CHAPTER 1

General introduction

Chemorepellent axon guidance molecules in spinal cord injury

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Abstract

Regenerating axons stop growing when they reach the border of the glial-fibrotic scar, presumably because they encounter a potent molecular barrier inhibiting growth cone advance. Chemorepulsive axon guidance molecules provide a non-permissive environment restricting and channeling axon growth in the developing nervous system. These molecules could also act as growth-inhibitory molecules in the regenerating nervous system. The receptors for repulsive guidance cues are expressed in the mature nervous system, suggesting that adult neurons are sensitive to the activity of developmentally active repulsive proteins. Here we summarize recent observations on semaphorins, ephrins and slits in the injured brain and spinal cord, providing evidence that these proteins are major players in inhibiting axonal regeneration and establishing the glial-fibrotic scar.

Introduction

It is now generally accepted that developing axons are guided to their appropriate targets by both attractive and repulsive molecules. Intriguingly, many repulsive axon guidance cues continue to be expressed during adulthood and are regulated after the nervous system is injured. Do proteins that guide developing axons to their targets by a repulsive mechanism restrict plasticity and inhibit axon growth during adulthood and following neural injury? This paper provides an overview of our current understanding of the role of repulsive axon guidance factors (semaphorins, ephrins, slits) in nervous system regeneration.

The function of chemorepellent guidance molecules in the adult and injured nervous system can be viewed from different perspectives, depending on the cellular context in which a particular protein and its receptor(s) are expressed. Four possible roles with regard to their involvement in central nervous system (CNS) regeneration have been postulated so far:

1. Repellent guidance molecules may contribute to the inhibitory properties of oligodendrocyte-lineage cells, thus emphasizing the initial concept of myelin-based inhibition of axon regeneration (Schwab and Bartholdi, 1996). The transmembrane semaphorins Sema4D/CD100 and Sema5A are expressed in oligodendrocytes (Moreau-Fauvarque et al., 2003, Goldberg et al., 2004) and may act in concert with the myelin-associated inhibitors Nogo, MAG and OMgp (reviewed by Schweigreiter and Bandtlow, 2006) to interfere with axon regeneration.

2. Repellent guidance molecules expressed at a CNS lesion site may form a molecular boundary for regenerating axons. This is likely to be the case for secreted semaphorins that are expressed by meningeal fibroblasts populating the lesion core upon mechanical injury to the brain and spinal cord (Pasterkamp et al., 1999b, Pasterkamp and Verhaagen, 2001). Certain
chondroitin sulphate proteoglycans (CSPGs) are also induced at sites of CNS injury (reviewed by Davies et al., 2006). Since Sema3A interacts with the glycosaminoglycan (GAG) chains of CSPGs (De Wit et al., 2005) these molecules could form highly repulsive extracellular protein deposits at CNS lesion sites.

3. Repellent guidance molecules could be involved in instigating or modulating the cellular response to injury, e.g. inducing astrogliosis, tissue compartmentalization and neovascularization in the scar. Recent evidence demonstrates a role for EphA4 in the astrocytic response to injury (Goldshmit et al., 2004). Neuropilin-1 (Npn-1), a receptor for secreted semaphorins, is involved in blood vessel formation during embryogenesis (Kitsukawa et al., 1995, Kawasaki et al., 1999, Takashima et al., 2002) and is present in newly formed blood vessels in a neural scar surrounded by meningeal cells expressing Sema3A (Pasterkamp et al., 1999a).

4. Guidance molecules may be re-expressed in the injured nervous system in order to enable correct pathfinding and target recognition. In the optic system of adult fish and mammals ephrins and their receptors are still expressed in gradients and at least in the regenerating fish optic nerve expression of these molecules is essential for correct pathfinding of regenerating axons (Rodger et al., 2004). Repulsive guidance cues may have a similar role in regenerating peripheral nerves in mammals.

Semaphorins in the mature intact and injured nervous system

Semaphorins comprise a large family of secreted and membrane-associated proteins. Secreted semaphorins bind and signal through receptor complexes comprised of neuropilins andplexins. Membrane-associated semaphorins bind directly to plexins and do not require neuropilins as co-receptors. Several additional proteins have been implicated as components or modulators of the semaphorin holoreceptor complex in vertebrates: the cell adhesion molecule L1, the Met receptor and integrins. CD72 and Tim-2 are alternative semaphorin receptors in the immune system. The intracellular signaling events that unfold after semaphorin-receptor interaction lead to depolymerization of F-actin and consequent growth cone collapse (reviewed by Pasterkamp and Kolodkin 2003).

