

Semaphorins in axon regeneration: developmental guidance molecules gone wrong?

R. Jeroen Pasterkamp^{1,*} and Joost Verhaagen^{2,*}

¹*Department of Pharmacology and Anatomy, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands*

²*Netherlands Institute for Neuroscience, Meibergdreef 33, 1105 AZ, Amsterdam, The Netherlands*

Semaphorins are developmental axon guidance cues that continue to be expressed during adulthood and are regulated by neural injury. During the formation of the nervous system, repulsive semaphorins guide axons to their targets by restricting and channelling their growth. They affect the growth cone cytoskeleton through interactions with receptor complexes that are linked to a complicated intracellular signal transduction network. Following injury, regenerating axons stop growing when they reach the border of the glial-fibrotic scar, in part because they encounter a potent molecular barrier that inhibits growth cone extension. A number of secreted semaphorins are expressed in the glial-fibrotic scar and at least one transmembrane semaphorin is upregulated in oligodendrocytes surrounding the lesion site. Semaphorin receptors, and many of the signal transduction components required for semaphorin signalling, are present in injured central nervous system neurons. Here, we review evidence that supports a critical role for semaphorin signalling in axon regeneration, and highlight a number of challenges that lie ahead with respect to advancing our understanding of semaphorin function in the normal and injured adult nervous system.

Keywords: axon growth inhibition; axon guidance; intracellular signalling; peripheral nervous system and central nervous system injury; neuromuscular junction; semaphorin

1. INTRODUCTION

It is now generally accepted that developing axons navigate to their targets by sensing attractive and repulsive molecules through receptors expressed on their growth cones. Binding of guidance molecules to receptors on the growth cone plasma membrane leads to the activation of intracellular signalling cascades. This results in dynamic changes in the cytoskeleton and subsequent directional axon extension and target recognition (Goodman 1996; Guan & Rao 2003; Huber *et al.* 2003). During the past 10–15 years, proteins instrumental in guiding developing axons by repulsive mechanisms have been proposed to also function as regulators of (structural) plasticity and inhibitors of axon regeneration. This idea is based on the initial observations that several repulsive axon guidance cues continue to be expressed in the adult nervous system and/or are induced following central nervous system (CNS) injury (De Winter *et al.* 2002a; Koeberle & Bahr 2004). This review will focus on our current understanding of the role of semaphorins, one of the largest families of axon guidance molecules (figure 1; Fiore & Puschel 2003), in adult nervous system regeneration. A summary of studies on the expression of semaphorins in various central and peripheral lesion paradigms (§2) will precede a detailed review of the remarkable progress that has been made with elucidating the complex intracellular signalling

pathways downstream of semaphorins and a discussion of their potential as targets for encouraging axon regeneration (§3). In §4, we will discuss future directions and speculate on potential strategies for counteracting the axon growth inhibitory effects of semaphorins following neural injury.

Semaphorins comprise a large family of secreted and membrane-associated proteins that can signal axon repulsion and/or attraction (figure 1; Fiore & Puschel 2003). Vertebrate secreted semaphorins (Sema3s) are among the best-characterized semaphorin family members and signal through neuronal receptor complexes that contain neuropilins (NPs) and plexins as ligand binding and signal transducing subunits, respectively (Raper 2000). In contrast, several membrane-associated semaphorins bind directly to plexins and do not require NPs as co-receptors (figure 2). Cell adhesion molecules, Off-track, Met receptor, VEGF-R2 and ErbB-2 have been identified as (modulatory) components of specific semaphorin receptor complexes. In addition, semaphorin receptors unrelated to plexins and NPs have been identified, such as Tim-2 and CD72 (figure 2; Castellani & Rougon 2002; Pasterkamp & Kolodkin 2003; Kruger *et al.* 2005).

Theories on semaphorin function in neural trauma have been formulated based on the cellular context in which semaphorins, their receptors and intracellular signalling molecules are (re)expressed following injury. At least four possible functions have been postulated. First, semaphorins may contribute to the inhibitory properties of oligodendrocyte-lineage cells and act in concert with the classical myelin-associated inhibitors Nogo, myelin-associated glycoprotein (MAG) and

* Authors for correspondence (j.pasterkamp@med.uu.nl; j.verhaagen@nih.knaw.nl).

One contribution of 13 to a Theme Issue 'The regenerating brain'.

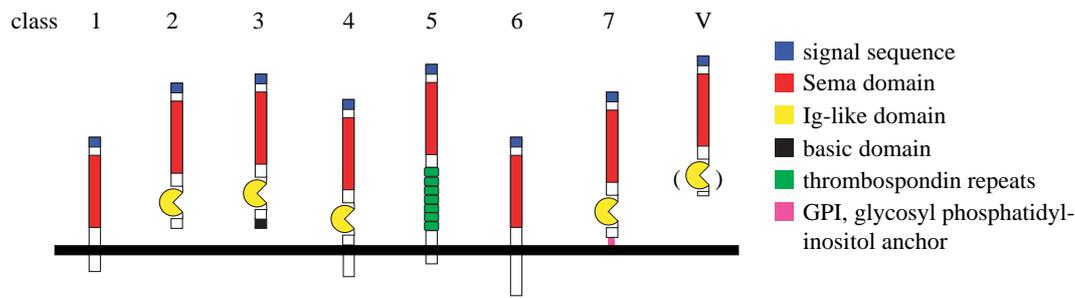


Figure 1. Semaphorins. The semaphorin gene family has been categorized into eight classes on the basis of sequence similarity and structural features. Semaphorins exist as secreted (classes 2, 3 and V) and membrane-associated proteins (classes 1, 4, 5, 6 and 7). Classes 1 and 2 contain invertebrate semaphorins, classes 3–7 vertebrate semaphorins, and class V viral semaphorins.

