Inhibitors of spinal cord regeneration
strategies to determine the contribution of class-3
semaphorins to the inhibitory properties of the glial scar
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Inhibitors of spinal cord regeneration: strategies to determine the contribution of class-3 semaphorins to the inhibitory properties of the glial scar

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Scope and outline

Erich M.E. Ehlert
Traumatic injury to the spinal cord often results in permanent loss of motor and sensory function. The extent of these functional impairments depends on the spinal level and severity of the injury. The inability of the central nervous system (CNS) to repair itself is due to the moderate intrinsic regenerative response of injured CNS neurons, limited neurotrophic support and an outgrowth inhibitory environment present at the injury site. Also the simple fact that injured axons have to re-find their proper target cells, cells that are often centimeters away from the site of the injury, is an important factor that limits successful regeneration. In the developing nervous system axons are effectively guided to their target cells by a myriad of axon guidance molecules. After a lesion in the adult CNS, however, some guidance cues are re-expressed at sites where they do no longer support but apparently inhibit axon regrowth.

In chapter 1 we give an overview of our current understanding of the role of repulsive axon guidance factors in nervous system regeneration. We discuss four possible roles. First, repellent axon guidance cues may contribute to the inhibitory properties of the oligodendrocyte-lineage cells, acting in concert with the classical myelin-associated inhibitors of axonal outgrowth. Second, repellent axon guidance cues expressed by the newly formed neural scar may form a molecular barrier for injured axons. Third, repellent axon guidance cues could be involved in modulation of the cellular response to an injury, e.g. at or around the injury site axon guidance molecules may have a role in neural scar and blood vessel formation. Finally, proper spatial re-expression of axon guidance molecules may facilitate proper target finding for outgrowing injured axons, e.g. in the peripheral nervous system.

Among the axonal guidance cues that are re-expressed after a lesion are members of the class-3 semaphorin protein family. Semaphorins were initially discovered as a family of axon guidance molecules that act as repulsive guidance proteins during development. Upon injury to the CNS, class 3 semaphorins are re-expressed by the meningeal fibroblasts present in the core of the neural scar.

The aim of the work described in this thesis was to develop methods to interfere with the chemorepulsive activity of secreted class 3 semaphorins to investigate their contribution to the inhibitory nature of the spinal cord injury site. We first studied the chemorepulsive activity of semaphorin 3A derived from cultured primary meningeal fibroblast. Subsequently, we initiated two different approaches to interfere with semaphorin signalling in vivo. First we explored the use of adeno-associated viral vector-mediated expression of shRNAs as a technique to knock down expression of the semaphorin receptors neuropilin-1 (Npn-1) and neuropilin-2 (Npn-2) in vivo. Second, we used Npn-1 and Npn-2 (conditional) knockout mice to analyze axonal regeneration and motor function following spinal cord injury (Fig. 1).
In **chapter 2** we first focussed on the meningeal fibroblast that form the core of the scar that is formed after penetrating injury to the spinal cord. We demonstrate that cultured meningeal cells express a repertoire of class 3 semaphorins similar to that previously reported in the in vivo situation. By using meningeal cell cultures from semaphorin 3A (Sema3A) knockout mice, we were able to show that Sema3a derived from meningeal cell membrane protein extracts is a potent growth cone-collapse factor for embryonal DRG growth cones. Studies with meningeal cells from Sema3A knockout mice also demonstrated that Sema3A contributes to the growth inhibitory properties of cultured meningeal cells. These data, together with the upregulation of sema3A in the fibroblast of the neural scar, suggest that Sema3A could be a potent scar-associated inhibitor of axonal regeneration in vivo and that removal of semaphorin signalling will result in improved regeneration (Fig. 1).

Subsequently we set out to develop methods to interfere with Sema-signalling in vivo. Adeno-associated virus (AAV) is a naturally occurring replication deficient single stranded DNA virus. AAV-based vectors are becoming increasingly popular as tools to direct foreign gene expression in many types of neurons due to their safety profile, stability in vivo and relatively easy production. In **chapter 3**, we first compared AAV vectors based on seven adeno-associated virus (AAV) serotypes (this are naturally occurring AAV variants) and lentivirus for their efficiency of transduction of adult dorsal root ganglion (DRG) neurons in vivo. AAV5 was the most effective serotype overall, followed by AAV1. With AAV5 we obtained transduction rates of up to 90%. After having established an effective technique to transduce DRG-neurons in vivo, we proceeded to evaluate the application of viral vector-mediated shRNA expression in vivo.

**Chapter 4** describes our efforts to develop shRNA expressing viral particles with the objective to knock down the expression of the semaphorin receptors, Npn-1 and Npn-2, in cultured neurons and subsequently in vivo. Although one of the shRNA sequences was successful in knocking down Npn-2 in DRG neurons, we unexpectedly observed severe toxicity when shRNAs were expressed in neurons of the red nucleus. As reported by others, shRNA induced toxicity is caused by saturation of the endogenous miRNA machinery. This saturation interferes with
normal miRNA processing and results in adverse effects in the shRNA over-expressing cells. Currently there are at least 8 publications that report problems with the in vivo expression of shRNA via AAV vectors (see also discussion in Chapter 7). We therefore conclude that, although this technology would have great potential to interfere with multiple inhibitory signaling pathways, the results in this chapter illustrate unanticipated problems with the application of shRNAs in vivo.

In **chapter 5** we examine the role of Npn-1 – Sema3A signalling in a corticospinal tract lesion by using conditional Npn-1 knock out mice. Since mice with a deletion of the Npn-1 gene are not viable, Npn-1 was conditionally mutated in postnatal neurons by crossbreeding animals with a floxed Npn-1 allele with mice that expressed the recombinase Cre via a neurofilament promoter. We evaluated motor function during recovery after spinal cord injury and analysed the distance corticospinal axons could advance towards the lesion site. We show, that following CST lesion, neuron-specific deletion of Npn-1 does not improve the outgrowth of CST axons. In addition, we do not observe enhanced recovery of motor function in lesioned conditional Npn-1 KO mice.

Corticospinal neurons express both Npn-1 and Npn-2, which together account for all scar-derived class 3 semaphorin (except Sema3A) signalling. Just knocking out Npn-1 only eliminates the Sema3A from the inhibitory milieu leaving all other inhibitory class 3 semaphorin signalling intact. In **chapter 6** we abolish all class 3 semaphorin signalling by making use of the Npn-2 constitutive knockout mice. In wild type animals, neurons of the red nucleus only express Npn-2. Consequently, in Npn-2 KO animals none of the scar derived class 3 semaphorins can have a direct effect on regenerating rubrospinal axons. Analogous to the Npn-1 KO mouse, we analyse motor function and performed fiber tract histology following injury to the rubrospinal tract. We did not observe enhanced regeneration of injured RST fibers or improved recovery in Npn-2 deficient mice.

In **chapter 7** we summarise the contents of this thesis and we discuss our efforts in neutralizing semaphorin signalling in animal models for spinal cord injury. First, we discuss the use of adeno associated viral vectors to deliver shRNAs to the CNS. As described in chapter 4, we found that over-expression of shRNAs in the red nucleus causes cellular toxicity. We discuss the mechanisms that underlie this toxicity and provide the reader with recommendations for experimental design to adequately detect these adverse effects. The problems with shRNA-induced toxic effects have to be resolved before this technique can be used routinely in neurobiological studies. Therefore, we review recent developments in gene silencing techniques, and discuss new strategies to safely knock down gene expression in vivo. Second, we discuss the role of semaphorins in spinal cord injury. The main result of our studies is that, in the models we used,
(conditional) knockout of semaphorin receptors Npn-1 or Npn-2 does not enhance regeneration of injured fibers and does not improve recovery of motor function. These findings are in line with a recent similar study that also used a genetic approach to interfere with semaphorin signalling. Interestingly, pharmacological intervention with semaphorin signalling has recently been shown to be effective in improving axonal regeneration and enhanced recovery of motor function. In chapter 7 we provide possible explanations that may account for the different outcomes of these genetic and pharmacological approaches to interfere with semaphorin signalling.

We propose that, within the biological context where a multitude of other growth-inhibitory molecules are still present and active, interfering with semaphorin signalling alone, has no impact on the recovery after spinal cord injury. Furthermore it is important to realize that there are multiple reasons for the failure of the CNS to regenerate after injury. Apart from inhibitory molecules in myelin and in the neural scar, the limited intrinsic capability of injured neurons to regenerate and the lack of growth supportive molecules contribute to the poor regenerative response. An approach that combines stimulation of the intrinsic growth-response of neurons and provides a permissive environment for regenerating axons, is the next complex but logical step towards improved functional recovery after CNS injury.
General introduction

Chemorepellent axon guidance molecules in spinal cord injury

Simone P. Niclou, Erich M.E. Ehlert and Joost Verhaagen

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 and plexins. 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, Skaliora 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 colocalized with markers of fibroblasts (fibronectin and vimentin) and do not colocalize 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).
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**

Transsection 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, tenascin-C and myelin-derived inhibitors (Pasterkamp and Verhaagen, 2001). CSPG and tenascin-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 tenascin-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 tenascin-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 cribriform 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 xanthofulvin 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 from 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.
Meningeal cell-derived semaphorin 3A inhibits neurite outgrowth

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MENINGEAL CELL-DERIVED SEMAPHORIN 3A

Abstract

The neural scar that forms after injury to the mammalian central nervous system is a barrier to sprouting and regenerating axons. In addition to reactive astrocytes that are present throughout the lesion site, leptomeningeal fibroblasts invade the lesion core. When isolated in vitro, these cells form a very poor substrate for growing neurites, even more so than reactive astrocytes. Nevertheless the molecular mechanisms involved in this growth inhibition are not well understood. Semaphorins have been reported to be upregulated in meningeal cells (MCs) on mechanical injury to the brain and spinal cord. In the present study, we show that Sema3A mRNA and active protein are produced by cultured meningeal cells. A protein extract from these cells induces the collapse of embryonic dorsal root ganglion (DRG) growth cones. This collapsing activity is partially blocked by neuropilin-1 antibodies and is absent in meningeal cells derived from Sema3A-knockout mice. In addition to growth cone collapse, recombinant Sema3A but not Sema3C inhibits neurite outgrowth of embryonic DRGs. Consistent with this result we find that the inhibitory effect of meningeal cells on neurite outgrowth is partially overcome on Sema3A-deficient MCs. Furthermore we show that the inhibitory effect of MC-derived Sema3A on neurite outgrowth is modulated by nerve growth factor. Our results show that Sema3A, a chemorepellent during nervous system development, is a major neurite growth-inhibitory molecule in meningeal fibroblasts and is therefore likely to contribute to the inhibitory properties of the neural scar.

Introduction

Regenerating neurons are unable to cross the glial/fibrotic scar that forms after penetrating injuries to the adult mammalian central nervous system (CNS), yet the molecular and cellular culprits responsible for this potent inhibition of axon growth are largely unknown. Although potent inhibitory proteins such as NogoA, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) are released from injured myelin (Spencer et al., 2003), these are largely excluded from the scar proper (Wang et al., 2002b) and therefore unlikely to contribute significantly to the barrier imposed by the scar.

The scar is made up primarily of reactive astrocytes characterized by enhanced glial fibrillary acidic protein (GFAP) expression and a variable number of other cell types including microglia/macrophages, oligodendrocyte precursors, and leptomeningeal fibroblasts. The latter migrate in from injured meninges and disrupted blood vessels to form the fibrotic scar (Ross, 1968, Berry et al., 1983, Carbonell and Boya, 1988). At the interface between glial and meningeal cells, an extensive basal lamina is deposited and astrocytes undergo morphological changes leading to the formation of a new glia limitans (Shearer and Fawcett, 2001). This process, which seals off the CNS tissue from the non-CNS...
environment, is thought to require the presence of leptomeningeal cells (Abnet et al., 1991, Sievers et al., 1994).