Semaphorins were first identified in invertebrates as proteins that promote nerve fasciculation (Kolodkin et al., 1992). The prototype vertebrate semaphorin, the secreted semaphorin 3A (Sema3A), was purified from adult chick brain membranes on basis of its growth cone collapse-inducing activity on cultured embryonic sensory neurons (Luo et al., 1993). Sema3A null-mutant mice display severe defasciculation of peripheral nerves and more subtle changes in the orientation or termination of fibers in the cortex, hippocampus, spinal cord and olfactory bulb (Behar et al., 1996, Taniguchi et al., 1997, White and Behar, 2000). During embryogenesis secreted semaphorins are expressed in non-neuronal cells along developing nerve tracts and in subpopulations of developing neurons.
Expression is widespread and changes rapidly as development proceeds (Luo et al., 1995, Giger et al., 1996, Shepherd et al., 1996). In the mature nervous system expression of the secreted semaphorins is largely restricted to particular populations of neurons (www.gensat.org/index.html). Sema3A expression is confined to cranial and spinal motor neurons, entorhinal cortex stellate cells, neurons in the amygdala, olfactory bulb mitral cells and subsets of retinal ganglion cells (Giger et al., 1998a, Skaliiora 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 covering the olfactory bulbs and frontal cortex (Pasterkamp et al., 1998a, Pasterkamp et al., 1999a). Several other semaphorins have been reported to be expressed in the adult rodent or human brain (Eckhardt and Meyerhans, 1998, Xu et al., 1998, Encinas et al., 1999, Hirsch et al., 1999).

Expression of secreted semaphorins after CNS injury

The neural scar that forms after penetrating brain or spinal cord injury is a major barrier to regeneration (reviewed by Silver and Miller, 2004). The cellular composition of the neural scar is complex (see Fig.1). Meningeal fibroblasts and blood-borne cells invade the center of the lesion. Astrocytes around the lesion core become hypertrophic and increase their expression of glial fibrillary acidic protein (GFAP). The astrocyte response to injury is usually referred to as reactive astrogliosis. Astrocytes and meningeal fibroblasts do engage in the formation of a new glia limitans and in the restoration of the blood brain barrier (Shearer and Fawcett, 2001). The formation of a neural scar can be regarded as a neural wound healing response that prevents further spread of damage to the uninjured nervous system. Axons of injured CNS neurons are unable to cross the fibrotic core of the scar and develop swollen endings, so-called dystrophic endbulbs, which are found just proximal to or within the zone of reactive astrocytes (Tom et al., 2004).

Three lines of evidence indicate that meningeal fibroblasts invading the core of the scar express the secreted semaphorins Sema3A, Sema3B, Sema3C, Sema3E and Sema3F (Pasterkamp et al., 1999a, De Winter et al., 2002b). Rodent Sema3D, which was not available at the time of these studies, has not been investigated so far. First, double labeling studies revealed that semaphorin transcripts co-localized with markers of fibroblasts (fibronectin and vimentin) and do not co-localize with markers of astrocytes (GFAP), macrophages/microglia (ED1, OX42), oligodendrocytes (MBP, Gal-C) and Schwann cells (S100). Second, the morphology of the semaphorin positive cells is consistent with previous descriptions of fibroblasts in the neural scar (Berry et al., 1983, Hirsch and Bahr, 1999). Third, semaphorin positive cells in the scar form typical strands that are sometimes clearly connected to the semaphorin positive lepto-meningeal sheet adjacent to the primary lesion site.
Interestingly, the distribution of semaphorin-positive fibroblasts differs markedly in spinal cord scars formed either after transection or contusion lesion (De Winter et al., 2002b). Penetrating injuries result in the proliferation and migration of meningeal fibroblasts deep into the lesion. In contrast, contusion injuries usually leave the meningeal sheet intact and a rim of spared white matter often remains present at the site of contusion. Contusion injuries do induce the proliferation of meningeal fibroblasts resulting in a thickening of the meningeal sheet but fibroblasts do not migrate into the center of the lesion. This explains that semaphorin positive cells are observed in the core of the lesion after a transection injury, but remain confined to a relatively thin layer of leptomeningeal sheet cells surrounding the injury site after a spinal cord contusion.

Sema3A mRNA expression was enhanced 3.8 fold in the denervated cervical spinal cord one week after a pyramidotomy, as determined by affimetrix gene arrays (Bareyre and Schwab, 2003). The cellular source of this lesion-induced increase is not clear. A recent study in completely transected rat spinal cord reportedly did not detect Sema3A in meningeal cells in the lesion, while Sema3A was found to be transiently up-regulated in injured neurons of the trigeminal nucleus and the cerebral cortex (Hashimoto et al., 2004).