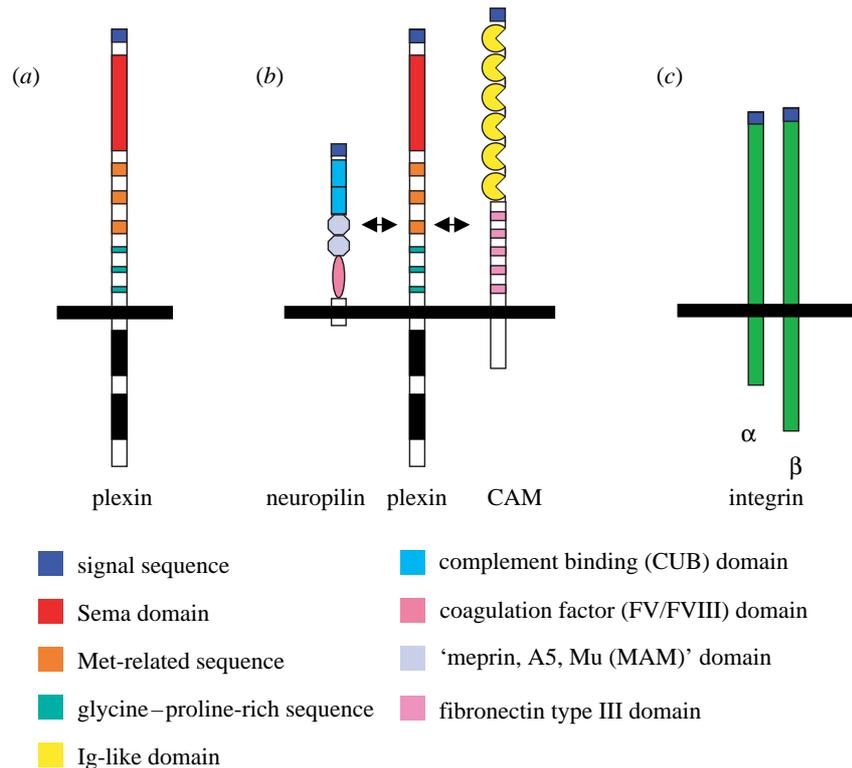


Figure 2. Semaphorin receptors. Functional semaphorin receptors in the vertebrate nervous system: (a) semaphorins belonging to classes 4–7 and Sema3E can directly interact with plexins, (b) secreted class 3 semaphorins can signal through a neuropilin/plexinA (cell adhesion molecule (CAM)) complex and (c) Sema7A requires a β 1-integrin for effects on axon outgrowth. α , alpha subunit, β , beta subunit.

oligodendrocyte-myelin glycoprotein (OMgp). Multiple membrane-associated semaphorins including Sema4D, Sema5A and Sema6A are expressed by oligodendrocytes, and Sema4D is upregulated in oligodendrocytes located in the vicinity of CNS lesions (Cohen *et al.* 2003; Moreau-Fauvarque *et al.* 2003; Goldberg *et al.* 2004; Kerjan *et al.* 2005). Second, secreted class 3 semaphorins (Sema3s) may act as repulsive signals in neural scar tissue since they are expressed in meningeal fibroblasts that populate the core of the scar (Pasterkamp *et al.* 1998a, 1999; De Winter *et al.* 2002b). This idea is supported by the observation that injured CNS neurons express Sema3 receptor components and several associated cytosolic signalling molecules and may therefore be sensitive to the repulsive effects of Sema3s (Pasterkamp & Verhaagen 2001; De Winter *et al.* 2002a). Third, blood vessels in the scar express a semaphorin receptor, neuropilin-1, and are surrounded by Sema3A-positive meningeal fibroblasts (Pasterkamp *et al.* 1999). In addition,

several scar-associated cells express semaphorin receptors and signalling molecules (Pasterkamp *et al.* 1999, 2005). Thus, secreted semaphorins could be involved in instigating or modulating the formation of the scar by influencing tissue compartmentalization or neovascularization. Fourth, in the peripheral nervous system (PNS) Sema3s are regulated by injury in lesioned motor neurons, in Schwann cells of the peripheral nerve and in specialized Schwann cells at neuromuscular junctions of fast fatigable muscle fibres (Pasterkamp *et al.* 1998c; Scarlato *et al.* 2003; Ara *et al.* 2004; De Winter *et al.* 2006). Interestingly, expression of semaphorin receptor proteins and signalling molecules is unchanged following PNS injury (Pasterkamp *et al.* 1998c; Gavazzi *et al.* 2000; R. J. Pasterkamp & J. Verhaagen 2006, unpublished observations). This suggests a role for semaphorins in motor neuron regeneration and plasticity at a subset of neuromuscular junctions. Ongoing and future studies will test the validity of the proposed involvement of semaphorins

in inhibiting axon regeneration, in regulating scar formation and in modulating plasticity of the neuromuscular system.

2. SEMAPHORIN EXPRESSION IN THE INJURED PERIPHERAL NERVOUS SYSTEM AND CENTRAL NERVOUS SYSTEM

(a) *Expression in the intact nervous system*

During embryogenesis, many semaphorins are expressed in non-neuronal cells along developing nerve tracts and in subpopulations of neurons. Expression is often widespread and changes rapidly as development proceeds. In the mature nervous system, expression of semaphorins is mostly restricted to specific populations of neurons (www.gensat.org/index.html). For example, the prototype semaphorin *Sema3A* is expressed in cranial and spinal motor neurons, entorhinal cortex stellate cells, neurons in the subiculum and amygdala, olfactory bulb mitral cells and subsets of retinal ganglion cells (RGCs) (Giger *et al.* 1998; Skalióra *et al.* 1998; de Winter *et al.* 2004). *Sema3A* expression is not detectable in glial cells in the intact adult brain, but low levels of expression are observed in the lepto-meningeal sheet (Giger *et al.* 1996; Pasterkamp *et al.* 1998*b*, 1999). In addition to *Sema3A*, several other secreted and membrane-associated semaphorins have been reported to be expressed in the adult rodent or human brain (Eckhardt & Meyerhans 1998; Xu *et al.* 1998; Encinas *et al.* 1999; Hirsch & Bahr 1999*b*).

(b) *Secreted semaphorins and central nervous system injury: expression in neural scar tissue*

The cellular composition of the neural scar that forms as a result of penetrating brain and spinal cord injuries is complex (Silver & Miller 2004). The formation of neural scar tissue is a normal neural wound healing response that prevents further spread of damage to the uninjured parts of the nervous system. Following injury, meningeal fibroblasts and blood-borne cells invade the centre of the lesion and astrocytes around the lesion core become hypertrophic. Interactions between astrocytes and meningeal fibroblasts result in the formation of a new glia limitans and in the restoration of the blood–brain barrier (Shearer & Fawcett 2001). The fibrotic core of the scar is characterized by the presence of an extensive meshwork of extracellular matrix (ECM) consisting of collagen and chondroitin sulphate proteoglycans (CSPGs). Axons of injured CNS neurons are unable to cross the fibrotic scar and develop swollen endings (also known as dystrophic endbulbs) just proximal to or within the zone of reactive astrocytes (Tom *et al.* 2004). Meningeal fibroblasts invading the lesion core express the secreted semaphorins *Sema3A*, *Sema3B*, *Sema3C*, *Sema3E* and *Sema3F* (Pasterkamp *et al.* 1999; De Winter *et al.* 2002*b*). Double labelling studies reveal that the cells that express these *Sema3* transcripts co-express proteins typical of fibroblasts (fibronectin and vimentin) and do not co-localize with markers of astrocytes (glial fibrillary acidic protein, GFAP), macrophages/microglia (ED1, OX42), oligodendrocytes (myelin basic protein (MBP), Gal-C) and

Schwann cells (S100). In addition, *Sema3*-positive cells have a morphology that is congruent with previous descriptions of fibroblasts in the neural scar (Hirsch & Bahr 1999*a*) and form typical strands that are clearly derived from the semaphorin-positive lepto-meningeal sheet close to the primary lesion site.