In vitro, astrocytes are normally considered to be a permissive substrate for neurite growth similar to Schwann cell cultures (Noble et al., 1984) and astrocyte conditioned medium is often used for the maintenance of cultured neurons. Although their role in regeneration is debated, astrocytes also display growth-inhibitory properties depending on culture type, time in culture, and source and age of the astrocyte (reviewed in Reier et al., 1989, Fawcett and Asher, 1999). Based on a large number of studies it appears that the main class of inhibitory molecules produced by astrocytes are chondroitin sulfate proteoglycans (CSPGs). These molecules are upregulated around CNS injuries and many of them have been shown to inhibit axon growth in vitro (reviewed in Asher et al., 2001). In vivo the treatment of the lesion site with chondroitinase ABC, which removes the glycosaminoglycan chain from CSPGs, allows some axon regeneration through the glial scar in brain and spinal cord injuries (Moon et al., 2001, Bradbury et al., 2002).

Meningeal cells (MCs) are clearly an unfavorable substrate for neurons in vitro (Noble et al., 1984, Hirsch and Bahr, 1999), but surprisingly little is known about the inhibitory molecules involved. The inhibition of neurite growth appears to result from cell surface interactions between neuronal and meningeal cells, as the effect was not apparent when neurons were cultured in conditioned medium of MCs (Noble et al., 1984). When neurons are seeded on a mixed substrate of astrocytes and MCs, neurite outgrowth decreases dramatically with increased numbers of fibroblasts, an effect that was attributed to the reduced availability of the permissive substrate (Rudge and Silver, 1990). MCs were also shown to negatively affect the growth-promoting characteristics of cultured astrocytes (Ness and David, 1997). In this case, the effect appears to be due to a soluble factor, as the changes in astrocyte morphology and growth promotion were also observed with MC-conditioned medium. MCs express keratan sulfates, versican, NG2, and possibly other proteoglycans but their contribution to the nonpermissiveness of MCs is poorly understood (Hirsch and Bahr, 1999, Asher et al., 2002, Morgenstern et al., 2002).

We have previously reported that MCs at the lesion site express the potent chemorepellent semaphorin 3A (Sema3A) in several models of brain and spinal cord injury (Pasterkamp et al., 1998a, a). Other members of the class 3 semaphorins including Sema3B, Sema3C, Sema3F, and Sema3E transcripts are also upregulated in MCs in the neural scar (De Winter et al., 2002b). Neuropilins and plexinA proteins, which form a receptor complex for secreted semaphorins, are expressed in injured and noninjured neurons in the adult brain (Reza et al., 1999, Gavazzi et al., 2000, Murakami et al., 2001, De Winter et al., 2002b). Of note is that in neonatal rats, where axon regeneration takes place, infiltration of meningeal fibroblasts into the scar does not occur (Berry et al., 1983) and Sema3A expression is detected only in the intact meninges (Pasterkamp et al.,
Thus Sema3A and other class 3 semaphorins are prominent candidates to mediate growth inhibition by MCs in the glial/fibrotic scar.

To investigate this possibility, we used cultured MCs and showed that a protein extract from MC membranes induces the collapse of dorsal root ganglion (DRG) growth cones. This collapsing activity is inhibited by neuropilin-1 (Npn-1) antibodies and is absent in MCs from Sema3A-knockout mice. We further show that MCs are an inhibitory substrate for growing axons and that their inhibitory effect is partially overcome on Sema3A-deficient MCs. Finally we show that the inhibitory effect of MC-derived Sema3A on neurite outgrowth is modulated by nerve growth factor (NGF).

**Results**

**Characterization of cultured meningeal cells**

*Immunocytochemical markers expressed by cultured meningeal cells*

MCs prepared from the leptomeninges of neonatal rat pups were used experimentally between passages 2 and 5. The cells were characterized by immunocytochemical staining for several marker proteins. As expected meningeal fibroblasts strongly express the extracellular matrix (ECM) molecule fibronectin and the cytoskeletal protein vimentin (Fig. 1A). They also stain positive with the CS-56 antibody recognizing chondroitin sulfates, although the staining intensity was variable from one cell to the other. In agreement with earlier studies (Avnur and Geiger, 1984), the CS-56 staining pattern was patchy, possibly representing agglomerates of CSPGs and other proteins (Fig. 1A). A novel marker for meningeal fibroblasts is the enzyme retinaldehyde dehydrogenase type 2 (Raldh2), an enzyme involved in the synthesis of retinoic acid (Zhao et al., 1996). This enzyme is widely expressed in the developing brain and spinal cord (Berggren et al., 1999), but is limited to the leptomeningeal sheet in the adult brain (J. Fawcett, personal communication). As shown in Fig. 1A, cultured MCs are also immunopositive for Raldh2. No staining for glial fibrillary acidic protein (GFAP) was detected in these cells (not shown). These data indicate that the MCs in culture are a pure cell population and express a very similar profile of marker proteins as described in vivo in the normal brain and the injured brain (Hirsch and Bahr, 1999, Pasterkamp et al., 1999a).

*Semaphorin expression in cultured meningeal cells*

The lack of appropriate antibodies to Sema3A precludes a direct detection of the protein. We therefore investigated Sema3A expression in cultured MCs using in situ hybridization and reverse transcription (RT)-PCR. The Sema3A riboprobe gives a relatively weak signal with in situ hybridization, while a strong signal
is obtained with the Sema3C probe (Fig. 1B). No in situ hybridization signal is seen with control sense probes (not shown). A Sema3A transcript is readily detected in MCs by RT-PCR (Fig. 1C). Again as seen with in situ hybridization, Sema3C expression is substantially higher compared with Sema3A (Fig. 1C). This is consistent with the in vivo observations after CNS injury, where MC-derived Sema3C expression is stronger than Sema3A expression (De Winter et al., 2002b). Expression of Sema3B, 3E, and 3F was detected at a very low level in cultured MCs using real-time quantitative PCR (not shown). Taken together, the data indicate that cultured MCs express a similar repertoire of semaphorins as they do in vivo and thus provide an adequate model to study the contribution of semaphorins to the inhibitory properties of these cells.

**Meningeal cells produce functional Sema3A protein**

Although Sema3A is a secreted protein, we did not detect significant collapsing activity in MC-conditioned medium on E15 rat DRGs. Since collapsin-1 (chicken Sema3A) was originally purified from brain membranes (Luo et al., 1993), we turned to a protein extract prepared from MC membranes and tested it for Sema3A collapsing activity. As shown in Fig. 2A, extracts from cultured rat MCs cause growth cone collapse in DRG neurons in a dose-dependent manner. Protein extracts from adult MCs contained a similar collapsing activity (not shown);
however, since these cells are more difficult to grow, the remaining experiments were carried out with MCs from newborn pups. To investigate whether the collapsing activity was due to Sema3A, we applied blocking antibodies against the Sema3A receptor component neuropilin-1 (Npn-1) concomitant with the protein extract. The collapsing activity is partially blocked on addition of anti-Npn-1 antibodies (Fig. 2B), indicating that at least part of the collapsing activity requires a functional Npn-1 receptor. The blocking effect of Npn-1 antibodies is dose-dependent (Fig. 2B); however, due to limited amounts of Npn-1 antibody available, 200 μg per well (400 μg/ml) was the highest dose tested, and did not result in full inhibition of growth cone collapse. Although the trend of the dose–response curve suggests that the blockade may be complete with a high enough antibody concentration or with earlier administration of the antibody, we cannot exclude that additional chemorepellents independent of Npn-1 receptor contribute to the collapsing activity detected in MCs.
To analyze this question further, we turned to MC cultures prepared from Sema3A-deficient MCs, derived from neonatal mouse pups, that were homozygous for a targeted mutation in the Sema3A gene (Taniguchi et al., 1997). MC extracts from wild-type littermates had a collapsing activity similar to that of extracts from rat MCs (compare Figs. 2A and C), while no collapsing activity could be detected in MC extracts prepared from Sema3A-knockout (KO) pups (Fig. 2C). Interestingly, the collapsing effect was completely abolished even after administering a high protein concentration, indicating that Sema3A is the sole responsible MC-derived collapsing agent measured in this assay. Sema3E has also been reported to collapse embryonic DRGs (Miyazaki et al., 1999a, Miyazaki et al., 1999b); however, our results suggest that its expression in MCs is not sufficient to contribute to the collapsing activity detected in these cells. It is noteworthy in this context that E15 DRGs are not responsive to Sema3C (Koppel et al., 1997, Chen et al., 1998) (see Fig. 4 below), presumably because at this stage of development they do not express detectable amounts of Npn-2 receptor (Chen et al., 1997, Kolodkin et al., 1997). Taken together, our results demonstrate that wildtype MCs produce functional Sema3A protein that is able to negatively affect growth cone behavior.

Comparison of neurite growth on a laminin, a meningeal cell, and an astrocyte substrate

It has long been established that MCs are an unfavorable substrate for growing neurites in culture, while astrocytes are permissive for such growth (Noble et al., 1984, Rudge and Silver, 1990). Yet the reason for this nonpermissive nature of MCs is poorly understood. Here we address the question of (i) how inhibitory an MC substrate is compared with more permissive substrates and (ii) whether Sema3A is involved in this neurite growth inhibition. To this end DRG explants were grown for 24 h on either laminin-coated glass coverslips or a monolayer of rat MCs or astrocytes.

Inhibition of neurite outgrowth on a meningeal cell substrate

As expected, neurite outgrowth was drastically reduced on a MC substrate compared with either laminin or astrocytes (Fig. 3A). Typically neurites on laminin grew out regularly, forming a thin homogenous sheet of individual neurites. Conversely, outgrowth on MCs was very sparse and much more variable, with neurites extending from the explant in an irregular pattern and often in thick fascicles. Fasciculation is often thought to be the result of a nonpermissive environment where neurites prefer to grow on each other rather than on the substrate (Kapfhammer et al., 1986). Moreover the number of neurites that grew out on MCs and the average neurite length were dramatically reduced. Interestingly, the morphology of neurites on an astrocyte monolayer was also significantly different from that of either laminin or MCs. Although the density
of neurites was high on astrocytes, they were also much fasciculated and the longest neurites never reached the length obtained on laminin. For quantification of neurite growth the surface area outside the ganglion that was covered with neurites was determined. This showed that neurite growth on a MC substrate was 32.9% of the growth obtained on laminin (P < 0.001) and about half the growth observed on an astrocyte monolayer (P < 0.05) (Fig. 3B). Although the growth pattern on astrocytes compared with laminin was qualitatively different and measured to be 62.3% of control, this difference was statistically not significant (0.05 < P < 0.1).

Enhanced neurite growth on meningeal cells from Sema3A-knockout mice

Sema3A inhibits neurite outgrowth
To determine whether Sema3A added to the medium of cultured DRGs could affect their outgrowth pattern, DRG explants were grown on a laminin substrate in the presence of Sema3A-containing conditioned medium (Sema3A-CM). Conditioned medium of GFP-transfected cells (GFP-CM) was used as a control. As seen in Fig. 4A, Sema3A-CM reduces neurite outgrowth from DRG neurons to
about 40% of control. Although Sema3C does not induce growth cone collapse in E15 DRGs (Koppel et al., 1997) (and data not shown), it was not clear whether it could affect neurite outgrowth of these cells. Since we found that MCs express high levels of Sema3C (Fig. 1B), we tested its effect on neurite outgrowth by adding Sema3C-CM to cultured DRGs. As expected from the result on growth cone collapse, Sema3C did not reduce neurite outgrowth of E15 DRG neurons; if anything it tended to increase growth (134% of control, 0.05 < P < 0.1). These data show that Sema3A, besides being a chemorepellent that deviates axon growth, is a bonafide inhibitor of axon outgrowth of E15 DRG neurons and this outgrowth is not inhibited by Sema3C.