Figure 1. Expression of chemorepulsive molecules in the injured spinal cord. Schematic representation of a sagittal section through a partially transected spinal cord. A penetrating injury to the spinal cord leads to infiltration of meningeal fibroblasts, Schwann cells and microglia/macrophages that occupy the lesion core. Astrocytes in the damaged area become hypertrophic and form a dense network of reactive astrocytes around the lesion core. At the interface between meningeal fibroblasts and reactive astrocytes, a new glia limitans is formed. Chemorepellent axon guidance cues are expressed at the injury site by meningeal fibroblasts and reactive astrocytes as shown. Receptors for repellent factors are present on regenerating axons and motoneurons. In-vading Schwann cells, oligodendrocyte precursors, and microglia/macrophages are omitted for simplicity.
Using semi-quantitative PCR Sema3A, Sema3B and Sema3E have been detected in cultured oligodendrocyte precursor cells and Sema3B and Sema3E but not Sema3A, were found in cultured astrocytes derived from optic nerves of one week old rats (Goldberg et al., 2004). In situ hybridization has not been employed yet to unequivocally confirm the presence and the cellular source of these semaphorins in the neonatal or injured adult optic nerve. Under conditions of penetrating injuries of the cortex, spinal cord and the primary olfactory pathway, expression of secreted semaphorins was confined to lepto-meningeal fibroblasts and neurons surrounding the lesion, and were not detectable in reactive astrocytes (Pasterkamp et al., 1998a, Pasterkamp et al., 1999a, Pasterkamp and Verhaagen, 2001, De Winter et al., 2002b). Discrepancies in the results described by Goldberg et al. and other labs (De Winter et al., 2002a, Moreau-Fauvarque et al., 2003, Hashimoto et al., 2004) are likely to be explained by the difference in sensitivity of PCR versus in situ hybridization and/or expression observed in cultured cells versus cells in vivo in tissue sections.

Are adult neurons sensitive to semaphorins?
Gene gun-mediated expression of Sema3A in the corneal epithelial cells of adult rabbits causes repulsion of trigeminal sensory fibers and prevents reinnervation of the cornea following epithelial wounding (Tanelian et al., 1997). This experiment provided the first indication that adult neurons exhibit an axonal withdrawal response from an area with enhanced expression of a semaphorin. Most adult rat dorsal root ganglion (DRG) neurons express Npn-1, Npn-2 and one or more plexins (Reza et al., 1999, Owesson et al., 2000, Pasterkamp and Verhaagen, 2001). Cultured adult NGF-responsive DRG neurons are repulsed by Sema3A, but not Sema3B or Sema3E (Reza et al., 1999, Owesson et al., 2000). Recently in vivo evidence for an inhibiting effect of Sema3A on NGF-mediated sensory nerve sprouting was obtained (Tang et al., 2004). Adenoviral vector-mediated expression of Sema3A in the intact spinal cord of adult rats counteracts the NGF-induced sprouting of central CGRP-positive sensory fibers, indicating that Sema3A may be effective in conditions where excessive sprouting is detrimental. Interestingly, Sema3A was only capable of restricting NGF-induced sprouting under conditions in which the expression of NGF was relatively low (Tang et al., 2004). These data support the observation that NGF can reduce Sema3A induced growth cone collapse and neurite growth inhibition in a dose dependent fashion (Dontchev and Letourneau, 2002, Niclou et al., 2003).

Following penetrating injuries to the spinal cord, injured corticospinal and rubrospinal axons form endbulbs at short distances from the semaphorin-positive scar cells. Corticospinal neurons express Npn-1 and Npn-2 and rubrospinal neurons express Npn-2 (De Winter et al., 2002b). Injured adult sensory neurons continue to express several receptor components required for secreted semaphorin signalling (Gavazzi et al., 2000, Pasterkamp and Verhaagen, 2001). The axons of these neurons do not penetrate the semaphorin positive
portion of the scar. Npn-1 expression is upregulated in OX42-positive microglial cells and spinal interneurons after spinal cord injury (Agudo et al., 2005). These observations suggest that injured neurons are capable of sensing the presence of semaphorins in the neural scar and are halted at sites of semaphorin expression.

Localization of secreted semaphorins in the extracellular matrix

Transection lesions of the spinal dorsal columns were used to study the expression patterns of Sema3A in relation to other proteins implicated in neurite outgrowth inhibition, the CSPGs, tenasin-C and myelin-derived inhibitors (Pasterkamp and Verhaagen, 2001). CSPG and tenasin-C expression overlap with Sema3A in the meninges and in the dorsolateral cap of scar tissue that is mostly comprised of meningeal fibroblasts. The area of expression of tenasin-C and CSPG extended deeper into the ventral (astrocytic) aspect of the lesion where no Sema3A positive cells were present. Conditioning lesions (a sciatic nerve crush prior to dorsal column transection) enable injured ascending sensory axons to regrow across areas of strong CSPG and tenasin-C expression, while areas containing Sema3A and CSPGs in the dorso-lateral portion of the scar and areas containing CNS myelin surrounding the scar were avoided by regenerating sensory fibers. Conditioning lesions enhance the growth state of ascending sensory neurites (Neumann and Woolf, 1999) and promote nerve sprouting into areas of CSPG expression but fail to induce growth into CSPG-Sema3A positive areas of the scar (Pasterkamp and Verhaagen, 2001).