Are injured adult neurons sensitive to the predicted repulsive actions of meningeal fibroblast-derived *Sema3s* in the lesion core? Several lines of evidence suggest that indeed injured neurons sense and respond to repulsive semaphorins. First, intact and injured adult CNS and PNS neurons express semaphorin receptors and associated intracellular signalling proteins. Second, sprouting sensory axons are responsive to *Sema3*-mediated axon repulsion *in vitro* and *in vivo*. Third, conditioning peripheral nerve injuries that allow dorsal root ganglia (DRG) axons to regenerate centrally do not promote regenerative axon growth through *Sema3*-expressing scar tissue (Wang & Strittmatter 1996; Tanelian *et al.* 1997; Pasterkamp *et al.* 1998*b,c*, 2000, 2001; Reza *et al.* 1999; Gavazzi *et al.* 2000; Owesson *et al.* 2000; De Winter *et al.* 2002*b*; Lindholm *et al.* 2004; Tang *et al.* 2004; Agudo *et al.* 2005). Although these results suggest that at least a subset of injured adult CNS neurons is inhibited by *Sema3s*, future studies employing genetically manipulated mice or other means of blocking semaphorin receptor/intracellular signalling will be needed to show unambiguously that injured neurons sense and respond to semaphorins following neural injury *in vivo*.

(c) *Secreted semaphorins and PNS injury: control of neuromuscular plasticity?*

Injured peripheral neurons are capable of regeneration and reinnervation of their distant target cells in skin and muscle. Schwann cells in the distal portion of the injured nerve support axonal regeneration by upregulating the expression of neurotrophic factors and cell adhesion molecules. This regeneration-promoting effect of Schwann cells is also clear following transplantation of peripheral nerve pieces in the injured CNS (Hirsch & Bahr 1999*a*). Traditionally, most studies on the mechanisms regulating peripheral nerve regeneration have focused on the expression of proteins that promote axon regeneration. However, understanding the role of neurite growth inhibitory proteins in the injured PNS may be equally important.

Adult motor and sensory neurons continue to express the receptors for secreted semaphorins, i.e. NP-1, NP-2 and several plexins (Pasterkamp *et al.* 1998*c*; Gavazzi *et al.* 2000; De Winter *et al.* 2002*b*; Lindholm *et al.* 2004). Furthermore, adult motor, but not sensory, neurons display persistent expression of *Sema3A* (Pasterkamp *et al.* 1998*c*). Interestingly, peripheral and central lesions of motor neuron axons affect *Sema3A* expression in opposite ways (Pasterkamp *et al.* 1998*c*; Lindholm *et al.* 2004). Peripheral nerve lesions at mid-thigh level result in a decline in *Sema3A* expression in motor neurons, while NP-1 continues to be expressed in motor neurons and is induced in small diameter DRG neurons (Pasterkamp *et al.* 1998*c*; Gavazzi *et al.* 2000). In contrast, lesions of the ventral funiculus, which lead to transection of the most proximal intraspinal segment of motor axons, result

in upregulation of *Sema3A* and *Sema3F* in motor neurons. In line with observations following penetrating spinal cord and brain injuries, *Sema3A* is induced in the neural scar after ventral funiculus lesions (Lindholm *et al.* 2004). Ventral funiculus lesions result in very poor regeneration of motor axons, while peripheral lesions are followed by vigorous spontaneous regeneration. The different regulation of semaphorin expression following these two types of motor axon injuries may contribute to this differential regenerative outcome.

Sema3A expression has been studied extensively in relation to the plasticity of neuromuscular synapses. Different subsets of neuromuscular synapses differ markedly in their ability to display anatomical plasticity. Nerve terminals that innervate slow (subtype I) muscle fibres sprout vigorously following blockage of synaptic transmission. In contrast, motor nerve terminals innervating fast-fatigable (subtype IIb/x) muscle fibres fail to exhibit extra-synaptic sprouting. Interestingly, *Sema3A* is differentially expressed in terminal Schwann cells (TSCs) of different populations of muscle fibres: postnatal, regenerative and paralysis induced remodelling of neuromuscular connections in rat gastrocnemius muscles is accompanied by increased expression of *Sema3A* in TSCs of subtype IIb/x muscle fibres, but not in TSCs at subtypes I and IIa muscle fibres (De Winter *et al.* 2006). This is the first known molecular difference between TSCs on neuromuscular junctions of different subtypes of muscle fibres. Overall, these results hint at the exciting possibility that TSCs not only support nerve terminal growth, but also restrict nerve terminal plasticity at specific neuromuscular junctions by secreting (a) chemorepulsive proteins.

(d) Are secreted semaphorins a component of the extracellular matrix?

Although numerous studies have used *in situ* hybridization to examine semaphorin mRNA expression in the intact and injured nervous system, the corresponding protein distribution patterns remain largely unknown. A few recent studies have begun to analyse *Sema3* protein expression in the human and rat brain. In the human subiculum, *Sema3A* protein shows a punctate surface distribution on cell bodies and dendrites (Good *et al.* 2004). In the human cerebellum, *Sema3A* displays a punctate distribution around Purkinje cells, suggestive of secretion of *Sema3A* from Purkinje cell dendrites into the ECM or association of *Sema3A* with presynaptic terminals originating from other sources (Eastwood *et al.* 2003). The same antibody produces a quite different, intracellular distribution in rat Purkinje cells (Eastwood *et al.* 2003). In a third study, *Sema3A* was shown to be expressed in numerous neurons located throughout the basal ganglia and thalamus (Majed *et al.* 2006). This is surprising since these neurons do not express *Sema3A* mRNA (Giger *et al.* 1998). *Sema3F* shows a vesicular distribution in human cell lines and is associated with processes and nerve terminals in adult human brain (Hirsch & Bahr 1999b). Overall, it is difficult to draw definitive conclusions about the localization of *Sema3s* in nervous system tissue. One possible explanation for the inconsistent results

outlined above is that the various *Sema3* antibodies, in particular those directed against the highly conserved sema-domain, recognize multiple semaphorins. The available evidence should therefore be interpreted with great caution, but hints at an association of *Sema3s* with intracellular organelles, nerve terminals, cell surfaces and the ECM.