**Figure 4.** MC-derived Sema3A inhibits neurite outgrowth, an effect that is modulated by NGF. (A) E15 DRGs were grown on laminin-coated coverslips in the presence of conditioned medium of 293T cells transfected with GFP (GFP-CM), Sema3A (Se3A-CM), or Sema3C (Se3C-CM). Outgrowth in GFP-CM was set to 100% (y axis). In the presence of Sema3A, outgrowth was reduced to 46.5% of control GFP-CM (P < 0.05), while in the presence of Sema3C outgrowth was not significantly different from control (134% with 0.05 < P < 0.1). (B) E15 DRGs were grown for 24 h on a monolayer of MCs derived from wild-type (wt MC) or Sema3A-knockout mice (ko MC). Growth was in the presence of 20 ng/ml NGF. Neurofilament staining (2H3 antibody) was used to visualize neurites. (C) Quantification of neurite outgrowth on wt MC and Sema3A ko MC in the presence of either 20 or 0.2 ng/ml NGF. Outgrowth is expressed as a percentage of growth on LN control in 20 ng/ml NGF (set at 100%). Outgrowth on LN in 0.2 ng/ml NGF was about 60% of LN control (not shown). Note that in 20 ng/ml NGF a 1.6-fold increase in outgrowth is observed on Sema3A-ko MC (P < 0.05), while this increase amounts to 3.8-fold in 0.2 ng/ml NGF (P < 0.001).
Neurite outgrowth is enhanced on Sema3A-deficient MCs

The data presented so far show that MCs poorly support neurite growth and suggest that Sema3A contributes to their inhibitory properties. To further address this, we performed the neurite outgrowth assay on Sema3A-deficient MCs. As seen in Fig. 4B, outgrowth on wild-type mouse MCs was strongly inhibited; the outgrowth measured was even less than that obtained on rat MCs (13% on mouse MCs and 33% on rat MCs as compared with laminin) (compare Figs. 3B and 4C). Instead, neurite outgrowth improved significantly (21%) when DRGs were seeded on Sema3A-deficient MCs (Fig. 4C). The increase was measured to be 160% under regular assay conditions (1.6-fold increase; P < 0.05), demonstrating that Sema3A is a growth-inhibitory molecule produced by MCs.

The response to MC-derived Sema3A is modulated by NGF

A recent study reported that NGF treatment increases resistance of sensory growth cones to Sema3A-induced collapse (Dontchev and Letourneau, 2002). Since in our assays neurons were routinely cultured in 20 ng/ml NGF (corresponding to 10^{-9} M NGF in their study), we wondered whether changing the NGF concentration would also modulate the effect of MC-derived Sema3A on neurite outgrowth. When the assay was performed under 100-fold lower NGF concentration (0.2 ng/ml), outgrowth on laminin was reduced to about 60% of that seen under high NGF (not shown). Growth on wild-type mouse MCs was almost completely abolished in the low NGF condition (4.1% of laminin control; Fig. 4C). Interestingly, on Sema3A-deficient MCs, neurite growth increased to 15.6% of laminin control, meaning an increase of 380% compared with growth on wild-type MCs (3.8-fold increase, P < 0.001). Thus the effect of Sema3A on neurite growth is more potent in a low NGF concentration, which can be seen by the increased release of the blockade on neurite growth in the absence of Sema3A. This result supports the notion that Sema3A activity is regulated by neurotrophin signaling and shows that this regulation occurs not only in short term responses, i.e., collapse response, but also in a long-term process such as neurite outgrowth.

Discussion

Class 3 (secreted) semaphorins were found to be upregulated in meningeal fibroblasts in a neural scar formed after mechanical injury to brain and spinal cord (Pasterkamp et al., 1999a, De Winter et al., 2002b). Semaphorins and, in particular, Sema3A are potent chemorepellents for growing axons during nervous system development (Kolodkin, 1998, Raper, 2000); they are therefore likely candidates to mediate neurite growth inhibition in the neural scar. In the present study, we show that Sema3A mRNA and active protein are produced by cultured MCs. Sema3A derived from MCs induces growth cone collapse in DRG
We further show that Sema3A contributes to the inhibitory properties of MCs, since neurite outgrowth is increased on MCs that lack Sema3A expression. Furthermore, we find that the inhibitory effect of MC-associated Sema3A on axon growth is modulated by NGF.

**Meningeal cells express functional Sema3A**

Protein extracts from MCs contain a collapsing activity on DRG growth cones that is blocked by Npn-1 antibodies and is absent in Sema3A-deficient MCs. This demonstrates for the first time that Sema3A protein is present in MCs in sufficient amounts to functionally affect growth cone behavior. Interestingly, since no residual collapsing activity could be measured in Sema3A-KO MCs using E15 DRGs as an assay system, our results indicate that this activity can be attributed solely to Sema3A. This indicates that Sema3A is a major MC-derived chemorepellent for sensory growth cones. Nevertheless we cannot formally rule out that the improved outgrowth observed on Sema3A-deficient MCs is an indirect consequence of the loss of Sema3A expression. In such a scenario, Sema3A deficiency would induce changes in the gene expression profile of MCs including, e.g., the downregulation of other repellent guidance factors.

Taking into account that our assay only reveals the activity of proteins that have their receptors expressed on DRG neurons, other semaphorins like Sema3C and additional chemorepellents expressed in MCs may exert their inhibitory effect on different neuronal populations. In an accompanying study by Shearer et al. (Shearer et al., 2003), it was found that an antibody to Npn-2 facilitates axon crossing of an astrocyte/meningeal cell border, suggesting that an Npn-2 dependent semaphorin such as Sema3C or Sema3F contributes to the repulsive nature of MCs. In their study DRGs from neonatal rats were used, which, in contrast to E15 DRGs (Chen et al., 1997, Kolodkin et al., 1997), do express the Npn-2 receptor (F. De Winter and J. Verhaagen, personal communication). Thus the sensitivity of neurons to different semaphorins is determined by the receptor repertoire expressed on their growth cone. Since neuropilin and plexin expression is still present in intact and injured neurons in the adult (Giger et al., 1998a, Reza et al., 1999, Gavazzi et al., 2000, De Winter et al., 2002b) and adult sensory neurons have been shown to remain sensitive to Sema3A (Tanelian et al., 1997, Owesson et al., 2000), it is reasonable to assume that injured neurons are repelled by MC-derived semaphorins in the neural scar. The response to neural trauma has been reported to be different in mice and rats, the most prevalent differences being the absence of cyst formation and the presence of a dense connective tissue matrix at the lesion site in mice (Steward et al., 1999). Despite these anatomical differences, our data indicate that the cellular and molecular components of neurite growth inhibition addressed in this article do not differ considerably between mice and rat. The bioassays performed with meningeal cells from either rat or mice revealed a similar growth cone collapsing activity...
Menin Geal cell-derived Sema3A (Fig. 2A and 2C) and a similar inhibition of neurite outgrowth (Figs. 3 and 4) from these cells independent of the species used.

**Sema3A inhibits neurite outgrowth**

It is often suggested that chemorepellents are functional only when they are sensed in a gradient but not when presented at a constant concentration or over a longer period (Bagnard et al., 2000, Loschinger et al., 2000). Since axon extension is a long-term phenomenon, it could be speculated that the continued presence of a chemorepellent leads to adaptation of the growth cone and therefore does not affect growth per se. Our findings that neurite outgrowth is enhanced on a Sema3A-deficient MC substrate and is inhibited on addition of exogenous Sema3A provide evidence that a chemorepellent, rather than merely deviating growing axons, can indeed act as an inhibitor of axon growth. This observation may also better describe the function of chemorepellents in the adult CNS where semaphorins and their receptors are still widely expressed (see references above) and where they are thought to be involved in limiting sprouting and plasticity and in inhibiting axon regeneration, in the intact and injured brain, respectively (De Winter et al., 2002a, Holtmaat et al., 2002, Holtmaat et al., 2003).

**Sema3A is associated with the MC cell surface**

In this study Sema3A collapsing activity was present in a crude preparation of membrane proteins while we did not detect significant collapsing activity in MC-conditioned medium. This might be explained by low expression levels compared with a cell line manipulated to express recombinant Sema3A protein. Nevertheless our neurite outgrowth assay shows that the endogenous expression level of Sema3A in MCs is high enough to exert a potent inhibitory effect on primary neurons. In the latter condition, the axons are in direct contact with the cell membrane and ECM, where, if retained locally, even a low expression level may lead to a high protein concentration. Therefore our data suggest that, although secreted, the vast majority of Sema3A stays associated with the cell surface and/or ECM components. Further support of this contention comes from the work of Shearer et al. (Shearer et al., 2003) who show an effect of semaphorins on axon crossing that is limited to a sharp boundary between MCs and astrocytes. This implies that after injury Sema3A will probably not disperse throughout the scar or the surrounding intact tissue, but rather form a barrier to regenerating axons at its site of production. The observed abrupt stopping behavior of sprouting axons abutting the lesion core is consistent with this notion (Davies et al., 1999). Moreover, considerable axonal regeneration is obtained after chemical disruption of the collagenous basal lamina in the neural scar, suggesting that together with the collagen network associated inhibitory proteins are eliminated by this treatment (Stichel et al., 1999a, Stichel et al., 1999b, Hermanns et al., 2001).
**Modulation of Sema3A sensitivity by NGF**

When embryonic DRGs were grown under our standard culture conditions (20 ng/ml NGF), their neurites grew poorly on a monolayer of MCs (see Figs. 3B and 4C). This growth capacity on MCs is virtually zero when the NGF concentration is lowered to 0.2 ng/ml (Fig. 4C), while on a laminin substrate this low NGF concentration is sufficient to support neuronal survival and trigger significant neurite outgrowth (60% of control). On removal of Sema3A the neurite outgrowth potential is more strongly increased in low NGF (3.8-fold compared with 1.6-fold in high NGF) (Fig. 4C), indicating that NGF can partly overcome the inhibitory effect of Sema3A. In other words under conditions of diminished neurotrophin support the effect of growth inhibition is amplified. Our data are in line with previous reports showing that DRGs exposed to high NGF are more resistant to Sema3A-induced growth cone collapse (Tuttle and O’Leary, 1998, Dontchev and Letourneau, 2002) and extend the observation to include a long-term regulatory effect of neurotrophins on Sema3A in neurite outgrowth. This has important implications for regeneration studies in the adult CNS where the presence of growth factors is limited and thus the sensibility to inhibitory chemorepellents may be particularly high.