Cultured meningeal fibroblasts are a poor substrate for growing neurites compared to astrocytes although both cell types express several CSPGs (Noble et al., 1984, Fawcett and Asher, 1999). The growth inhibitory properties of meningeal fibroblasts appear to rely on cell-associated rather than soluble factors, since conditioned media from cultured meningeal fibroblasts do not block neurite outgrowth (Noble et al., 1984) and do not induce growth cone collapse (Niclou et al., 2003). However, membrane extracts from cultured adult and neonatal meningeal fibroblasts induce the collapse of embryonic DRG growth cones (Niclou et al., 2003). This collapsing activity is blocked by Npn-1 antibodies and is absent in membrane extracts from Sema3A-deficient meningeal fibroblasts. Similarly, meningeal fibroblasts prepared from Sema3A knockout mice provide a more permissive substrate than cells prepared from wild-type littermates. Thus in vitro Sema3A is a major neurite growth-inhibitory factor in meningeal fibroblasts and appears to be presented not in soluble form but as a substrate-bound molecule associated with the cell membrane or the extracellular matrix (Niclou et al., 2003). In co-cultures of astrocytes and meningeal fibroblasts, the two cell types cluster in separate patches and form distinct territories. Postnatal DRG neurons plated on these co-cultures grow well on the astrocyte patches but do not cross the interface between astrocytes and meningeal fibroblasts (Shearer et al., 2003), again indicating that inhibitory factors are localized to the cell membrane or extracellular matrix rather than freely diffusing in the...
medium. Blocking semaphorin signaling by application of Npn-2 antibodies partially overcomes the repellent effect of the meningeal boundary (Shearer et al., 2003). A Npn-1 blocking antibody had no effect in this assay suggesting that Npn-2 ligands are selectively concentrated at the astrocyte-meningeal fibroblast interface.

Interestingly, Sema3A expressed by neurons localises in discrete patches reminiscent of proteoglycan distribution in the extracellular matrix (De Wit et al., 2005). Under these conditions removal of GAG chains with chondroitinaseABC or competing for binding with soluble chondroitin sulphate releases Sema3A into the medium, indicating that Sema3A binds chondroitin sulphate GAG chains. The mechanisms by which CSPGs inhibit neurite outgrowth are poorly understood. Receptors for CSPGs have not been identified on the growth cone. One explanation for failure of regenerating sensory axons to grow into CSPG-Sema3A positive areas may be that repulsive proteins that bind to the GAG-chains of CSPG, e.g. Sema3A, significantly enhance the inhibitory properties of CSPGs. 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 and Lander, 1996). In the developing fiber tract of the circulus retroflexus heparan sulphate proteoglycans (HSPGs) and CSPGs convert the transmembrane Sema5A in an attractive or an inhibitory axon guidance cue, respectively (Kantor et al., 2004). Whether CSPG-semaphorin interactions also occur in the scar and how this affects their inhibitory activity towards regenerating axons is currently under investigation.

Secreted semaphorin expression in the injured peripheral nervous system

In contrast to most mature central neurons, injured peripheral neurons are capable of regeneration and reinnervation of their distant target cells. Traditionally, most studies on the mechanisms that regulate peripheral nerve regeneration have focused on the expression of proteins that would promote axonal regeneration. Peripherally injured motor neurons upregulate specific transcription factors (e.g. c-jun, Atf-3) and growth-associated proteins (e.g. GAP-43, CAP-23, juvenile tubulin), some of which have been positively linked to peripheral nerve regeneration (Aigner et al., 1995, Raivich et al., 2004). 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 also becomes apparent following peripheral nerve transplantation in the injured CNS (David and Aguayo, 1985).

Understanding the role of neurite growth inhibitory proteins in the injured peripheral nervous system is as important as insight into the function of neurite outgrowth promoting molecules. Adult motoneurons and sensory neurons continue to express the receptors for secreted semaphorins, Npn-1, Npn-2, and
several plexins (Giger et al., 1998a, Pasterkamp et al., 1998b, Gavazzi et al., 2000, De Winter et al., 2002b, Lindholm et al., 2004). Adult motoneurons, but not sensory neurons, also have continued expression of Sema3A (Pasterkamp et al., 1998b) and several other members of the class 3 semaphorins (De Winter and Verhaagen, unpublished). Peripheral and central lesions of motoneuron axons affect the expression of Sema3A in opposite ways (Pasterkamp et al., 1998b, Lindholm et al., 2004). Peripheral nerve lesions at the mid-thigh level result in a decline in Sema3A expression in motoneurons while Npn-1 continues to be expressed in motoneurons and is induced in small diameter sensory neurons (Pasterkamp et al., 1998b, 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 motoneurons. In line with observations following penetrating spinal cord and brain injuries, Sema3A is induced in the neural scar in elongated fibroblast-like cells after ventral funiculus lesions (Lindholm et al., 2004). This lesion results in very poor regeneration of motor axons while peripheral lesions are followed by vigorous spontaneous regeneration. It is tempting to speculate that the different response of semaphorins in meningeal fibroblasts and motoneurons contributes to the differential regenerative response of motoneurons after these two types of lesion.