Support for a cell surface distribution of *Sema3s* also comes from bioassays on the inhibitory properties of meningeal fibroblasts. *Sema3A* is a major meningeal fibroblast-derived neurite growth-inhibitory factor *in vitro* and appears to be presented not in a soluble form but as a substrate-bound molecule associated with the cell membrane or ECM (Niclou *et al.* 2003). In co-cultures of astrocytes and meningeal fibroblasts, these two cell types cluster together and form more or less distinct territories as seen in the neural scar. Postnatal DRG neurons plated on these co-cultures grow quite well on the astrocytic compartment but do not cross the interface between astrocytes and meningeal fibroblasts (Shearer *et al.* 2003). This indicates that inhibitory factors are localized at the cell membrane or in the ECM rather than freely diffusing in the medium. Perturbation of *Sema3* signalling by application of NP-2 antibodies partially overcomes the repellent effect of the meningeal boundary (Shearer *et al.* 2003). Taken together, this suggests that secreted *Sema3s* expressed by meningeal fibroblasts are presented to growth cones in a substrate-bound form.

How are *Sema3s* tethered to the cell membrane/ECM? It has been hypothesized that the C-terminal basic region present in *Sema3s* could mediate their binding to negative charges on the cell surface and ECM (Luo *et al.* 1993). Interestingly, GFP-tagged *Sema3A* associates with the cell surface of cultured neurons and is released in the medium by treatment with excess glycosaminoglycans or a glycosaminoglycan-degrading enzyme (chondroitinase ABC, chABC). This indicates that *Sema3s* may be connected to the ECM through binding of CSPGs (De Wit *et al.* 2005). To date, there is no direct evidence that proteoglycans modulate axon guidance by *Sema3s*, but there are some indications that heparan sulphate proteoglycans (HSPGs) can potentiate *Sema3A*-mediated growth cone collapse. Addition of heparin to *Sema3A* in a collapse assay results in enhanced growth cone collapse, while heparin alone has no effect. This suggests that association of *Sema3A* with heparan sulphate can potentiate *Sema3A* signalling (De Wit *et al.* 2005). Whether or not heparan sulphate can modulate *Sema3s* activity *in vivo* remains to be determined. Examples from the developing nervous system provide evidence that rather than proteoglycan expression *per se*, it is the presence of differentially localized chondroitin sulphate-binding molecules that confers specific inhibitory or stimulatory activity (Emerling & Lander 1996). Interestingly, an impressive study by Kantor *et al.* (2004) shows that HSPGs and CSPGs convert the transmembrane *Sema5A* in an attractive or an inhibitory axon guidance cue, respectively. Whether other classes of semaphorins are affected by proteoglycans in a similar fashion remains to be determined.

Are the above described semaphorin–proteoglycan interactions likely to occur following injury? The expression pattern of *Sema3A* mRNA in relation to neurite growth inhibitors, i.e. CSPGs, tenascin-C and myelin-derived inhibitors, has been studied in transection lesions of the spinal dorsal columns (Pasterkamp *et al.* 2001). CSPG and tenascin-C expression overlap with *Sema3A*-positive cells in the meninges and in the dorsolateral cap of scar tissue that is mostly comprised of meningeal fibroblasts. The area of expression of tenascin-C and CSPG extends deeper into the ventral (astrocytic) aspect of the lesion where no *Sema3A* positive cells were present. Conditioning lesions of the sciatic nerve enhance the growth state of ascending sensory neurites (Neumann & Woolf 1999) and promote their sprouting into areas of CSPG expression but fail to induce growth into CSPG-*Sema3A* positive areas of the scar (Pasterkamp *et al.* 2001). This observation suggests the exciting possibility that *Sema3A* interacts with CSPGs in the neural scar to form a highly repulsive protein complex. In light of these observations it is interesting that chABC treatment promotes regeneration of spinal axons (Bradbury *et al.* 2002) and reinstates synaptic plasticity in the visual cortex after the critical period (Pizzorusso *et al.* 2002). The release of cell surface bound *Sema3A* following chABC treatment *in vitro* (De Wit *et al.* 2005) may imply that this inhibitory cue is also released from the GAG chains of CSPG in the scar. The putative release of *Sema3A* from CSPGs in a neural scar could be a mechanism that contributes to the beneficial effect of chABC treatment on axon regeneration.

(e) Transmembrane semaphorins and central nervous system injury: oligodendrocyte-associated inhibition?

Oligodendrocytes express a number of myelin-associated inhibitory proteins, including Nogo, MAG and OMpg (Filbin 2003; McGee & Strittmatter 2003; He & Koprivica 2004; Schwab 2004). Recently, membrane-bound semaphorins have been added to the list of oligodendrocyte-associated inhibitors. Together with the classical myelin-associated inhibitors these semaphorins are thought to inhibit axonal regeneration in the injured CNS. *Sema4D*, a transmembrane semaphorin also known as CD100, is expressed in a subpopulation of oligodendrocytes and is upregulated transiently after spinal cord injury in oligodendrocytes in white matter areas close to the lesion site (Moreau-Fauvarque *et al.* 2003). Plexin-B1, a *Sema4D* receptor, displays very limited expression in the adult brain. Remarkably, CD72, a *Sema4D* receptor originally identified in the immune system, is broadly expressed in the mature CNS and could thus mediate the inhibitory effect of *Sema4D* on adult neurons (Moreau-Fauvarque *et al.* 2003). *Sema5A* is a member of the thrombospondin repeat-containing semaphorins (class 5) expressed in oligodendrocytes and oligodendrocyte precursor cells in the optic nerve (Goldberg *et al.* 2004). *Sema5A* induces the collapse of cultured RGC growth cones and inhibits neurite growth from RGCs. Axon outgrowth on optic nerve explants is enhanced by application of *Sema5A* function blocking antibodies. In contrast to the

upregulation of *Sema4D* after a spinal cord lesion, expression of *Sema5A* does not change after optic nerve axotomy but its constitutive expression may be sufficient to render the optic nerve inhibitory for regenerative outgrowth. The role of *Sema5A* after CNS injury is particularly intriguing in view of the recent finding that *Sema5A* is a bi-functional molecule that is growth-inhibitory in the presence of CSPGs and growth-permissive in the presence of HSPGs (Kantor *et al.* 2004). Since optic nerve lesions lead to the induction of CSPG expression at the lesion site this may result in a relative increase in inhibitory *Sema5A*–CSPG molecular complexes at the site of injury.

Our picture of the injury-induced (changes in) expression of membrane-bound semaphorins is still far from complete. Given the large number of membrane-associated semaphorins that has been identified and studied in developmental processes, we expect that more information will become available on their expression, localization and function following neural injury in the near future.