In this context it is noteworthy that the repulsive effect of myelin-associated inhibitors such as MAG and NogoA is also modulated by neurotrophin treatment through a mechanism that involves cAMP and protein kinase A (Cai et al., 1999). Neurotrophins are known to increase cAMP which in turn can inhibit the small GTPase rhoA, an important effector of cytoskeletal changes in response to inhibitory cues (Luo, 2000). Since intracellular levels of cyclic nucleotides can affect the response to guidance molecules (Song et al., 1998), it is likely that neurotrophin-induced elevation of cAMP interferes with signaling by chemorepellents. Interestingly it was shown that increased levels of cGMP convert the response to Sema3A from repulsion to attraction in *Xenopus* neurons (Song et al., 1998), while a recent study shows that the chemokine SDF-1 reduces growth cone responsiveness to multiple chemorepellents, including Sema3A, through elevated cAMP levels (Chalasani et al., 2003). Therefore the elevation of cAMP levels may be a general mechanism in mammalian neurons to reduce their sensitivity to inhibitory guidance cues. Indeed, recent work has shown that increasing cAMP levels or inhibiting rhoA by the enzyme C3 transferase enhances axon extension in cultured neurons (Niederost et al., 2002) and stimulates regenerative fiber growth in vivo (Lehmann et al., 1999, Dergham et al., 2002, Neumann et al., 2002, Qiu et al., 2002).

In conclusion, our results show that Sema3A, a chemoattractant during nervous system development, is a major neurite growth-inhibitory molecule in meningeal fibroblasts and is therefore likely to contribute to the inhibitory properties of the neural scar. Similar to axon growth in the developing nervous system, it appears that the balance between growth inhibition and growth promotion regulates the regenerative capability of injured neurons. We propose that a therapeutic approach to stimulate axon regeneration should combine the stimulation of
neurite extension while at the same time specifically interfere with growth inhibition.

**Experimental methods**

**Cell culture**

Meningeal cells and astrocytes were prepared from newborn rat pups (P0–P2). Briefly, meninges were removed from the cortices and incubated for 30 min at 37°C in Ca\(^{2+}\) and Mg\(^{2+}\)-free Hanks' buffered salt solution (HBSS) containing 0.125% trypsin, 0.5 mM EDTA, and 0.25% collagenase. Meninges were triturated gently in the presence of 80 μg/ml DNase and 100 μg/ml soybean trypsin inhibitor, centrifuged, and resuspended in DMEM containing 10% FCS and penicillin/streptomycin (PS). The suspension was passed several times through 19 and 25-gauge needles and seeded at a density of two brains per 25-cm\(^2\) flask. Medium was refreshed the next day; the first cell passage was carried out after 7 to 10 days. Cells were used for experiments between passages 2 and 5. Astrocytes were prepared from the cortices following the same procedure as described for meningeal cells, except that collagenase was omitted. Meningeal cells from mouse pups were isolated as above, except that they were prepared individually per pup and seeded at a density of 1 brain per 3.5-cm dish. Sema3A-knockout mice were from either a C57BL/6 or a CD1 background. The pups were from heterozygous litters and were genotyped by PCR on tail DNA as described (Taniguchi et al., 1997). Only cultures from wild-type and homozygous knockout mice were carried further.

**Immunocytochemistry**

For immunocytochemistry cells were fixed 30 min in 4% PFA, rinsed, blocked for 30 min in Tris-buffered saline containing 0.1% Triton X-100 (T-TBS) and 2% FCS, incubated with the primary antibody in blocking solution overnight at 4°C, and washed in T-TBS followed by incubation with a Cy3-labeled secondary antibody (Jackson). The following antibodies were used: anti-vimentin (mAB clone V9, Roche 814318); anti-fibronectin (goat, Calbiochem 341653), anti-chondroitin sulfate (clone CS-56, Sigma C8035), anti-GFAP (cow, Dako Z0334), anti-neurofilament (mAB, 2H3 clone, Developmental Studies Hybridoma Bank, University of Iowa), anti-Raldh2 (rabbit polyclonal; a gift from Dr. P. McCafferey, Harvard University) (Berggren et al., 1999).
In situ hybridization

For in situ hybridization, meningeal cells were plated at low density on poly-L-lysine-coated glass coverslips. Cells were fixed for 12 min in 4% PFA, 0.2% Triton X-100, washed, permeabilized, and prehybridized for 3 h at room temperature in hybridization buffer (50% formamide, 5x SSC, 5x Denhardt’s, 200 μg/ml hsDNA, and 250 μg/ml tRNA). The buffer was replaced with hybridization buffer containing digoxigenin-labeled Sema3A or Sema3C riboprobes and incubated overnight at 60°C. Probes were prepared as described previously (De Winter et al., 2002b). Coverslips were washed for 30 min at 60°C in 0.2x SSC/50% formamide, brought to room temperature by rinsing in 150 mM NaCl, 100 mM Tris, pH 7.5, and incubated for 30 min in the same buffer containing 1% blocking reagent (Roche, No. 1096176) and 0.3% Triton X-100. A horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody (1:100; Roche, No. 1207733) was applied for 1 h in blocking buffer, followed by 3 x 5-min rinses. An amplification step was performed with biotinyl-tyramide (1:50; TSA Biotin System NEL700A, NEN Life Science Products) following the manufacturer’s instructions. Finally, a streptavidin–alkaline phosphatase complex was applied and the color reaction was developed with NBT and BCIP substrates. The amplification step leads to a characteristic staining pattern of large dots surrounding the cell nucleus, which results from the high concentration of alkaline phosphatase in one particular spot.

RT-PCR

Total RNA was isolated from cultured newborn rat meningeal cells and from rat brain (P9) using TRIzol reagent (Invitrogen) following the manufacturer’s guidelines. First strand DNA was synthesized with 1 μg total RNA using Superscript II (Invitrogen) and oligo(dT) primers. Control PCRs were performed with RNA samples where the reverse transcriptase step was omitted. A 25-cycle PCR was carried out using Super Taq (Sphaero Q) and supplemented with 2.5 mM MgCl2. The primers were as follows: Sema3A, 5’ agaaggttgatctgtgctg; Sema3A, 3’ cactgggttatagtgtgctg; Sema3C, 5’ ctgcatcgctgccatatct; Sema3C, 3’ gggagcacactcaaggaag. The expected amplicon sizes were 194 bp for Sema3A and 252 bp for Sema3C. The PCR fragments were sequenced to ensure specificity of the PCR.

Calcium phosphate transfection

Sema3A and Sema3C proteins were obtained by transient Ca2+ phosphate transfection of 293T cells using myc-tagged chicken Sema3A or Sema3C constructs (a gift of J.A. Raper) (Koppel et al., 1997). In brief, for a 6-cm dish 6 μg DNA was vigorously mixed with 0.1x TE, 2.5 M CaCl2, and 2x HBS (281 mM NaCl, 1.5 mM Na2HPO4, pH 7.12) and immediately added to the culture dish. The transfection proceeded for 12–16 h, medium was replaced with DMEM containing PS and 2% FCS, and conditioned medium (CM) was harvested after another 24 h.
Semaphorin expression in conditioned medium was verified by Western blot analysis using an anti-myc antibody (Santa Cruz, clone 9E10) (not shown). For the neurite outgrowth assay, CM from transfected cells was applied to the DRG culture at a 1:1 dilution with the neuronal culture medium (see below).

**DRG culture and collapse assay**

Dorsal root ganglia (DRGs) were isolated from E15 rat embryos, stripped from surrounding roots, and plated as whole explants on laminin-coated glass coverslips. For the collapse assay they were cultured for 24 h in DMEM/F12 containing N2 supplements (Sigma, No. 17502), 2 mM glutamax, 20 ng/ml NGF (Roche, No. 1014331), and PS. Membranes from meningeal cells were prepared by lysing a confluent 10-cm dish in 1 ml cold lysis buffer (20 mM Hepes, pH 7.4, 2 mM MgCl₂, 1 mM EDTA, pH 8, and protease inhibitor cocktail) (Roche, No. 1697498). The cells were allowed to swell for 5 min, then passed three times through a 20-gauge needle. Lysed cells were spun briefly (1000 g for 5 min at 4°C) to pellet unbroken cells and nuclei. The membranes were pelleted at 100,000g for 1 h at 4°C, and the pellet was washed twice with lysis buffer, carefully adding and removing the buffer without disturbing the pellet. Proteins were extracted by resuspending the membrane pellet in 200 – 400 µl extraction buffer (0.5x PBS, 10 mM Tris 7.4, 3% sodium cholate) and passing it several times through a 23-gauge needle. Undissolved material was spun down and the supernatant was dialyzed overnight in 1x PBS, 5 mM Tris, pH 7.4, 0.01% sodium cholate at 4°C. Protein concentration was determined using a Lowry assay and the extract was applied to E15 DRG cultures at the appropriate concentrations. After a 30-min incubation DRGs were fixed in 4% PFA, 10% sucrose in PBS. The percentage of collapse was determined by counting the number of collapsed versus noncollapsed growth cones. Routinely about 80 growth cones per DRG and 3 DRGs per condition were counted. Each experiment was replicated at least three times.

**Neuropilin-1 blocking experiment**

The neuropilin-1 blocking antibody was a gift from Dr. A. Kolodkin (Baltimore, MD, USA). The antiserum was purified by protein A-Sepharose chromatography as described (Kolodkin et al., 1997). For the collapse assay, the IgG fraction was added to the DRGs simultaneously with the protein extract at the indicated concentration. As a control the IgG fraction of a nonblocking Npn-1 antibody generated in our laboratory was used (Pasterkamp et al., 1998a).

**Neurite outgrowth assay**

Meningeal cells and astrocytes were plated on poly-L-lysine-coated glass coverslips (12 mm) at a density of 2 x 10⁵ cells/coverslip for MCs and 4 x 10⁵ cells/coverslip for astrocytes, and grown for 48 h in DMEM containing 10% FCS to reach confluency. Two days after preparation of the coverslips, E15 rat DRGs were
isolated and seeded on a confluent monolayer of meningeal cells or astrocytes or on control laminin-coated glass coverslips. DRGs were grown in DMEM/F12 containing N2 supplements (Sigma, No. 17502), 2 mM glutamax, 20 ng/ml NGF (Roche, No. 1014331), and PS. After fixation with 4% PFA in PBS containing 10% sucrose, neurites were visualized with the 2H3 anti-neurofilament antibody (2H3 ascites 1:1000; Dev. Stud. Hybridoma Bank, University of Iowa) as described. Fluorescence photographs were taken with the same intensity settings for all DRGs and quantification of neurite growth was performed with ImagePro software. The outgrowth area of each ganglion and the ganglion itself were outlined manually and the area of fluorescence above background level was determined within the marked area. To normalize the data the fluorescence area obtained on a laminin substrate was set at 100% for each experiment and the fluorescent area obtained for each experimental condition was calculated as a percentage thereof. Each experiment was carried out at least three times, and in each experiment 8 to 12 ganglia per condition were measured. Results were analyzed statistically using combined Student t-tests. Significance was set at P < 0.05.

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Comparison of AAV serotypes for gene delivery to dorsal root ganglion neurons

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Abstract

For many experiments in the study of the peripheral nervous system, it would be useful to genetically manipulate primary sensory neurons. We have compared vectors based on AAV serotypes 1, 2, 3, 4, 5, 6, and 8, and lentivirus, all expressing GFP, for efficiency of transduction of sensory neurons, expression level, cellular tropism and persistence of transgene expression following direct injection into the dorsal root ganglia (DRG), using histological quantification and qPCR. Two weeks after injection, AAV1, AAV5 and AAV6 had transduced the most neurons. The time course of GFP expression from these three vectors was studied from 1 to 12 weeks post-injection. AAV5 was the most effective serotype overall, followed by AAV1. Both these serotypes showed increasing neuronal transduction rates at later time-points, with some injections of AAV5 yielding over 90% of DRG neurons GFP-positive at 12 weeks. AAV6 performed well initially but transduction rates declined dramatically between 4 and 12 weeks. AAV1 and AAV5 both transduced large diameter neurons, IB4-positive neurons and CGRP-positive neurons. In conclusion, AAV5 is a highly effective gene therapy vector for primary sensory neurons following direct injection into the DRG.