Do de-differentiated Schwann cells in the distal portion of an injured peripheral nerve express semaphorins? Using RT-PCR the expression of class 3 semaphorins was found to be induced in the peripheral nerve distal to a crush or transection lesion (Ara et al., 2004). The cellular origin of these semaphorin transcripts is unclear. Low magnification photomicrographs of in situ hybridization experiments suggest that most of these transcripts are expressed in the epineurium and perineurium (Scarlato et al., 2003). We have not been able to detect Sema3A by in situ hybridization in cells in peripheral nerve stumps distal to a transection or crush lesion. This is unlikely to be due to a lack of sensitivity of our in situ hybridization procedure, since we do detect Sema3A transcripts in subpopulations of terminal Schwann cells on type IIb muscle fibers after denervation (De Winter and Verhaagen, unpublished). In these same experiments the dedifferentiated myelin forming Schwann cells in the intramuscular peripheral nerve trunks remain unstained for Sema3A. Moreover Sema3B transcripts are clearly detectable in a subpopulation of peripheral nerve Schwann cells that invade penetrating spinal injuries (De Winter et al., 2002b).

**Expression of transmembrane semaphorins after CNS injury**

Recent reports identify transmembrane semaphorins as novel oligodendrocyte-associated inhibitory factors that may contribute to restricting axonal regeneration in the injured CNS. Sema4D a transmembrane semaphorin (also known as CD100) is expressed in a subpopulation of myelinating oligodendrocytes and is transiently upregulated after spinal cord hemisection...
in oligodendrocytes in white matter areas immediately surrounding the lesion site (Moreau-Fauvarque et al., 2003). Sema4D is not detected in astrocytes, microglia/macrophages or oligodendrocyte precursor cells. Cultured postnatal sensory and cerebellar neurons avoid a Sema4D substrate, though their growth cones are not collapsed by this molecule. Plexin-B1 a bonafide Sema4D receptor, has very limited expression in the adult brain. CD72 a Sema4D receptor in the immune system, is however broadly expressed in the mature CNS and could thus mediate the effect of Sema4D in adult neurons (Moreau-Fauvarque et al., 2003).

Sema5A is a member of the thrombospondin repeat-containing semaphorins (class 5) that is expressed in oligodendrocytes and oligodendrocyte precursor cells in the optic nerve (Goldberg et al., 2004). In vitro Sema5A induces the collapse of retinal ganglion cell growth cones and inhibits neurite outgrowth from retinal ganglion cells, both of embryonic (50% inhibition) and postnatal origin (20% inhibition). Axon outgrowth is also blocked on optic nerve explants and can be induced by application of Sema5A blocking antibodies. Expression of Sema5A does not change however after optic nerve axotomy (Goldberg et al., 2004). The role of Sema5A after CNS injury is particularly intriguing in view of the recent finding that Sema5A is a bifunctional molecule that is growth-inhibitory in the presence of CSPGs and growth-permissive in the presence of HSPGs (Kantor et al., 2004). The distribution of Sema5A after spinal cord injury has not been investigated yet.

Gene expression profiling of the lesion site revealed a 3 fold upregulation of Sema6B at 3 weeks after fimbria fornix transection (Kury et al., 2004). The cellular source of this semaphorin and its regulation in spinal cord injury is not known.

**How can semaphorin activity be neutralized?**

We predict that perturbation of injury-induced semaphorin signaling could result in enhanced regeneration of injured spinal neurons. Null mutant mice for individual semaphorins (Sema3A, Sema3C, Sema3F, Sema4D) and semaphorin receptor components (Npn-1, Npn-2, PlexA3, PlexB3, CD72) implicated in the inhibition of regeneration are available but have so far been of little use in regeneration studies. Some of these mice (Sema3A, Sema3C, Npn-1) die before birth or early postnatally and can not be used in regeneration experiments (Behar et al., 1996, Taniguchi et al., 1997, Kawasaki et al., 1999, Feiner et al., 2001). A very small proportion of one strain of homozygous Sema3A null mutants (Taniguchi et al., 1997) survive into adulthood but the number of survivors is very small making it unrealistic to perform regeneration studies in these mice. Moreover the neutralization of a single semaphorin may not be sufficient to induce a strong regenerative response. The use of receptor knockouts is more promising because this could render neurons insensitive to multiple semaphorins simultaneously. Npn-2 knockout mice do survive into adulthood (Giger et al., 2000) and we and others are currently investigating the regenerative response in these mice. A
small proportion of Npn-1 knock-in mice expressing a Npn-1 variant with an altered ligand binding site that has retained the capacity to bind VEGF but lacks binding to Sema3A apparently survive into adulthood (Gu et al., 2003) and would be an interesting strain for regeneration studies.

An alternative to mutation of semaphorin or semaphorin receptor genes is the use of small interfering RNAs (siRNA). siRNA allows sequence specific gene silencing by targeted degradation of mRNA (reviewed by Genc et al., 2004). siRNA-mediated knockdown of the expression of Npn-1 in dorsal root ganglia of chick embryos results in the abolition of Sema3A-induced growth cone collapse and DRG axons that prematurely enter the dorsal horn (Bron et al., 2004). In combination with adeno-associated or lentiviral vector technology, siRNA can be used to locally suppress the expression of multiple genes (Grimm et al., 2005, Li and Rossi, 2005). This would overcome the developmental lethality observed in many semaphorin/semaphorin receptor null mutants and would allow the simultaneous knockdown of e.g. Npn-1 and Npn-2 in mature, injured spinal neurons.

