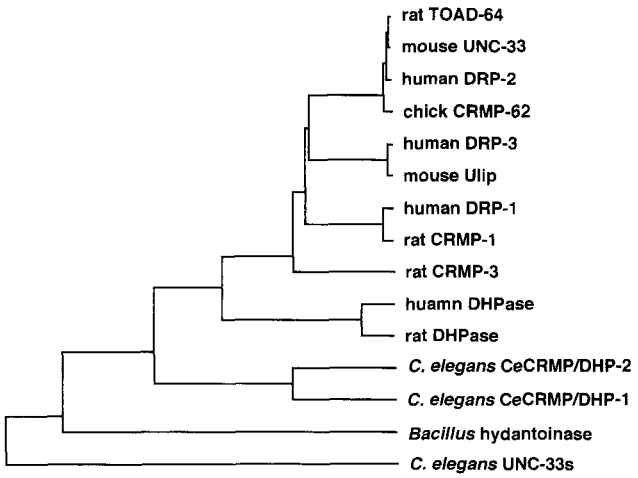

Fig. 3. Phylogenic tree of CRMP/DHP/UNC-33s. TOAD, turn on after division; UNC, uncordinated; CRMP, collapsin response mediator protein; DRP, dihydropyridine related protein; Ulip: unc like protein; DHP, dihydropyrimidinase.

Isolation and characterization of CRMP

What are the molecular bases for signal transduction mechanisms in nerve growth cone which is highly sensitive to extracellular cues? We have provided evidence that pertussis toxin (PTX)-sensitive heteromeric guanosine triphosphate (GTP)-binding protein mediates collapsin-1 /Sema3A-induced growth cone collapse (8, 9, 10). We analyzed a *Xenopus laevis* oocyte expression system in an attempt to characterize components of the collapsin /Sema3A pathway, and the isolated a collapsin response mediator protein (CRMP-62) from chick dorsal root ganglion. CRMP is an intracellular protein that seems to be required for Sema3A signaling. CRMP-62 shares homology with the *C. elegans* protein UNC-33. Although its mechanisms of action remain ill-defined (11, 26, 27), CRMP-related proteins have been identified in chick, rat, human, bovine and *Xenopus laevis* (11, 26 – 32) (Fig. 3). In the rat, rCRMP-1, -2, -3 and -4 have been isolated, being differentially expressed almost solely in the nervous system (28). rCRMP-2 is most closely related to chick CRMP-62 and is the most widely expressed CRMP within the nervous system. The only exception of the neuronal specificity of CRMP expression is a substantial level of rCRMP-2 in lung epithelial cells. Together with Npn-1 expressed in the lung epithelial cells, the rCRMP-2 may be functional, because Sema3A inhibits branching morphogenesis of embryonic mouse lung (3). rCRMP-1 and CRMP-4 are expressed during discrete periods of neuronal development and are not found in the adult nervous system. rCRMP-3 has a distinct distribution, being expressed transiently in developing spinal cord and selectively in the postnatal cerebellum (28). The expression of the *Xenopus* homologue of the CRMP-2 (XCRMP-2) has been shown to be induced at the midgastrula stage and increases through the early neural developmental stage. XCRMP-2 expression is induced by neural inducers noggin and chordin which antagonize the neural inhibitor BMP4 (32). A dominant negative BMP receptor also induces XCRMP-2 expression, suggesting that transcription of XCRMP-2 is negatively regulated by the BMP4 signaling. XCRMP-2 is thus an early response marking neural commitment and that transcriptional control of the XCRMP-2 gene is one of the targets of BMP4 signaling. We have recently identified CeCRMP-1 and CeCRMP-2 in *C. elegans* (Sasaki et al., 12th *C. elegans* Meeting, 724P, 1999; Takemoto et al., ibid., 826P). These two gene products and the mammalian CRMPs share similar amino acid identities with rat dihydropyrimidinase (DHP). As a minor but significant level of DHPase activity is detected with recombinant worm CRMPs, we newly term these CRMPs CeCRMP/DHP-1 and -2. CeCRMP/DHP-1::GFP (green fluorescent protein) is in some neuronal cells and hypodermis in the larval stage, but downregulated in the adult stage; on the other hand, CeCRMP/DHP-2::GFP is seen predominantly in body wall muscle cells at the 180-cell stage and the expression continues throughout life. Whether or not there exist genetic interactions of CRMP/DHPs and semaphorins in worms is now under investigation in our laboratory. The other interesting features of CRMP molecules are as follows: 1) CRMP-2 is a major phosphoprotein in developing neurons (27); 2) human CRMP-2 is associated with a fraction of paired helical filaments in brains of patients affected with Alzheimer's disease (30); 3) CRMP-2 gene maps to the region of mouse chromosome 14 syntenic with human chromosome 8p21, which is in the vicinity of the wabbler-lethal mutation, related to neurological and neuromuscular disorders (31); 4) CRMP-2 is upregulated in spinal motor neurons by sciatic nerve injury (26) and upregulated in olfactory receptor neurons by axotomy of the primary olfactory nerve and

bulbectomy (33).