Introduction

Adeno-associated virus (AAV) is increasingly regarded as one of the most useful gene therapy vectors, due to its low immunogenicity and apparent absence of toxicity. AAV-based vectors have been used successfully to direct transgene expression in a variety of tissues including the nervous system (Kaplitt et al., 1994, McCown et al., 1996, Peel et al., 1997, Klein et al., 1998); reviewed in Peel and Klein, 2000, Ruitenberg et al., 2002) and a number of early phase clinical trials for neurological or ophthalmic diseases have been conducted or are underway (Mandel and Burger, 2004, Kaplitt et al., 2007, Bainbridge et al., 2008, Arvanitakis et al., 2009). These early AAV vectors were based on AAV serotype 2, but subsequently vectors have also been generated based on novel serotypes of AAV derived from primate and human populations (Rutledge et al., 1998, Xiao et al., 1999, Chao et al., 2000, Gao et al., 2002, Rabinowitz et al., 2002). Usually these utilize the AAV2 inverted terminal repeats (ITRs), but are packaged with the capsid proteins of the other serotypes. These vectors have different cellular tropism and transduction properties to AAV2, e.g. following injection into mouse or rat brains, AAV1 and AAV5 have consistently shown transduction of greater numbers of neurons, improved spread through the parenchyma, and transduction of areas that were not transduced by AAV2 (Wang et al., 2003, Burger et al., 2004, Paterna et al., 2004).
For many studies of the peripheral nervous system, it is desirable to be able to direct transgene expression to the primary sensory neurons in the dorsal root ganglia (DRG). Over-expression or viral-vector mediated knockdown of genes of interest would allow a large range of investigations into gene function during, among other things, regeneration after peripheral nerve injury and neuropathic pain. The central branches of these neurons also project into the spinal cord and, in the case of large-diameter neurons, up to the brainstem, making them also a useful model in the study of regeneration in the central nervous system. HSV-based vectors and rabies-pseudotyped lentivirus have been used successfully (Lilley et al., 2001, Glorioso et al., 2003, Wong et al., 2006) but transduction with these vectors may sometimes be inefficient or transient. AAV vectors based on AAV2 were shown to be effective by direct injection of the ganglion (Glatzel et al., 2000, Xu et al., 2003a) and were used to deliver an antinociceptive gene (Xu et al., 2003b). Storek et al. tested several serotypes for gene delivery following delivery in the intrathecal space, and found that self-complementary AAV8 was the most effective in transducing DRG neurons (Storek et al., 2006, Storek et al., 2008), using it to deliver analgesic genes. AAV6 delivered intrathecally or via nerve injection was also recently shown to be effective at delivery of GFP to DRG neurons (Towne et al., 2009).

Direct injection of DRG for gene delivery may be preferable for some experiments where transduction of specific ganglia is required, and might lead to higher transduction rates. However the transduction profiles of the different AAV serotypes using this delivery method are not known. We have compared AAV vectors based on serotypes 1, 2, 3, 4, 5, 6 and 8 for transduction of DRG neurons following injection of titre-matched viral vector stocks directly into the DRG. Viral vectors were prepared using serotype-specific packaging plasmids, but for all vectors we used an identical transfer plasmid consisting of a CMV-GFP-WPRE expression cassette flanked by AAV2 ITRs. It is also not known whether VSV-G pseudotyped lentiviral (LV) vectors are able to transduce DRG neurons in vivo. Since these vectors are widely used in the nervous system, the performance of the AAV serotype vectors was also compared with an LV vector. We have also examined the time-course of transgene expression of the three most successful vectors (AAV1, AAV5 and AAV6) up to three months after injection. To accurately compare the serotypes, we used a semi-automated method to quantify GFP expression in histological sections of DRG, allowing unbiased measurement of transgene expression in large numbers of sensory neurons. Additionally, to compare the vectors’ efficacies at the level of transcription, we measured total GFP mRNA expression levels by qPCR.
Results

Comparison of AAV serotypes and lentivirus for gene delivery in dorsal root ganglia

Vectors based on AAV serotypes 1, 2, 3, 4, 5, 6 and 8, and lentivirus, were injected into the left L4 and L5 dorsal root ganglia of four animals per group. Two weeks after virus injection, all AAV serotypes except AAV3 had transduced significant numbers of DRG neurons (Fig 1). Transduction appeared to be neuron specific from all AAV vectors except AAV6, as all transduced cells were TuJ1 positive and no other cells expressing GFP were observed. In the case of AAV6 we frequently observed satellite cells within the ganglion which had also been transduced. These cells were S100 positive (Fig. 2). To confirm the neuronal specificity of transduction in the other AAV serotypes, sections from each serotype were also co-stained for GFP, TuJ1 and S100 and examined by confocal microscopy. Only neuronal transduction was seen and no satellite cells were transduced (Fig. S1). LV-GFP transduced only a handful of neurons, although it did produce GFP expression in non-neuronal cells predominantly located in the intra-ganglionic nerve bundles (Fig. 1).

GFP expression in the DRG neurons was quantified using a semi-automated histological method, using ImagePro Plus to identify neuronal nuclei visible after βIII tubulin staining. An example of automated identification of DRG nuclei is shown in Fig. 3a-c. The algorithm identified an average of 830 neurons from 11-12 sections per DRG, and in this study a total of 160,000 neurons were quantified. The percentage of neurons expressing GFP 2 weeks after injection are shown in Fig. 4a. AAV5 shows the highest average transduction rate of 48.7±4.1%, followed by AAV6 and AAV1 at 37.3±7.4% and 33.5±3.5% respectively. AAV5 was significantly higher than all other vectors except AAV1 and AAV6 (ANOVA followed by Dunnett’s T3 post-hoc test; p<0.01). AAV8, AAV2 and AAV4 were the next best serotypes, in that order, while AAV3 performed poorly. The average expression levels of GFP in transduced cells per DRG were not significantly different between vectors (Fig.4b).

Expression levels of GFP mRNA from all injected ganglia were also quantified by qPCR. eGFP values were normalized to neuron-specific enolase (NSE). As shown in Fig. 4c, the results were similar to those of the histological quantification method. Serotypes 1, 5 and 6 showed the highest expression. Total GFP mRNA expression from AAV1, 5 and 6 was very similar, while as before AAV3 and 4 were the poorest performing serotypes and AAV2 and AAV8 gave expression levels in between. AAV5 was significantly better than AAVs 1, 3, 4, 8 and LV (p<0.01) and AAV2 (p<0.05) (ANOVA with Dunnett’s T3 post-hoc test).
Replicate viral batches

Although the viral stocks were carefully titre-matched and confirmed to be active using in vitro transduction assays, the possibility remained that some of the differences we observed between AAV serotypes may have been due to variation in production quality of viral batches. To rule out this possibility we performed a replicate set of injections for AAV serotypes AAV 1, 2, 5, 6 and 8 using independently made batches. These serotypes represent the best performing serotypes from the first comparison (AAV 1, 5 and 6), the classical and most widely used serotype AAV2, and AAV8 which was previously reported effective.
These data are shown in Fig. S2. The findings are highly similar to the initial screen, AAV1, 5 and 6 being superior to AAV2 and 8. As before, AAV6, but not other serotypes, transduced satellite cells as well as neurons (data not shown). qPCR expression data from the replicate virus batches similarly confirmed the findings of the initial comparison. Relative expression levels are shown in Fig. S2c. Again, AAVs 1, 5 and 6 performed better than AAVs 2 and 8.
Time-course of expression from AAV1, AAV5 and AAV6

We took the three serotypes which performed best at 2 weeks post-injection and examined the time-course of GFP expression. Left L4 and L5 DRGs were injected in 4 animals for each virus and each time-point. Survival times were 1 week, 4 weeks and 12 weeks after injection. Fig. 4d shows the transduction rates over the time course for these three serotypes. The percentage of GFP-positive cells
and the amount of GFP per cell expressed from AAV1 and AAV5 (Fig. 4e) clearly increased over the initial 4-week period and AAV5 showed a further increase in both measures at 12 weeks. This increase in expression was also clearly visible (Fig. 5a, c, e). AAV5 was consistently the most effective serotype of these three. Over the time-course, the transduction rates delivered by AAV5 were significantly greater than those of both AAV1 and AAV6 (2-way ANOVA, Dunnett’s post-hoc test; p<0.05). GFP-positive cell numbers from AAV6 expression peaked at 4 weeks at 75.5±6.6% but then declined markedly between 4 weeks and 12 weeks. At 12 weeks the transduction rate from AAV6 was significantly lower than that of AAV5 (Student’s t-test; p<0.001).

eGFP mRNA expression was also quantified over the time-course by qPCR. Relative expression is shown in Fig. 4f. Expression was highest overall from AAV5. Expression from AAV1 slowly increased over the time course, reaching its maximum at 12 weeks. mRNA expression by AAV5, however, was already high at 1 week after injection and stayed at this level for the time-course. Expression from AAV6 increases from 1 to 4 weeks and then declines at 12 weeks, reflecting the histological expression data. The maximum RNA levels attained by each serotype were similar. AAV5 expression was significantly higher over the time-course than AAV1 or AAV6 (2-way ANOVA, Dunnett’s post-hoc test; p<0.01).

High-titre AAV5

Although the average AAV5 transduction rate at 12 weeks was as high as 77%, at 2 weeks only 49% of neurons were detectably GFP positive. We made a higher titre stock of AAV5 to determine if we could achieve a higher transduction rate at this time-point. We injected approximately a 10-fold higher number of viral genomes ($5.5 \times 10^9$) into left L4 and L5 DRGs of four animals. The mean transduction rate 2 weeks after injection was significantly increased, at 61.9±4.4% as compared to 48.7±4.1% after injection of the lower titre stock (Student’s t-test; p<0.05) (Fig. S3a). The expression level of the GFP positive cells was also significantly increased from 5.0±0.3 times background with the initial titre to 6.7±0.4 times background with the higher titre (Student’s t-test; p<0.01; Fig. S3b). qPCR quantification of eGFP expression revealed that mRNA expression was 5.6-fold higher from high titre virus injections than the standard titre (Student’s t-test; p<0.001); shown on a log-2 scale in Fig. S3c.

Histology of injected ganglia

To determine if the injection procedure causes significant damage to the DRG we stained sections of injected ganglia with haemotoxylin and eosin. Two weeks after injection we could find little evidence for tissue damage (Fig. S4). Because we also observed an unexpected reduction in expression from AAV6 between 4 and 12 weeks we also carefully examined sections of 12-week injected DRG with H&E and double staining with S100 and GFP (Fig. S5). We observed that in AAV6-injected DRG an area of apparent tissue damage had formed at 12 weeks.
Figure 5. Histology of time-course of GFP expression after injection AAV5. (a-f) Sections were stained with GFP (green) and βIII tubulin (red). (a, c, e) DRG neurons expressing GFP, 1 week (a), 4 weeks (c), and 12 weeks (e) after viral injection. (b, d, f) Sciatic nerves containing GFP-positive fibres, again 1 week (b), 4 weeks (d), and 12 weeks (f) after injection. At 1 week almost no GFP positive axons are present, while at 4 weeks and 12 weeks many axons are visible. (g) Horizontal section of superficial lumbar spinal cord, 4 weeks after AAV5 was injected into the left L4-5 DRG, stained for GFP (green) and GFAP (red). Many GFP-positive fibres are visible in the dorsal roots and can be traced over long distances within the spinal cord. Scale bars 100µm (a, c, e), 50µm (b, d, f), 250µm (g).
around the injection site, while with the other serotypes little or no evidence of damage was visible. GFP-positive neurons appeared to be often near the edge of the damaged area.