Recently two ways to functionally block Sema3A in vivo have been reported in the optic and in the olfactory system. Following optic nerve axotomy Sema3A expression is upregulated in the retina peaking after 2 to 3 days. Sema3A induces apoptosis of retinal ganglion cells in culture (Shirvan et al., 2000). A marked inhibition of retinal ganglion cell death was observed after intravitreous infusion of a function blocking antibody into rat eyes prior to optic nerve injury (Shirvan et al., 2002). In the primary olfactory system axotomy leads to the induction of Sema3A in fibroblast-like cells at the injury site (Pasterkamp et al., 1998a). Although newly formed primary olfactory neurons regenerate successfully through an axotomy lesion placed caudally from the cribiform plate, application of a Sema3A blocking agent stimulated the regrowth of primary olfactory axons into the olfactory bulb (Kikuchi et al., 2003). The Sema3A blocking agent (SM-216289 or xanthofulvin) is isolated from Penicillum and inhibits the interaction of Sema3A with Npn-1 in vitro. Whether this compound binds to other semaphorins is not clear (Kikuchi et al., 2003). It would be very interesting to examine the effect of function blocking antibodies and xantofulvin on regeneration of the injured spinal cord neurons.

**Ephrins and Eph receptors after CNS injury**

Ephrins are membrane-tethered repellent guidance molecules that bind to Eph receptors, a large family of tyrosine kinase receptors. The receptors are divided into an A-subclass (EphA1-A8) and a B-subclass (EphB1-B6). A-type receptors typically bind to glycosylphosphatidylinositol (GPI)-linked A-type ligands (ephrinsA1-A5), whereas B-type receptors bind to transmembrane B-type ligands (ephrinsB1-B3). However receptor-ligand interactions between subclasses also occur (Kullander and Klein, 2002, Himanen et al., 2004). The Eph/ephrin system plays crucial and versatile roles in many tissues of the developing...
and adult organism, including the nervous system. It is implicated in boundary formation in developing somites and hindbrain rhombomeres, in cell migration and vasculogenesis, in axonal pathfinding and topographic mapping, in the regulation of structural and functional properties of synapses and the control of dendritic spine morphology (Klein, 2004, Yamaguchi and Pasquale, 2004).

An exquisite characteristic of the Eph/ephrin system is its ability to elicit bi-directional signaling, that is classical ligand-induced forward signaling by the Eph receptor via its intrinsic kinase activity and ‘receptor’-induced reverse signaling by the membrane-bound ephrin ligand via recruitment of intracellular signaling molecules. Such reverse signaling mechanisms have been described both for transmembrane B-class ephrins as well as for GPI-linked A-class ephrins (Murai and Pasquale, 2003). This characteristic together with the promiscuity of ligand/receptor interactions make it a daunting task to define the exact role of each molecule and ligand/receptor pair. Nevertheless with the generation of genetically modified mice of individual family members including single and double knockouts as well as truncation mutants, their specific roles in particular processes, including neuroregeneration, are being elucidated.

Expression of Eph/ephrin molecules after spinal cord injury

Increased expression of several members of the Eph receptor family is observed at 7 days after thoracic spinal cord injury. In particular a strong upregulation of EphB3 mRNA and protein is seen in reactive astrocytes in the lesion epicentre and in the rostral spinal cord white matter (Miranda et al., 1999, Willson et al., 2003). Similarly members of the EphA family of receptors (EphA3, A4, A6, A7, A8) are induced in astrocytes at the lesion centre and in astrocytes and oligodendrocytes in the ventral white matter, although their is some discrepancy in mRNA and protein levels (Willson et al., 2002). This increased expression of EphA and EphB3 in astrocytes may indicate a repellent effect on regenerating axons through reverse signaling. Alternatively Eph receptors may be involved in reactive astrogliosis and scar formation.

A role for the Eph/ephrin system in scar formation?