Signal transduction cascade for Sema3A-Npn-1 system

Although the entire picture for the Sema3A-Npn-1 system remains obscure, essential and/or modulatory components for the signaling machinery are now emerging from current studies. We have recently obtained evidence that certain types of tyrosine kinase are likely to be involved in the Sema3A-induced responses (34). An inhibitor of tyrosine kinase(s), lavendustin A, but not genestein, inhibits Sema3A-induced growth cone collapse. Several findings on ligand-receptor interaction of repulsive molecules also support the above-mentioned idea. Npn-1, a Sema3A receptor, can be associated with tyrosine kinases, KDR and/or Flt-1, forming a receptor complex for VEGF (4). Ephrins, another large family of repulsive molecules, act as ligands for receptor tyrosine kinases Ephs (1). On the other hand, we found that olomoucine, an inhibitor the serine/threonine kinase cdk-5, inhibits mastoparan (a G-protein activator)-induced growth cone collapse in chick retinal ganglion cells (35). Immunocytochemical analysis revealed that the growth cone collapse induced by mastoparan is associated with accumulation of phosphorylated tau, a substrate for cdk-5, in growth cone. Rac-1 and CRMP have been implicated in Sema3A-induced growth cone collapse (11, 36), but the molecular interaction and mechanism of these intracellular components are largely unknown (Fig. 2).

Different signaling pathway of Sema3A may contribute to its functions as an axon guidance cue

Apparently, activities of repulsive molecules to inhibit axon outgrowth parallel those to induce growth cone collapse. However, guidance molecules having repulsive effects on a subset of neurons are not necessarily capable of inducing collapse of the growth cone (1). This is probably related to the diverse functions of these molecules which have their own specific receptor, signaling system and different cellular effects on developing and/or adult neurons. For example, NI (neurite outgrowth inhibiting protein)-35, a CNS myelin-derived protein(s) (see below), -induced growth cone collapse is associated with a rise in intracellular Ca^{2+} concentration in the growth cone (37), while Sema3A appears not to affect the level of Ca^{2+} (7). Growth cone collapse responses by Sema3A and NI-35 are both sensitive to PTX, but only Sema3A has an effect via growth cone Npn-1 to facilitate antero- and retrograde axoplasmic transport in mouse dorsal root ganglion (8, 9).

In contrast to the effect of Sema3A on growth cone morphology, the Sema3A action on axoplasmic transport is insensitive to PTX (8). These findings suggest that growth cone Npn-1 initiates two different signal transduction cascades, eliciting PTX-sensitive growth cone collapse and PTX-insensitive axoplasmic transport. The biological relevance of local regulation by Sema3A of axoplasmic transport, however, remains unclear. The bidirectional axoplasmic transport may have some effects on the process of neurite branching or remodeling of neuronal architecture. Recently, it has been reported that cultured retinal ganglion cells (RGC) growth cone contact with repellent material from posterior tectum affects lateral extension from the axons (38). After temporal RGC growth cones contact posterior target cells, most growth cones collapse. Within minutes of contact, new lateral extensions of outgrowth appear along the shaft of the retracting fiber. These new extensions from the fiber appear frequently if contact results in growth cone collapse, but infrequently otherwise, in a manner that is highly correlated with growth cone behavior (38). An analogous finding was obtained with the cultured dorsal root ganglion cells, where accumulations of transported organelle are seen at the future branching points (39). It is also possible that the bidirectional axoplasmic transport elicited by Sema3A might be a signal between the growth cone and the cell body. Elucidating the signal transduction pathway activated in response to external guidance cues will tell us whether one or both of these mechanisms are at work.

Roles of semaphorins and Npn-1 in axonal regeneration

Although PNS neurons show vigorous regrowth after injury, mammalian CNS neurons can not regenerate once heavily damaged in the adult. This, for example, causes tragic prognosis after traumatic injury of the CNS. The biochemical identification of neurite outgrowth inhibitory proteins, NI-35 and NI-250, associated with oligodendrocyte membranes and CNS myelin (37) has provided evidence that growth inhibitory and repulsive molecules contribute at least in part to the regenerative failure observed following CNS injury. The neurtralization of NI-35 and NI-250 by a specific monoclonal antibody (IN-1) results in enhanced axonal regeneration along the injured corticospinal tract and recovery of contact-placing responses in rats (37). This can provide strong evidence that inhibition of repulsive activity can enhance regeneration of CNS neurons. However, even after neutralization of myelin-associated inhibitory activities, only a small number of regenerative sprouts is observed, and functional recovery is always limited to rather simple behavioral parameters. This suggests that additional factors or mechanisms are involved in the failure of CNS regeneration. Indeed, some semaphorins have been observed to be highly expressed in the adult CNS, and the expression could contribute to the inability of CNS neurons to regenerate (33). Recently, it was shown that after bulbectomy or surgical removal of the olfactory bulb, Sema3A mRNA is highly expressed in the glial scar cells, which is a major obstacle for the

regeneration, surrounding Npn-1-positive olfactory axon bundles, and no axon bundles were observed beyond the Sema3A-positive area. Similar inductions of Sema3A are also observed in the lesions damaging other regions of the brain. In contrast, in the PNS, spinal and facial motor neurons down regulate Sema3A during axonal regeneration (33). It is thus of great interest to determine if inhibition of the repulsive signal of the semaphorin would enhance regeneration of CNS neurons. Considering the diversity and complexity of the neuron networks, however, it is plausible that there still remains to be discovered a wide variety of chemorepellent repulsive molecules being expressed and playing a role in the CNS.

Conclusion and future aspects

Whether interfering signal transduction pathways of repulsive molecules constitute novel and rational approaches to promote neural recovery following injury is an intriguing issue to be determined. There appear to be various types of repulsive molecules that exert their functions by diverse mechanisms. Identifying repulsive guidance cues, the signal-transduction components downstream of each molecule, and evaluating inhibition of the repulsive signal in in vivo animals remain some of the most important tasks ahead.

Appendix

During the editorial process of this manuscript, Npn-1 and plexin 1 have been demonstrated to form a stable receptor complex with Sema3A (Cell **99**, 59–69, 1999; ibid., 71–80).

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