3. INTRACELLULAR SIGNALLING COMPONENTS: TARGETS FOR PROMOTING CENTRAL NERVOUS SYSTEM REGENERATION?

During the past 10–15 years, considerable progress has been made in unravelling the intracellular signal transduction pathways that function downstream of repulsive semaphorins and their receptors (for review see Castellani & Rougon 2002; Pasterkamp & Kolodkin 2003; Kruger *et al.* 2005). Interestingly, the sustained sensitivity of sprouting adult axons to *Sema3A* *in vivo* (Tanelian *et al.* 1997; Tang *et al.* 2004) supports the idea that these intracellular signalling pathways also operate in injured adult neurons and may represent valuable targets for blocking semaphorin repulsion following injury. Here, we review some of the recent progress in understanding repulsive semaphorin signalling and discuss the potential of individual semaphorin signalling components as targets for reducing semaphorin-mediated axon repulsion in the injured CNS (figure 3).

(a) RhoGTPases

Binding of semaphorins to their receptors triggers a series of intracellular signalling events that ultimately affect the growth cone's actin cytoskeleton. One of the key regulators of actin dynamics is the Rho family of monomeric GTPases. Both RhoGTPases, including Rac, RhoA, RhoD and Rnd1, and the proteins that regulate their activity, such as guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs), have been implicated in semaphorin signalling (Castellani & Rougon 2002; Pasterkamp & Kolodkin 2003; Kruger *et al.* 2005). Interestingly, semaphorin receptors themselves serve as GAPs. The *Sema3A* and *Sema4D* receptors plexinA1 and plexinB1, respectively, directly stimulate the intrinsic GTPase activity of R-Ras, a member of the Ras superfamily of small GTP binding proteins. This activity requires the interaction of plexins with another small GTP binding protein, Rnd1 (Oinuma *et al.* 2004; Negishi *et al.* 2005; Pasterkamp 2005; Toyofuku *et al.* 2005).

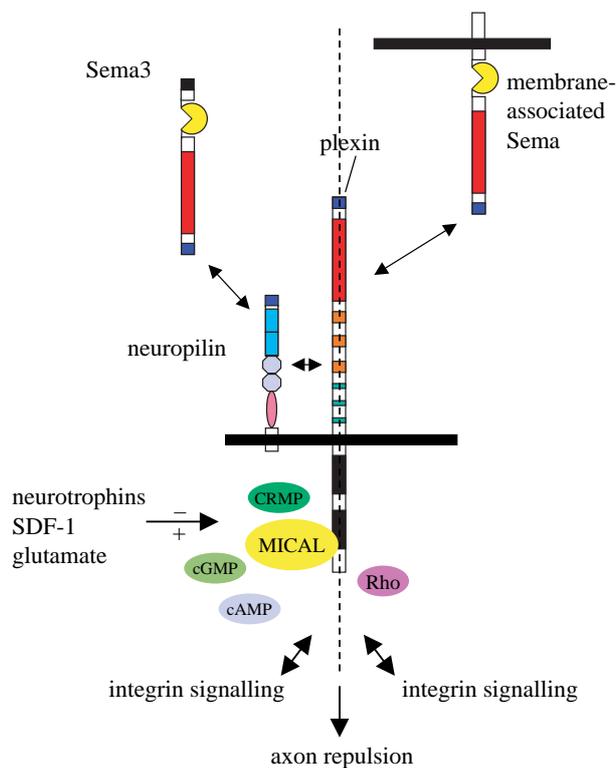


Figure 3. Intracellular semaphorin signalling. Signalling molecules such as Rho, CRMP and MICAL may serve as targets for reducing the repulsive actions of vertebrate secreted (Sema3) and membrane-associated semaphorins in the injured CNS. See text for more detail.

Several studies have explored Rho as a potential target for encouraging CNS regeneration. RhoGTPases and their downstream effector proteins are expressed in lesioned neurons, and blockage of Rho, by treatment with C3 transferase or Rho kinase (ROCK) inhibitors (Y27632), induces impressive anatomical and functional recovery in different models of spinal cord injury (Cai *et al.* 1999; Lehmann *et al.* 1999; Dergham *et al.* 2002; Fournier *et al.* 2003; Sivasankaran *et al.* 2004; Bertrand *et al.* 2005). Rho is thought to function downstream of several, but not all (e.g. class 3), classes of semaphorins (Castellani & Rougon 2002; Pasterkamp *et al.* 2003; Kruger *et al.* 2005). Current therapeutic strategies aimed at inactivating Rho or its downstream effectors are, therefore, likely to neutralize the repulsive effects of only a subset of repulsive semaphorins.

(b) Collapsin response mediator protein

One of the first cytosolic proteins shown to link semaphorin receptor complexes to the actin cytoskeleton is collapsin response mediator protein (CRMP)-2, a member of a small family of cytosolic phosphoproteins (Goshima *et al.* 1995; Wang & Strittmatter 1996). A number of different *in vitro* approaches have firmly established the role of CRMPs in semaphorin-induced (growth cone) collapse. CRMPs can associate with tubulin heterodimers, F-actin and several cytosolic proteins, including CRMP-associated molecule (CRAM), α 2-chimaerin, kinesin-1, phospholipaseD₂, Numb and Fes/Fps tyrosine kinase. Sequential phosphorylation of CRMP-2 by cyclin-dependent kinase-5 (Cdk-5) and glycogen synthase kinase3 β (GSK3 β) is believed to be critical for

Sema3A-induced growth cone collapse (Schmidt & Strittmatter *in press*). Furthermore, a recent study by Kaibuchi and colleagues suggests that phosphorylation of CRMP-2 by GSK3 β is also critical for the regulation of neuron polarity (Yoshimura *et al.* 2005). The effects of CRMPs on neuron polarity and axon growth are thought to result from direct interactions between CRMPs and the microtubule cytoskeleton (Arimura *et al.* 2004).

CRMP expression has been observed in different populations of PNS and CNS neurons following injury (Pasterkamp *et al.* 1998b; De Winter *et al.* 2002b; Suzuki *et al.* 2003). In addition, adenoviral vector-mediated overexpression of CRMP-2 in lesioned hypoglossal motor neurons accelerates peripheral nerve regeneration, probably through effects on axon growth/elongation (Suzuki *et al.* 2003). Whether increasing CRMP levels could also help to facilitate regeneration of CNS projections is currently unknown. It should be noted, however, that increases in CRMP expression in injured CNS neurons may also render them more sensitive to repulsive semaphorins expressed by CNS scar tissue. Recent insights into how the phosphorylation state of CRMP proteins relates to their cellular function (Schmidt & Strittmatter *in press*) may allow for the design of CRMP molecules that stimulate axon growth but do not affect axon responsiveness to repulsive semaphorins. Neuronal application of mutated CRMP proteins that can mediate axon growth but are unable to transduce axon repulsion may help to stimulate regenerative axon growth.