The establishment of tissue boundaries is a well known function of Eph/ephrin molecules in the developing embryo. A similar role is now being proposed in the neural scar. ephrinB2 and EphB2 are constitutively expressed at low level in adult spinal cord astrocytes and meningeal fibroblasts, respectively (Bundesen et al., 2003). This expression is strongly induced after complete spinal cord transection over a period of 14 days. EphrinB2 protein levels increase to about 80% over control at 7, 10 and 14 days post-injury, while its phosphorylation state peaks at about 440% at 3 days post-injury. In parallel, EphB2 protein levels increase dramatically from 7 days onwards and its phosphorylation state reaches a maximum of 1000% already at 3 days post-injury. This dual phosphorylation of ligand and receptor suggests a bi-directional signaling event.
Immunohistochemical analysis showed that ephrinB2 is confined to reactive astrocytes and EphB2 is present in meningeal fibroblasts invading the lesion site form the meninges (Bundesen et al., 2003). The expression is highest during the early period of astrocyte-meningeal fibroblast intermingling at the lesion site and decreases when a clear boundary is formed between the two cell types. Although this study supports a role for ephrinB2/EphB2 in astrocyte-meningeal cell interaction and compartmentalization of the scar, functional data to support this is presently lacking. The initial intermingling and subsequent segregation of astrocytes and meningeal fibroblasts observed during maturation of the glial-fibrotic scar can be recapitulated in a co-culture model leading to clearly separated patches of the two cell types (Abnet et al., 1991, Shearer et al., 2003). It would be interesting to use this model in combination with inactivation or elimination of ephrinB2 in astrocytes or EphB2 in meningeal fibroblasts or both, to reveal a functional involvement of these molecules in boundary formation in the CNS scar.

**Enhanced regenerative response in EphA4 deficient mice**

Strong evidence for a role of Eph/ephrin signaling in scar formation and axonal regeneration comes from a recent study in EphA4 deficient mice (Goldshmit et al., 2004). Thoracic spinal cord hemisection performed in these mice led to significant functional recovery and abundant/impressive long-distance regeneration of long spinal motor pathways. Although spinal hemisection in mice is characterized by a large extent of spontaneous recovery, EphA4-deficient mice also regained functionality in two behavioural tests (ability to walk and climb on a grid and ability to grasp with the affected hindpaw) that did not improve in wildtype mice at 3 months post-injury. Histological analysis revealed that numerous axons had crossed the lesion site, including corticospinal and rubrospinal tract axons. The most remarkable finding however was that the glial scar was severely disturbed in the EphA4 knockout mice. The astrocytic reaction to spinal injury was virtually absent and thus glial scar formation was drastically reduced (Goldshmit et al., 2004).

These data provide additional evidence for the contention that the scar is a major barrier to regenerating axons. However several mechanisms appear to cooperate in the improved regeneration in EphA4 null mice. First, EphA4 is normally expressed in astrocytes and is upregulated upon injury and cultured neurons grew 2-3 fold longer neurites on EphA4 deficient astrocytes, indicating that EphA4 acts via reverse signaling as a scar-derived inhibitory factor on regenerating axons. Second, EphA4-deficient neurons grew even better than wildtype neurons suggesting the presence of an additional molecule(s) in reactive astrocytes that induces an inhibitory forward signal in EphA4 bearing axons. A possible ligand on (reactive) astrocytes may be ephrinB2 which is known to interact with EphA4 (Klein, 2001). Although the authors doubt the involvement of the neuronal EphA4 pathway in the injured spinal axons due to limited axonal
expression in adult neurons, it is important to note that EphA4 null mice show severe defects in the projection of corticospinal axons due to loss of sensitivity towards the midline repellent ephrinB3 during development (Kullander et al., 2001, Yokoyama et al., 2001). It remains possible that in wildtype animals EphA4 is re-expressed at the regenerating growth cone, thereby rendering injured axons sensitive to scar-derived ephrinB2/B3 ligands.

Third, in addition to these direct effects on axonal growth via EphA4 on astrocytes or on the axon itself, the main regenerative effect observed in the knockout mouse is likely to be indirect and attributable to the lack of astrocytic gliosis and limited scar formation. The size of the scar is drastically reduced in these animals as judged by GFAP and CSPG staining, suggesting that apart from EphA4 several other major growth-inhibiting molecules are missing in this scar. It will be important to examine the fate of other cellular and molecular components in this perturbed scar, in particular oligoendrocyte precursor cells, meningeal fibroblasts and their associated inhibitors.

A possible explanation for the lack of gliosis is given by the observation that EphA4-deficient astrocytes do not respond to inflammatory cytokines such as interferon γ and leukemia inhibitory factor by hypertrophy and proliferation. Moreover these cells fail to repair a scratch wound in vitro. This suggests that induction of injury-related gene expression (upregulation of GFAP), proliferation and migration are impaired in EphA4-deficient astrocytes thus leading to limited scar formation. Noteworthy in this context is another model of scar reduction, where inhibiting the assembly of the collagenIV network, leads to significant axon growth through the lesion core in the injured fimbria fornix (Stichel et al., 1999a) (reviewed by Klapka and Muller, 2006). These data strongly suggest that controlled reduction of scar tissue is a very promising approach to achieve long-distance regeneration in the injured spinal cord. The timing and methodology used to limit scar formation is crucial however, since the complete ablation of reactive astrocytes has detrimental effects (Faulkner et al., 2004).