**GFP in sciatic nerve and spinal cord**

Spinal cords and sciatic nerves of animals injected with AAV5 were processed for GFP immunohistochemistry. At 1 week, only occasional single GFP-positive axons could be seen in the sciatic nerve but at later time-points many fibres were labelled (Fig. 5b, d, f), with the most intense labelling seen at 12 weeks. Sections of lumbar spinal cord from 4 week AAV5-injected animals were also stained for GFP and again many GFP-positive axons were visible in the dorsal roots, and entering the cord (Fig. 5g).

**Transduction of neuronal sub-types**

The primary sensory neurons of the DRG consist of several subtypes, which can be broadly classified as large-diameter, small-diameter peptidergic and small-diameter non-peptidergic. To determine if AAV1 and AAV5 transduce the different subtypes equally, we examined a subset of sections from 4 injected DRGs from the 4-week time-point. These were co-stained with CGRP, a marker for the small peptidergic neurons, or IB4, a marker for small non-peptidergic neurons, alongside GFP and βIII tubulin. CGRP-positive and IB4 positive neurons were also transduced by AAV1 and AAV5 (see Fig. 6a-f for examples). A total of 33% of neurons were CGRP positive and 48% were IB4 positive. Transduction rates and mean expression levels of CGRP positive neurons were not significantly different.
to the overall population. However, the transduction rates of IB4 positive neurons
were significantly lower than the overall transduction rates, for both serotypes
(p<0.01; Student's t-test, paired). Mean expression levels per DRG were also
lower in the case of IB4+ cells transduced by both serotypes, although this was
only significant in the case of AAV5 (p<0.01; Student's t-test, paired). Figure 7
shows the transduction rates and mean expression levels for each serotype in
CGRP+ and IB4+ cells, expressed as a percentage of the overall transduction rate
in the sample sections.

**AAV5 transduction of contralateral DRGs**

Sections of contralateral uninjected DRGs were used to calibrate the background
fluorescence level for subsequent quantification of GFP expression. However we
noticed that following injection of AAV5 into the left L4 and L5 DRGs, a small
number of neurons in the contralateral DRGs were transduced (not shown).
The transduction rate was low, less than 1 neuron per section. Transduction of
contralateral DRG neurons was not seen with any of the other serotypes. Because
of this transduction at a distant site we also looked for GFP immunolabelling in
meninges and sections of lumbar spinal cord, brain stem and forebrain of AAV5
injected animals. A very few transduced neurons were observed in the spinal
cord, and no transduced cells were observed in the brainstem, forebrain or
meninges.

Figure 7. Quantification of transduction rates (a) and expression levels (b) in
CGRP- and IB4-positive neurons, relative to the whole neuronal population, 4
weeks after injection of AAV1 or AAV5. Transduction rates and expression
levels of CGRP positive cells are not different to the overall population.
However both serotypes show reduced transduction rates and expression
levels of IB4 positive cells. ** p<0.01 compared to the whole population.
Discussion

We have compared viral vectors based on AAV serotypes 1, 2, 3, 4, 5, 6 and 8 and lentivirus for gene delivery to primary sensory neurons of the adult rat following direct injection of the DRG. We have accurately quantified both transduction rate and expression level from these vectors using automated image analysis, and mRNA expression by qPCR, and we show that AAV5 is the most effective in this model. The maximum transduction rate that we obtained, from AAV5, was 95%, which is remarkably high for in vivo viral vector delivery. AAV1 is also effective, but the transduction rate and protein expression level from this vector were consistently below AAV5. AAV6 also performs well at time-points up to 4 weeks after injection but its usefulness is limited by a severe decline in transgene expressing cells between 4 and 12 weeks. The onset of transcription from AAV5 also appears to be the most rapid, reaching its maximum already at 1 week, while mRNA levels from AAV1 and AAV6 increased more slowly. Protein levels, on the other hand, as measured by histological quantification, appear to increase more gradually, which may be because eGFP protein accumulates slowly. This effect was also seen by Xu et al. (Xu et al., 2003a) who found a large increase in the number of apparently transduced cells, between 1 and 3 weeks, following direct DRG injection of AAV2. The observations in DRG 12 weeks post-injection suggests that in the best injections nearly all neurons are transduced, but at earlier time points not enough GFP has been produced to be detectable. This may explain why injection of a near ten-fold higher titre of AAV5 resulted in only a modest increase in the average transduction rate at 2 weeks, compared to the standard titre we used for the serotype comparisons, although substantial increases in cellular expression level and mRNA were found. Collectively these observations indicate that with the current injection procedure, titres and the use of the AAV5 serotype we have achieved close to the maximum possible transduction rate of DRG neurons in vivo.

Several groups have reported transduction of DRG neurons with other AAV serotypes and delivery methods. Storek et al compared AAV serotypes 1, 3 and 5 by intrathecal delivery and although the DRG were not examined directly, only AAV1 produced strong GFP expression in cauda equine (Storek et al., 2006). The same group compared AAV8 and AAV1 using this delivery route and found the former to be effective at transducing DRG neurons, and the better of the two serotypes (Storek et al., 2008). While these findings differ from ours this is likely to be largely because of the very different delivery methods (intrathecal vs. direct injection). The AAV8 vector used by this group was also, unlike ours, self-complementary, which may explain why AAV8 was less effective in our hands than in their study. Foust et al. (Foust et al., 2008) reported sensory fibre labelling following intraperitoneal or intravenous injection of AAV8 in neonatal animals. AAV6 was also found to be effective for GFP delivery via intrathecal injection and sciatic nerve injection (Towne et al., 2009). Several groups have used AAV2 to deliver gene expression to DRG neurons by direct injection and it
has been used to deliver therapeutic genes (Glatzel et al., 2000, Xu et al., 2003a, Xu et al., 2003b). However, as we now show, much more robust expression should be attainable with AAV5 compared to AAV2. Our findings are consistent with studies which have tested various AAV serotype vectors following injection in the adult rodent brain or spinal cord, where AAV1 and AAV5 have been shown to be effective delivery vectors (Wang et al., 2003, Burger et al., 2004, Paterna et al., 2004, Klein et al., 2006, Blits et al., 2010). However, AAV8 was reported to outperform AAV1, 2 and 5 in hippocampus and substantia nigra (Klein et al., 2006). Which vector performs best depends on the area being injected within the CNS and the cell-type being targeted; e.g. in the retina, AAV2 and 6 were the best and AAV5 and 8 were the least effective following intravitreal injection (Hellstrom et al., 2009). AAV4, which performed poorly in the DRG, was found to efficiently transduce ependymal cells (Davidson et al., 2000). Lentivirus vectors, which did not transduce neurons efficiently in this study, do transduce neurons and astrocytes in the CNS, astrocytes in the neural scar (Naldini et al., 1996a, Blomer et al., 1997, Ahmed et al., 2004, Taylor et al., 2006, Hendriks et al., 2007b) and Schwann cells in peripheral nerve (Eggers et al., 2008). In the DRG, the choice of AAV serotype for experimental study will depend on the experiment. Direct injection of AAV5 into DRG has the advantage of targeting specific ganglia and may also be desirable where maximal transduction rates, high expression levels and early onset of expression are required. However, the surgery is more invasive and time-consuming compared to intrathecal delivery of AAV8 or AAV6.

AAV6 appears to transduce neurons with a similar efficiency to AAV5 after direct injection, as indicated by counts of GFP positive cells that were comparable to those of AAV5 up to 4 weeks after injection. However, this vector shows a remarkable decline in expression between 4 and 12 weeks after injection with the average transduction rate falling back to a similar level to that seen at 1-2 weeks. This was associated with a notable area of damage around the injection site, visible at 12 weeks. This suggests that GFP expressing cells may be lost between 4 and 12 weeks post-injection due to tissue loss around the injection site. This effect may be caused by an immune response, either to AAV6 capsid or to GFP. AAV6 was the only serotype to also express GFP in satellite cells, and as we have previously found that GFP expression in Schwann cells provokes an immune response (which is ameliorated by the use of a non-immunogenic ‘stealth GFP' (Hendriks et al., 2007a)), it is possible that this loss of expression is a consequence of the transgene expression in the satellite cells leading to an immune reaction in a similar way. We speculate that this does not occur with the other serotypes because transduction is neuron specific and neurons are poor at antigen presentation. This point would be interesting to investigate further in future studies. Lastly, Towne et al. recently showed AAV6 to be effective at transducing DRG neurons after intrathecal delivery or sciatic nerve injection (Towne et al., 2009) and we note that these authors did not find transduction of satellite cells, so for this reason intrathecal delivery may be preferable to direct injection when AAV6 is used.
Unexpectedly, we saw GFP-expressing neurons distant from the injection site following delivery of AAV5, in contralateral DRG and occasionally in lumbar spinal cord. This may be due to travel of the virus within the blood stream or CSF, and it may also indicate retrograde transport of AAV5 by these neurons. In other studies neuronal retrograde take-up of AAV5 has been observed (Wang et al., 2003, Burger et al., 2004, Taymans et al., 2007) although, unlike AAV1, it was not effective at transduction of motor neurons by retrograde transport from the sciatic nerve (Hollis et al., 2008). Care should be taken if GFP is to be considered for use as an axonal tracer, for example in spinal cord lesion models, as the transduction of neurons away from the injection site may lead to misleading results, if fibres from these neurons are also present at the site of interest.

In conclusion, we find that AAV5 is an excellent vector for gene delivery to dorsal root ganglion neurons, if direct injection is used. AAV1 is also effective but overall was out-performed by AAV5. Transduction of the great majority of neurons in the DRG is possible. The reliably high transduction rate will make this vector attractive for the over-expression of genes of interest in the DRG or, potentially, knockdown of specific genes by expression of hort hairin RNAs.

**Methods**

**Production of viral particles**

AAV vectors encoding enhanced green fluorescent protein (GFP) were all generated using the plasmid pTRCGW (Ruitenberg et al., 2002, Ahmed et al., 2004). This contains the inverted terminal repeats of AAV2 flanking a CMV promoter driving expression of GFP, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a polyadenylation signal. Helper plasmids used for cross-packaging were generously provided by Dr Kleinschmidt (AAV1-6) and J. Wilson (AAV8) and used as described previously (Gao et al., 2002, Grimm et al., 2003). For each AAV serotype, 30 roller bottles of 293T cells were transfected with the respective plasmids using the calcium phosphate method. For AAV1-6, pTR-CGW was co-transfected with the serotype-specific helper plasmid (ratio 1:3; total DNA 200ug/bottle). For AAV8, pΔF6, p5E18 and pTRCGW were co-transfected (ratio 2:1:1, total DNA 200 ug/bottle). Cells were grown in 50 ml Iscoves Modified Eagle Medium (IMEM) without phenol red and pyruvate, with 10% fetal calf serum and penicillin/streptomycin (Invitrogen). Three days after transfection AAV was harvested in 5 ml lysis buffer (50 mM Tris pH 8.5, 2 mM MgCl, 150 mM NaCl, 0.1 % Triton X-100) per roller bottle, incubated for 1 h with 10 μg/ml DNase, and spun down (30 min 4000G). The supernatant was filtered (0.45 μm), to yield crude lysate containing the AAV particles. AAV serotypes 1 and 5 were purified using affinity chromatography (Oranje et al., 2004), serotypes 2, 3 and 4 were purified using iodixanol gradient centrifugation (Zolotukhin et al., 1999), and serotypes 6 and 8 were purified using anion exchange chromatography (Zolotukhin et al., 2002) followed by iodixanol purification. For all vector
stocks, the buffer was changed to DPBS / 5% sucrose using Amicon® Ultra-15 (Millipore) concentrators and the viral stock concentrated to the appropriate volume. High titre stock of AAV5 was made by subsequent further concentration of 1 ml of vector stock of titre 1x10^{12} genomic copies (GC)/ml to approximately 150µl using a Centricon YM-100 (Millipore b.v., Amsterdam, the Netherlands). All vector stocks were kept at -80 °C until use. AAV stocks were assessed by SDS-PAGE analysis for visualization of the three capsid proteins (Grimm et al., 2003). Infectivity was assayed by application of viral stocks to camptothecin-treated (4.8 µg/ml for 4 h) 293T cells, and inspection after 3 days under a fluorescence microscope. Titres were determined by quantitative PCR for viral genomic copies extracted from DNase-treated viral particles. Titres were in the range of 6.6x10^{11} – 1.5x10^{12} GC/ml. Prior to injection, all vectors were titre-matched by dilution to 6.6x10^{11} GC/ml with D-PBS/5% sucrose. Lentiviral vectors were produced as described previously (Naldini et al., 1996b, Ahmed et al., 2004). The lentivirus expression cassette (pRRLsin-PPT-CMV-GFP), like the AAV cassette, contained the CMV promoter driving GFP expression, and a WPRE. Titre of the lentiviral vector encoding GFP was 1x10^9 transducing units (TU)/ml.