In addition to intact and injured neurons, CRMPs are expressed by oligodendrocytes in CNS white matter regions. In addition to CRMPs, oligodendrocytes express a broad spectrum of semaphorins, NPs, plexins and molecule interacting with *CasL* (MICAL; Ricard *et al.* 2000, 2001; Spassky *et al.* 2002; Cohen *et al.* 2003; Moreau-Fauvarque *et al.* 2003; Goldberg *et al.* 2004). In line with these expression patterns, semaphorins belonging to different subclasses, including class 3, can induce process retraction in oligodendrocyte progenitors, and oligodendrocytes and can also orient the migration of oligodendrocyte progenitors *in vitro* (Ricard *et al.* 2000, 2001; Cohen *et al.* 2003; Giraudon *et al.* 2004; Goldberg *et al.* 2004). Whether CRMPs are involved in mediating semaphorin-induced effects on oligodendrocyte morphology is unknown. Also, it remains to be determined whether neural injuries affect oligodendroglial CRMP expression.

(c) Cyclic nucleotides

Cyclic nucleotides, such as 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP), have strong regulatory effects on growth cone behaviour. Depending on the intracellular concentration of cyclic nucleotides, an axon guidance molecule can act as a repellent or attractant for the same growth cone. Elevation of cGMP in *Xenopus* neurons converts Sema3A-induced repulsive growth cone responses into attraction, while changes in cAMP have no effect (Song *et al.* 1997). However, cGMP-dependent switching of Sema3A repulsion into attraction can be blocked by cAMP antagonists (Song *et al.* 1998), suggesting an interplay between the different cyclic nucleotide pathways. In line with this idea, recent

evidence suggests that the ratio of cAMP and cGMP may be critical for determining the polarity of turning responses (Nishiyama *et al.* 2003).

Two recent studies have begun to shed light on how physiological changes in cyclic nucleotide levels may affect semaphorin signalling: (i) the *Drosophila* A-kinase anchoring protein (AKAP) Nervy physically interacts with plexinA and couples this plexin to protein kinase A (PKA). It is thought that Nervy-tethered PKA is activated by high cAMP levels to inhibit plexin signalling by phosphorylating plexin and/or downstream signalling components (Terman & Kolodkin 2004) and (ii) the *Drosophila* receptor guanylyl cyclase Gyc76C is required for semaphorin-mediated axon repulsion *in vivo* (Ayoob *et al.* 2004). Although it is currently unknown whether Gyc76C is truly a semaphorin receptor component, this observation may provide a functional link between local cGMP production and semaphorin-mediated axon repulsion.

Similar to RhoGTPases, cAMP has been targeted to enhance functional regeneration in the injured CNS. The initial observation that increases in cAMP may render cultured neurons less sensitive to the actions of several growth inhibitory proteins (Cai *et al.* 1999; Neumann *et al.* 2002; Qiu *et al.* 2002; Nikulina *et al.* 2004) supported the exciting possibility that functional CNS regeneration may be achieved by manipulating neuronal cAMP signalling. Indeed, several independent studies have shown that administration of cAMP analogues or rolipram following injury stimulates regeneration of axonal projections in the rat spinal cord (Neumann *et al.* 2002; Qiu *et al.* 2002; Lu *et al.* 2004; Nikulina *et al.* 2004; Pearse *et al.* 2004) and regeneration of the rat optic nerve (Cui *et al.* 2003). It should be noted, however, that conditioning nerve injuries which allow DRG axons to regenerate their centrally projecting neurites, an effect thought to be dependent on increases in endogenous cAMP, do not promote regenerative growth through *Sema3*-positive scar tissue (Pasterkamp *et al.* 2001). This is consistent with the original observation that cAMP has only a small effect on semaphorin responses in *Xenopus* turning assays (Song *et al.* 1998). More recent evidence suggests, however, that cAMP may be able to repress repulsive semaphorin responses rather than converting them into attraction. In addition, the ratio of cAMP/cGMP rather than the absolute levels of the individual cyclic nucleotides appears to be important in determining growth cone responses (Castellani & Rougon 2002; Pasterkamp & Kolodkin 2003; Bashaw 2004). Therefore, further studies are needed to better comprehend the modulation of semaphorin signalling by cyclic nucleotide pathways *in vivo* and to determine whether or how cyclic nucleotides can be targeted to specifically relieve repulsive semaphorin signals in the injured CNS.

(d) Molecule interacting with *CasL* (MICAL)

In a search for plexin-interacting proteins, MICAL (Suzuki *et al.* 2002) was identified (Terman *et al.* 2002). MICALs comprise a small family of phylogenetically conserved multidomain cytosolic proteins. *Drosophila* (D) MICAL binds the intracellular region of the *Sema-1a* receptor plexinA, and D-MICAL-plexinA interactions are required *in vivo* for *Sema-1a*-induced motor axon repulsion in the developing *Drosophila*

neuromuscular system (Terman *et al.* 2002). In vertebrates, three MICAL genes have been identified (MICAL-1, -2 and -3; Suzuki *et al.* 2002; Terman *et al.* 2002; Weide *et al.* 2003; Fischer *et al.* 2005; Pasterkamp *et al.* 2005). Interestingly, both vertebrate and invertebrate MICALs contain a NH₂-terminal flavoprotein monooxygenase domain of about 500 amino acids (Nadella *et al.* 2005; Siebold *et al.* 2005). Site-directed mutagenesis of the D-MICAL monooxygenase region attenuates repulsive *Sema-1a* signalling *in vivo* while monooxygenase inhibitors neutralize *Sema3*-mediated axon repulsion *in vitro* (Terman *et al.* 2002; Pasterkamp *et al.* 2005). These observations suggest that the MICAL monooxygenase enzyme participates in repulsive semaphorin signalling.