Finally it is important to note that EphA4-null mice show several neurological and neuroanatomical deficits that need to be taken into account in the interpretation of the response of these mice to spinal injury. Indeed EphA4-deficient mice display an unusual gait movement characterized by synchronous movement of the hindlimbs, a phenotype which can be largely explained by defects in the central pattern generator (Kullander et al., 2003). Moreover, along the whole length of the spinal cord many corticospinal axons project aberrantly across the midline, terminating ipsilateral to their cells of origin (Coonan et al., 2001). In addition EphA4 deficient mice frequently have no anterior commissure (Dottori et al., 1998, Helmbacher et al., 2000) and at least in one strain the peroneal nerve is missing leading to peroneal muscular atrophy and abnormal hindlimb position (club-foot) (Helmbacher et al., 2000). It is important to keep these data in mind when interpreting the improved regenerative response observed in spinal cord injured EphA4 deficient mice. In particular the involvement of un-injured
ipsilateral corticospinal fibers in the functional recovery of these hemisected mice needs careful examination.

**Eph/ephrin-mediated pathfinding in the injured optic nerve**

EphA expression in retinal ganglion cells and ephrinA2/A5 expression in retinal projection areas (tectum in chick and fish; superior colliculus in rodents) are essential for the establishment of retino-tectal topography during development. Several groups have investigated the distribution of Eph/ephrin proteins during optic nerve regeneration in adult animals, based on the idea that axon guidance information is a prerequisite for appropriate target finding by regenerating axons. In goldfish, where continued retinal and tectal neurogenesis and axon elongation occur, gradients of Eph/ephrin proteins remain present during adulthood. One month after optic nerve crush ephrinA2 is strongly increased in the tectum (Rodger et al., 2000, King et al., 2004), while EphA3 and EphA5 receptors are transiently upregulated in the retina (King et al., 2003). Blocking ephrinA signaling in the goldfish tectum by intracranial injection of recombinant EphA receptor or phosphoinositol-phospholipase C (to remove GPI-linked proteins) following optic nerve crush leads to multiple aberrant tectal projections as revealed by electrophysiological and immunohistochemical methods (Rodger et al., 2004). Thus at least in goldfish EphA/ephrinA interactions appear to be required for the restoration of retino-tectal topography during optic nerve regeneration. Interestingly in the adult mouse graded ephrinA expression in the superior colliculus is also similar to that found during development. In contrast to fish however, ephrinA expression in the colliculus does not change upon optic nerve transection and retinal EphA expression is reduced (Knoll et al., 2001).

**Slits in the injured CNS**

The slit family of repellent axon guidance molecules is composed of 3 members (slit 1-3). These are large secreted extracellular matrix proteins that repel axons from retinal ganglion cells (Erskine et al., 2000, Niclou et al., 2000), olfactory bulb (Li et al., 1999), spinal cord, forebrain and hippocampus (Nguyen Ba-Charvet et al., 1999, Patel et al., 2001). In addition slit proteins regulate cell migration in neuronal and non-neuronal tissues (reviewed by Wong et al., 2002). In vertebrates two transmembrane proteins termed roundabout (robo1 and robo2) function as receptors for slit-mediated chemorepulsion and, similar to semaphorins and ephrins, receptor activation leads to remodeling of the actin cytoskeleton via Rho GTPases (reviewed by Huber et al., 2003). Slit2 also binds the heparan sulphate proteoglycan glypican-1 with high affinity (Liang et al., 1999, Ronca et al., 2001).
Slit expression in the adult intact and injured brain

Slit proteins show dynamic spatiotemporal expression patterns in the developing nervous system and are also broadly expressed in the adult brain (Marillat et al., 2002). In many brain areas their expression is upregulated postnatally and is largely neuronal. The expression pattern of robo1 and robo2 is also broad and largely similar in the embryonic and the adult brain (Marillat et al., 2002). Little is known about the response of slit-robo proteins to CNS injury. 7 days after cryo-injury of the brain, Slit1-3 and glypican-1 were detected in a subpopulation of reactive astrocytes surrounding the necrotic tissue (Hagino et al., 2003). Expression of slits in injured spinal cord has not been reported.

Future directions

Taken together the data summarized here indicate that repellent axon guidance molecules are not only involved in developmental processes, but continue to be expressed in the adult and injured nervous system, where they are present at the right time and the right place to limit axonal regeneration (Fig.1). In addition to a direct inhibitory effect on axon growth, some of them also regulate the process of astrogliosis and the formation of the glial-fibrotic scar.

A key issue for future studies lies in the exact localization of repulsive proteins in relationship to regenerating axons. It should be noted that due to the lack of reliable antibodies the expression of secreted semaphorins has largely relied on mRNA detection. Direct protein localization using immunohistochemistry will be necessary. This is particularly important in order to determine the interaction of repulsive molecules with other inhibitory extracellular matrix proteins (e.g. CSPGs) and the effect of such interactions on regenerating axons.

Finally, the demonstration of a causal relationship between the expression of repulsive guidance cues and the failure of axon regeneration has just begun (Goldshmit et al., 2004). Determining this relationship will be the primary focus in future studies on secreted and transmembrane semaphorins. The application of neutralization strategies (conditional knockout mice, viral vector-directed siRNA expression, chemical inhibitors) to interfere with receptor or ligand activity in the injured spinal cord will be of great importance to resolve this.