Experimental animals and surgical procedures

In this study a total of 72 female Wistar rats (200-250 g; Harlan, Horst, the Netherlands) were used. Animals were housed under standard conditions with food and water ad libitum, and a 12h:12h light/dark cycle. All experimental procedures and post-operative care were carried out with approval from the local animal experimentation ethical committee. The injection was performed with a borosilicate glass capillary (0.78mm/1mm internal/external diameters; Harvard Apparatus, Holliston, MA) pulled to a fine point, attached by Portex polythene tubing (0.4mm/0.8mm internal/external diameter; Jencon’s Scientific Ltd, East Grinstead, UK) to a Hamilton syringe mounted in a Harvard PHD2000 microinjection pump (Harvard Apparatus, Holliston, MA). The needle was mounted on an extended arm of a stereotaxic frame swung to the outside (used to hold and manipulate the needle only). Tubing, syringe and needle were all filled with water. The viral vector solution was supplemented with 0.1 volumes of 1% Fast Green FCF (to allow monitoring of the injection of the viral vector solution into the ganglion). 1µl air was taken up in to the needle followed by 1.1µl of the viral vector solution. The needle was loaded separately with this volume for each injection. Animals were anaesthetized using Hypnorm (Fentanyl/Fluanisone; 0.08 ml/100 g body weight, i.m.; Janssen Pharmaceuticals, Beerse, Belgium) and Dormicum (Midazolam; 0.05 ml/100 g body weight s.c.; Roche, Almere, the Netherlands). Following an incision along the dorsal midline, the L4 and L5 DRG were exposed by removal of the lateral processes of the vertebrae. The epineurium lying over the DRG was opened, and the glass needle inserted into the ganglion, to a depth of 400µm from the surface of the exposed ganglion. After a 3 minute delay to allow sealing of the tissue around the glass capillary tip,
1.1µl virus solution was injected at a rate of 0.2 µl/min. After a further delay of 2 minutes the needle was removed. The L4 ganglion was injected first followed by L5. The muscles overlying the spinal cord were loosely sutured together with a 5/0 suture and the wound closed. Animals were allowed to recover at 37 °C and received post-operative analgesia (Temgesic 0.03 ml/100 g body weight s.c., Schering-Plough B.V., Maarssen, the Netherlands).

**Comparison of AAV serotypes and lentivirus**

Vectors based on AAV serotypes 1, 2, 3, 4, 5, 6 and 8 were injected into the left L4 and L5 dorsal root ganglia of four animals per vector. 6.6x10⁸ genomic copies were delivered per injection. A further group of 4 animals received 1x10⁶ transducing units (TU) per DRG of lentivirus expressing GFP, in the same volume. For AAV the ratio of GC to TU is in the range 500-1500 (Mayginnes et al., 2006), so this titre of LV is comparable to the AAV and has been routinely used to transduced large numbers of cells in vivo (Hendriks et al., 2007b, Eggers et al., 2008). After 2 weeks, animals were injected with a lethal dose of pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. DRG were post-fixed for 3 to 4 hours and transferred to 30% sucrose in PBS, and the following day were frozen in TissueTek OCT (Sakura Finetek Holland BV, Zoeterwoude, Netherlands). The three most successful serotypes derived from the first experiment, AAV 1, 5 and 6 were studied in more detail. For each of these vectors, 12 further animals received injections in the left L4 and L5 DRG, with identical titre and injection volume, as before. After survival times of 1 week, 4 weeks and 12 weeks (n=4 per vector and time-point), animals were sacrificed and tissue processed as before. In one further group of 4 animals, 5.5x10⁹ genomic copies of AAV5-CMV-GFP-WPRE were injected into the DRGs as above, again in 1.1µl total volume. A further 20 animals received injections of separately produced batches of serotypes 1, 2, 5, 6 and 8 (n=4 per serotype). The survival time for these groups was 2 weeks.

**Immunohistochemistry**

DRG were sectioned at 20µm thickness on a cryostat onto gelatinized slides. Every fourth section was stained with mouse anti-β III tubulin (clone TuJ1; Covance, Berkeley, CA) at 1:500, and rabbit anti-GFP (Abcam, Cambridge, UK) at 1:4000, followed by donkey anti-mouse Alexa594 (1:400) and biotinylated goat anti-rabbit (1:300; Vector, Burlingame, CA), and then streptavidin-Alexa488 (1:400). Co-localization of GFP and βIII-tubulin with CGRP, IB4 or S100 was performed on sections adjacent to those used for quantification: chicken anti-GFP (Millipore b.v., Amsterdam, the Netherlands) was used at 1:4000 followed by anti-chicken-Cy2 (1:400). Anti-tubulin TuJ1 and anti-mouse A594 were used as above. Anti-CGRP (Millipore; 1:1500) was followed by anti-rabbit Cy5 (1:400), and IB4-biotin (1:250; Vector, Burlingame, CA) was followed by streptavidin-Cy5 (1:400).
Anti-S100 (1:600, DakoCytomation B.V., Heverlee, Belgium) was followed by ant-rabbit A594. On spinal cord sections, rabbit anti-GFP was used as above with mouse anti-GFAP (Sigma, St Louis, MO; 1:400) followed by anti-mouse Alexa594. Fluorescent secondaries were from Invitrogen (Carlsbad, CA; Alexafluor labels) or Jackson ImmunoResearch (Newmarket, UK; Cy-dye labels).

**Histological quantification**

Every section was photographed at fixed exposure settings at 10x magnification on an Axioplan microscope (Zeiss, Sliedrecht, the Netherlands). GFP staining was imaged twice with different exposure times; a short exposure (25ms) for quantification and long exposure time (150ms) for visualization. The short exposure time used for quantification prevents saturation in the green fluorescence channel. Image analysis and quantification were performed in ImagePro Plus (MediaCybernetics, Bethesda, USA). When the DRG are stained for βIII tubulin, the nuclei are unstained and appear as a dark round object against the red signal of the cytoplasm. An algorithm was used to identify neuronal nuclei in the red channel based on roundness and size. GFP expression was quantified by measurement of green fluorescent intensity within the area of the nucleus. Uninjected contralateral DRG were also processed and quantified in the same way to provide a background measurement. For classification as a GFP-positive cell, a threshold of 2.25x the average background level was chosen. On sections co-stained with GFP and CGRP or IB4, thresholds were determined by visual inspection. Mean expression levels were calculated per injected ganglion as the mean log fluorescence level in GFP positive cells in the ganglion, and expressed as a multiple of background fluorescence.

**qPCR**

Gene expression was quantified using qPCR for eGFP mRNA. RNA extraction and qPCR on fixed material requires digestion of the tissue with proteinase K and the use of short (<80bp) amplicons (Lehmann and Kreipe, 2001, Specht et al., 2001). Sections of DRG were removed from slides and RNA extracted as follows: sections were digested overnight in 100µl 25mM sodium citrate pH 7, 25mM EDTA 1% SDS, 4M urea and 12.5mg/ml proteinase K at 55°C. 0.1 volumes sodium acetate pH4 was added and the RNA was phenol/chloroform extracted, precipitated and DNasel treated (15 minutes at 25°C). 750ng RNA of each sample was used as input for reverse transcription (RT) and a no RT control reaction. RT was performed using random hexamers and MMLV and the resulting cDNA treated with RNaseA. Primers for eGFP were: GTCTATATCATGGCCGACAA and CTTGAAGTTCACCTTGATGC. All expression values were normalized to that of neuron-specific enolase (NSE). NSE primers were: GGCAAGGATGCCACTAA and CGCTGTTCTCCAGGATATTG.
Figure S1. AAV serotypes 1, 2, 5 and 8 do not transduce satellite cells. Sections of DRG injected with each serotype were stained with S100 (red) and GFP (green). Double labelled cells were not found, while GFP-positive neurons are present. Shown are example confocal microscopy images of double labelling. Scale bar 20µm.
Figure S2. Replicate viral batch injections. Batches of independently produced batches of AAV serotypes 1, 2, 5, 6 and 8 were injected into lumbar DRGs and GFP expression quantified after 2 weeks using the histological algorithm (a, b) and qPCR (c). (a) shows the percentage of transduced neurons per DRG, (b) shows the mean expression level in transduced cells as a multiple of background fluorescence, and (c) shows relative total eGFP mRNA (log-2 scale). **p<0.01, ***p<0.001 (ANOVA and Dunnett’s T3 post-hoc test).

Figure S3. Effect of raising the titre of AAV5 on transduction efficiency. GFP expression was quantified after injection of a high titre stock (5.5\times10^{12}GC/ml) and is compared with the standard titre AAV5 (6.6\times10^{11}GC/ml). (a) transduction rate; (b) expression level in transduced cells; (c) total GFP mRNA expression determined by qPCR, shown on a log-2 scale. *p<0.05, ** p<0.01, ***p<0.001 (Student’s t-test).
Figure S4. Haemotoxylin and eosin staining of dorsal root ganglia (DRG). (a) Control uninjected DRG (b, c) DRG 2 weeks after injection with AAV5 (b) or AAV6 (b). Scale bar 100μm.

Figure S5. AAV6-injected DRGs 12 weeks after injection. (a) Haematoxylin and eosin staining. (b, c) Sections are labelled with S100 (red) and GFP (green). The decline in GFP expression from AAV6 is associated with an area of damage around the injection site (asterisks), not seen in the other serotypes. Scale bar 100μm.
Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system

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Abstract

Background: After a spinal cord lesion, axon regeneration is inhibited by the presence of a diversity of inhibitory molecules in the lesion environment. At and around the lesion site myelin-associated inhibitors, chondroitin sulfate proteoglycans (CSPGs) and several axon guidance molecules, including all members of the secreted (class 3) semaphorins, are expressed. Interfering with multiple inhibitory signals could potentially enhance the previously reported beneficial effects of blocking single molecules. RNA interference (RNAi) is a tool that can be used to simultaneously silence expression of multiple genes. In this study we aimed to employ adeno-associated virus (AAV) mediated expression of short hairpin RNAs (shRNAs) to target all semaphorin class 3 signaling by knocking down its receptors, neuropilin 1 (Npn-1) and neuropilin 2 (Npn-2).