The biological role of vertebrate MICALs is currently unknown. Expression analysis and yeast interaction experiments hint at a role downstream of *Sema3s* and their receptors, plexinAs (Terman *et al.* 2002; Pasterkamp *et al.* 2005). This idea gains further support from the observation that certain green tea polyphenols with the ability to block flavoprotein monooxygenases, (–)-epigallocatechin gallate (EGCG) and (–)-epicatechin (EC) (Abe *et al.* 2000a,b), abrogate *Sema3A*- and *Sema3F*-mediated repulsion of rat sensory axons *in vitro* (Terman *et al.* 2002; Pasterkamp *et al.* 2005). Vertebrate MICALs are expressed during neural development, in the adult nervous system and following injury. Contusion and hemisection injuries of the rat spinal cord induce expression of all three MICAL genes in meningeal fibroblasts that contribute to CNS scar tissue. In addition, MICAL-1 and -3 are expressed in oligodendrocytes in intact spinal cord white matter and in oligodendroglia present around the site of injury (Pasterkamp *et al.* 2005; figure 4). Although their role in non-neuronal cells in the intact and injured CNS requires further study, MICALs could function to transduce the effects of semaphorins on oligodendrocytes and meningeal fibroblasts. The concomitant expression of semaphorins, their receptors and associated cytosolic signalling molecules in oligodendroglia and meningeal fibroblasts suggests that semaphorins may act in an auto- and/or paracrine fashion in these cell types. Alternatively, oligodendroglia and meningeal fibroblasts could respond to semaphorins released in their environment. For example, *Sema3s* released from fibroblasts in the lesion core in the injured spinal cord may function to affect the migration and morphology of MICAL-positive oligodendrocytes surrounding the lesion and vice versa.

In line with the idea that injured adult neurons express *Sema3* signalling components, expression of all three MICAL genes was found in corticospinal tract neurons following spinal hemisection and contusion injuries (Pasterkamp *et al.* 2005). Sustained MICAL neuronal expression following CNS injury, coupled with the potential for *Sema3s* to mediate glial scar inhibition of regenerative axon growth, suggest that MICALs may serve as therapeutic targets for decreasing axon responsiveness to scar-derived *Sema3s* following spinal cord injury. Strategies aimed at inactivating MICAL (monooxygenase enzymatic) activity may, therefore, help to paralyse repulsive semaphorin

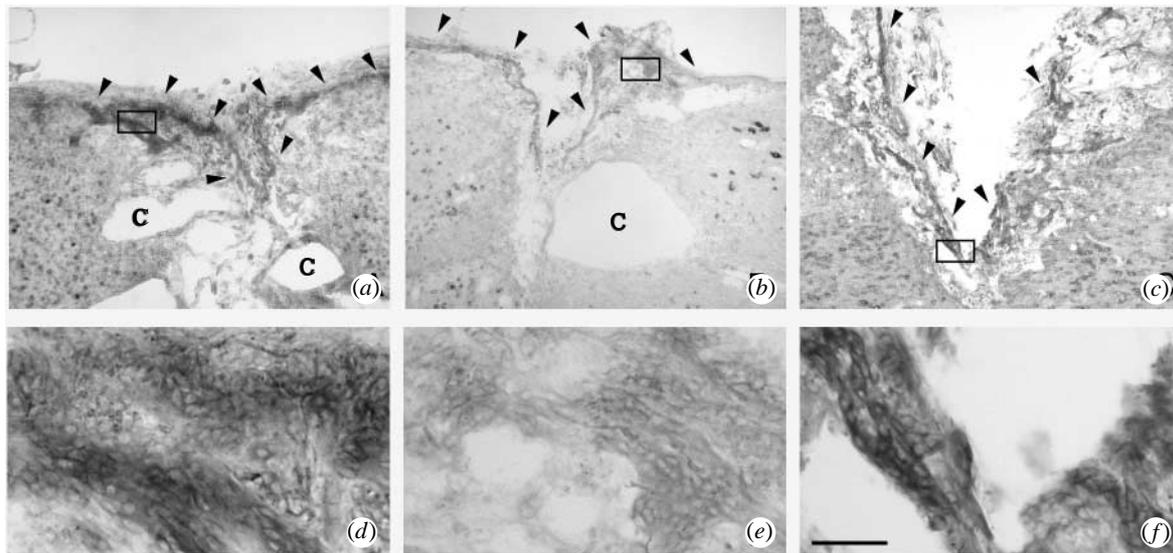


Figure 4. MICALs are expressed in the injured rat spinal cord. Horizontal sections of a spinal cord lesion site at 56 days following overhemisection injury were subjected to *in situ* hybridization for *MICAL-1* (a, d), *MICAL-2* (b, e), or *MICAL-3* (c, f). Panels d–f are higher magnifications of the boxed areas in a–c, respectively. *MICALs* are expressed in small, elongated meningeal cells in the lesion core and adjacent meningeal sheath (arrowheads in a–c) and in oligodendrocytes surrounding the lesion (not shown). c, cavity. Scale bar, 360 μm (a–c), 45 μm (d–f). Modified from Pasterkamp *et al.* (2005) with permission from Elsevier.

signalling. The robust effect of the monooxygenase inhibitor flavonoid EGCG on *Sema3A*- and *Sema3F*-mediated sensory neuron repulsion *in vitro* suggests that monooxygenase inhibitors may be used to reduce *Sema3* signalling following injury (Pasterkamp *et al.* 2005; figure 5). However, the molecular mechanism(s) by which EGCG affects *Sema3* signalling requires further research. Interestingly, EGCG also has neuroprotective and anti-inflammatory effects (Levites *et al.* 2001; Lee *et al.* 2003; Koh *et al.* 2004; Mandel *et al.* 2004). Another beneficial characteristic of EGCG in light of its potential application following neural injury is its access to the brain and other organs following peripheral administration owing to the ability of this flavonoid to efficiently cross the blood–brain barrier (Nakagawa & Miyazawa 1997; Suganuma *et al.* 1998). Future studies will determine whether EGCG can be used as a multiple-approach therapeutic agent for treating CNS injury.

(e) *Intracellular modulation of semaphorin signalling*

Our limited understanding of the signalling pathways downstream of repulsive semaphorins makes it difficult to predict with precision where intracellular semaphorin signalling pathways intersect with other signalling cascades. Nevertheless, several studies have begun to provide clues as to how semaphorin signalling may be modulated through interactions with other intracellular signal cascades. Intracellular signalling pathways that modulate semaphorin signalling could be excellent targets for manipulating semaphorin responsiveness following injury.

(i) *Neurotrophins*

Neurotrophic factors are widely used to promote CNS regeneration *in vivo*. It has become clear that in addition to their axon outgrowth promoting effects, neurotrophic factors may positively influence axon

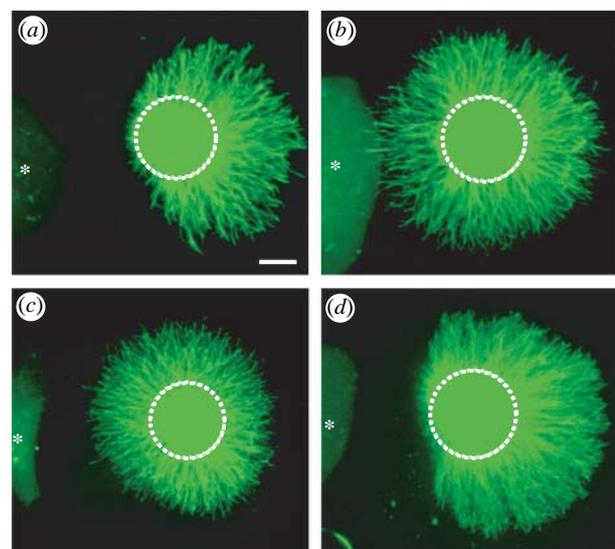


Figure 5. Flavoprotein monooxygenase inhibitors block *Sema3A*-induced sensory axon repulsion. E14 rat DRG explants were co-cultured with 293 cells expressing *Sema3A* (asterisks) and grown for 48 h in the presence of (a) vehicle or (b–d) an inhibitor. (a) *Sema3A* repels rat sensory neurons. The monooxygenase inhibitors EC (b) and EGCG (c), but not the xanthine oxidase inhibitor allopurinol (d), inhibit this *Sema3A*-dependent axon repulsion. Modified from Terman *et al.* (2002) with permission from Elsevier.

regeneration by changing the responsiveness of regenerating fibres to outgrowth inhibitory molecules. Neurotrophin-3 (NT-3), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) modulate growth collapse responses to *Sema3A* (Tuttle & O'Leary 1998; Dontchev & Letourneau 2002). Both NGF and BDNF can protect embryonic chick DRG growth cones from *Sema3A*-induced collapse in a concentration-dependent manner (Dontchev & Letourneau 2002). Vice versa, *Sema3F* antagonizes NGF-induced TrkA signalling (Atwal *et al.* 2003). It is not precisely clear how and where

NT and Sema3 signalling pathways intersect, but pharmacological studies suggest roles for the cyclic-nucleotide-dependent kinases PKA and PKG, phosphatidylinositol-3 kinase (PI-3), mitogen-activated protein kinase (MEK) and Rho-associated kinase (Tuttle & O'Leary 1998; Ming *et al.* 1999; Dontchev & Letourneau 2002; Atwal *et al.* 2003).

(ii) Integrins

Several reports have shown that semaphorins and plexins regulate integrin function in cell adhesion, migration and axon outgrowth (Pasterkamp *et al.* 2003; Serini *et al.* 2003; Barberis *et al.* 2004). Recent evidence suggests that MICALs and/or R-Ras may be involved in cross-talk between these signalling cascades. MICAL was originally identified as a molecule that interacts with CasL (Suzuki *et al.* 2002). CasL is important for β 1-integrin-induced signal transduction and cytoskeletal reorganization. This may indicate that semaphorin (axon repulsion) and integrin pathways (cell adhesion) converge on CasL. It is believed that sequestering of CasL by MICAL may locally decrease cell adhesion and thereby allow semaphorin-induced collapse. Similarly, binding of Sema3A and Sema4D to plexinA1/neuropilin-1 and plexinB1, respectively, leads to binding and inactivation of R-Ras (Oinuma *et al.* 2004; Toyofuku *et al.* 2005). Since R-Ras is a central player in integrin signalling this may mean that plexins shut down integrin signals by binding and inactivating this small GTPase.

(iii) Stromal cell-derived factor and glutamate

The chemokine stromal cell-derived factor (SDF)-1 and the neurotransmitter glutamate modulate the responsiveness of axons to Sema3s. The modulatory effects of SDF-1 are mediated through CXCR4 and a pertussis toxin-sensitive G-protein coupled signalling pathway that induces an elevation of cAMP (Chalasanani *et al.* 2003). Similarly, the effects of glutamate are mediated by the metabotropic glutamate receptor 1 (mGluR1) and a pathway that involves the pertussis toxin-sensitive activation of PKA and inactivation of Rho (Kreibich *et al.* 2004).

Our understanding of how semaphorin signalling pathways are modulated by other unrelated signalling pathways and vice versa, is still rudimentary. However, further insights into this process of molecular cross-talk may provide unique opportunities for designing therapeutic strategies aimed at tackling multiple aspects of regeneration failure simultaneously (e.g. increasing both axonal growth and neuronal survival, and decreasing the inhibitory properties of the injured CNS by targeting one signalling pathway).

4. PERSPECTIVES

Several challenges lie ahead with respect to advancing our insight into the role of repulsive semaphorins in the intact and injured adult nervous system. First, expression studies of most semaphorins have relied on *in situ* hybridization, while their protein localization remains largely unknown. This is the result of a lack of specific and reliable semaphorin antibodies. Determining the precise intracellular and extracellular distribution of individual

secreted and membrane-associated semaphorin proteins using well-characterized antibodies will be crucial to understand their role in axon plasticity, regeneration and degeneration. Furthermore, live imaging studies employing XFP-tagged semaphorin proteins will be required to reveal their axonal and dendritic transport and secretion and deposition characteristics at extracellular sites. This is particularly important in view of the proposed interaction of these repulsive molecules with the ECM, e.g. during the inhibition of axon regeneration or while modulating synaptic plasticity. Second, the demonstration of a causal relationship between the expression of semaphorins and the failure of axon regeneration has only just begun. Several approaches can be envisioned here: genetic ablation of specific semaphorin, semaphorin receptor or intracellular signalling cue genes, antibody perturbation or pharmacological inhibitor studies. The ECM appears to be a particularly important target for reducing the inhibitory nature of the injured CNS since multiple inhibitory molecules may be affected simultaneously by interfering with the chondroitin moieties of the proteoglycans or by inhibiting the synthesis of collagen in the ECM (Bradbury *et al.* 2002; Klapka *et al.* 2005). Targeting the ECM will also help to reveal the relative contribution of the different inhibitory factors (Nogo, MAG, OMpg, CSPG, semaphorins) to the failure of CNS regeneration and should lead the way to more effective and realistic interference strategies. Third, Sema3F modulates synaptic transmission in the adult mouse hippocampus (Sahay *et al.* 2005). This finding illustrates the diverse functional repertoire of these guidance molecules and suggests involvement in both the formation and synaptic efficacy of neural circuits. Finally, single-nucleotide polymorphisms in Sema5A and the Sema3 receptor plexinA2 have recently been linked to Parkinson disease and schizophrenia, respectively (Maraganore *et al.* 2005; Mah *et al.* 2006). This nominates these genes as novel susceptibility factors in these diseases and warrants more work on these molecules in relation to adult brain function and neurodegeneration. As the biological functions of semaphorins continue to be dissected at multiple levels we are likely to answer fascinating questions about their involvement in neuronal plasticity, regeneration and neurological disease and come up with ways to modulate semaphorin function to treat injury or disease.

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