Results: We have successfully generated shRNAs that knock down Npn-1 and Npn-2 in a neuronal cell line. We detected substantial knockdown of Npn-2 mRNA when AAV5 viral vector particles expressing Npn-2 specific shRNAs were injected in dorsal root ganglia (DRG) of the rat. Unexpectedly however, AAV1-mediated expression of Npn-2 shRNAs and a control shRNA in the red nucleus resulted in an adverse tissue response and neuronal degeneration. The observed toxicity was dose dependent and was not seen with control GFP expressing AAV vectors, implicating the shRNAs as the causative toxic agents.

Conclusions: RNAi is a powerful tool to knock down semaphorin receptor expression in neuronal cells in vitro and in vivo. However, when shRNAs are expressed at high levels in CNS neurons, they trigger an adverse tissue response leading to neuronal degradation.

Background

The lesion environment of the injured spinal cord constitutes an impediment to regenerating axons (Schwab and Bartholdi, 1996, Silver and Miller, 2004, Niclou et al., 2006). A number of neurite growth inhibitors expressed in and around the lesion area have been identified, including the myelin-associated inhibitors NogoA, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), ephrinB3 and semaphorin4D as well as scar-derived factors such as CSPGs, secreted semaphorins, ephrins, slits and Wnts (reviewed by Harel and Strittmatter, 2006, Bolsover et al., 2008, Giger et al., 2008). These proteins act through multimeric receptors expressed at the surface of injured axons. Functional interference with NogoA or its receptor stimulated the recovery of function after spinal cord lesion (Giger et al., 2008). Neutralizing inhibitory molecules in the injured cord would be an important component of a multifaceted therapeutic strategy to promote axonal regeneration. Given the diversity of repulsive proteins, targeting of multiple ligands or their receptors...
will be required to produce extensive repair after CNS trauma. RNAi is a relatively new tool to silence gene expression in a sequence-specific manner. shRNAs can be used to simultaneously silence the expression of multiple genes (Brummelkamp et al., 2002, McManus et al., 2002, Paddison et al., 2002, Sui et al., 2002). We investigated whether this technology could be applied in the CNS to render injured neurons insensitive to multiple repulsive signals. As a first step in this direction we explored the feasibility to apply RNAi to interfere with the signaling of secreted chemorepulsive semaphorins in vivo.

Semaphorins are potent chemorepulsive axon guidance cues. Secreted semaphorins are expressed by meningeal fibroblasts invading the spinal cord lesion site (Pasterkamp et al., 2001, De Winter et al., 2002b). The receptor for secreted semaphorins is composed of a semaphorin binding subunit (neuropilin-1 or neuropilin-2) and a plexin signaling subunit (reviewed by Zhou et al., 2008). These receptors persist in corticospinal tract and rubrospinal tract (RST) neurons after injury (Gavazzi et al., 2000, De Winter et al., 2002b). Rubrospinal neurons express Npn-2 but not Npn-1. The signaling component plexin A1 and the intracellular signaling molecule CRMP2 are present in rubrospinal neurons (De Winter et al., 2002b). Following injury of the RST, the expression of plexin A1 and A4 persist, whereas plexin A2 is upregulated and A3 is undetectable in the red nucleus (Spinelli et al., 2007). Thus, this descending motor tract in the spinal cord is potentially sensitive to semaphorins in the lesion core. Axon outgrowth is considerably improved when neurons are cultured on semaphorin3A (Sema3A)-deficient meningeal cells (Niclou et al., 2003) and axon crossing from an astrocyte to a meningeal cell substrate is enhanced by blocking Npn-2 (Shearer et al., 2003). Recently, an inhibitor of Sema3A was successfully used to enhance regeneration and to produce a certain degree of functional recovery of the injured spinal cord (Kaneko et al., 2006). Interfering with semaphorin-neuropilin signaling would therefore be a promising strategy to overcome inhibition of axonal regeneration.

The potential of RNAi-based therapies as well as the utility of RNAi for basic research is widely recognized. A persistent question in the field of RNAi is how the efficiency and specificity of RNAi-mediated knockdown of gene expression can be improved. The development of RNAi has been hampered by cellular toxicity, which can be the result of interference with the endogenous miRNA machinery, the induction of innate immune responses, and off-target effects (Bridge et al., 2003, Sledz et al., 2003, Grimm et al., 2006, McBride et al., 2008, Boudreau et al., 2009a, Ulusoy et al., 2009). Here we document our attempts to block semaphorin receptor expression by expressing shRNA molecules in neuronal cells in vitro and in the red nucleus and DRG neurons in vivo. We show that shRNA-mediated knockdown of the semaphorin receptor Npn-1 and Npn-2 can be achieved in cultured neuronal cells by lentiviral vector derived shRNAs. One of two shRNA sequences was effective in AAV-mediated knockdown of Npn-2 in DRG neurons in vivo. Unexpectedly, AAV1-mediated expression of shRNAs in the red nucleus resulted in an adverse tissue response and neuronal degeneration.
We conclude that, although this technology has great potential to interfere with multiple inhibitory signaling pathways, the present results illustrate unanticipated problems related to the in vivo delivery of shRNA. We discuss a number of solutions that have to be implemented before this technology can be routinely applied to interfere with chemorepulsive signaling following neurotrauma.

**Results**

*Efficient in vitro knockdown of Npn-1 and Npn-2 by lentiviral delivery of shRNA*

As a primary screening method to assess knockdown efficiency of endogenous Npn-1 and Npn-2 expression levels, F11 cells, a fusion cell line derived from of rat embryonal DRG and mouse neuroblastoma cells (Platika et al., 1985), were transduced with lentiviral vectors encoding green fluorescent protein (GFP) and an shRNAs directed against Npn-1 or Npn-2 (Fig. 1a). Four days after transduction, total RNA was isolated. QPCR analysis revealed that Npn-1 expression after transduction with two shRNA sequences was significantly reduced to 31.4 ± 1.8 % and 17.5 ± 3.4 % respectively (Fig. 2a). Western blot analysis confirmed Npn-1 knockdown at the protein level by showing that expression was reduced to 39.2 ± 9.7 % and 5.7 ± 2.6 % (Fig. 2b,d). Two out of seven Npn-2 shRNA sequences successfully reduced Npn-2 mRNA expression to 7.8 ±1.1 % and 13.3 ± 1.0 % respectively (Fig. 2c).

![Diagram A](image1.png)

**Figure 1 - Schematic representation of lentiviral and adeno associated viral constructs.** All viral particles express green fluorescent protein (GFP) under the cytomegalovirus (CMV) promoter flanked by the Woodchuck hepatitis posttranscriptional regulatory element (WPRE). In the lentiviral (LV) transfer vector (A) the shRNA expression cassette, driven by the H1 RNA promoter (H1), is placed back to back with the GFP expression cassette. In the adeno associated viral vector (AAV) the shRNA expression cassette is placed upstream of the CMV promoter (B). The packaging cassettes are flanked by inverted terminal repeats (ITR) or long terminal repeats (LTR) for the AAV and LV cassettes respectively.
AAV1-mediated overexpression of shRNA in the red nucleus results in a dose dependent adverse tissue response and neuronal degeneration

Recent data from our lab has shown that lentiviral vectors are suboptimal transducers of rubrospinal neurons. The efficiency of transduction of red nucleus neurons is much better when using AAV1 viral particles (Blits et al., 2003). Therefore, the control shRNA and the two shRNA cassettes that were effective in knocking down Npn-2 expression in vitro were cloned in the pTR-CGW AAV2 backbone (Fig. 1b). After packaging, the resulting AAV1 particles mediate both GFP and shRNA expression. These vectors were stereotactically injected in the red nucleus of rats. Animals were sacrificed 3 weeks after the injection and processed for GFP-immunohistochemistry. GFP immunohistochemistry was detected in the red nucleus, demonstrating efficient transduction efficiency. However all animals injected with AAV1 shRNA vectors displayed neuronal degeneration and an adverse tissue response (Fig. 3a and 3a’) as compared to the uninjected contralateral nucleus (Fig. 3a and 3a’’). High magnification

Figure 2 - Efficient lentivirus mediated knockdown in F11 cells. F11 cells were infected with a lentiviral vector expressing GFP and a control shRNA and two shRNAs directed against Npn-1 or Npn-2. Two days after infection Npn-1 mRNA expression was reduced to 31.4 ± 1.8% and 17.5 ± 3.4% (mean ± SEM)(A). Western blot (D) and quantification thereof (B) for Npn-1 showed a similar knockdown efficiency 39.2 ± 9.7% and 5.7 ± 2.6% (mean ± SEM). Npn-2 mRNA expression was knocked down to 7.8 ± 1.1% and 13.3 ± 1.0% (mean ± SEM)(C). (* <0.05, ** p < 0.005)
photomicrographs consistently showed atrophic morphology of neurons that were transduced with an AAV1 vector encoding shRNA (Fig. 3b and 4b). This adverse tissue response and aberrant cellular morphology was not present in AAV1-GFP transduced neurons (Fig. 3c and 4a), indicating that the effect was not due to GFP overexpression or AAV transduction per se.

The adverse tissue response was partially alleviated by injecting a 10 fold lower viral titer (Fig. 4c). Although more neurons appear to survive under these conditions, many still have an irregular and vacuolar morphology. An alternative strategy to attenuate the level of transgene expression in rubrospinal
neurons is the use of AAV2 particles. Previous experiments from our laboratory have shown that both spread and expression levels are reduced when using AAV2 as compared to AAV1 vector particles (Blits et al., 2003). When control shRNAs were expressed by injection of AAV2 vectors in the red nucleus, GFP immunohistochemistry showed a confined population of GFP positive neurons within the red nucleus (Fig. 4d). Lowering shRNA expression by reducing the injected AAV1 particles 10 fold (C) or using AAV2 particles (D) nearly completely alleviates toxicity. Scale bar A: 50µm

Figure 4 – Reduced toxicity by decreasing shRNA expression levels in the red nucleus. GFP expressing neurons in the red nucleus shows normal morphology after injection with AAV1-GFP throughout the red nucleus (A). AAV1-shRNA injection results in profound neuronal degeneration and only occasional GFP-positive profiles of transduced neurons are observed usually at some distance from the injection site (arrow heads) (B). Lowering shRNA expression by reducing the injected AAV1 particles 10 fold (C) or using AAV2 particles (D) nearly completely alleviates toxicity. Scale bar A: 50µm.
Figure 5 - AAV5 mediated knockdown of Npn-2 in DRG in vivo. AAV5 particles expressing GFP and a control shRNA (A, B, C) or shRNA directed against Npn-2 (D, E, F) were injected in the L4 and L5 DRG of 3 adult female wistar rats. GFP immunoreactivity (green: A, B, D, E) and Npn-2 mRNA (red: A, C, D, F) were visualized in the same section three weeks after AAV injection. A marked reduction in the percentage of Npn-2 expressing cells among the GFP positive cells was observed after shRNA expression: 16.5 ± 5.0 % versus 37.2 ± 6.6 % in control animals (G) (mean ± SEM, * p<0.05). Scalebar A: 100 µm
AAV5 mediated knockdown of Npn-2 in rat dorsal root ganglia

We also studied Npn-2 knockdown in a separate model often used for neuroregeneration studies, the rat dorsal root ganglia. Previous results showed that up to 80% of the DRG sensory neurons can be readily transduced by a single injection of AAV5 packaged viral genomes (Mason et al., 2010). We therefore packaged our Npn-2 targeting and control shRNA vectors in AAV5 particles and injected this vector in the L4 and L5 DRG of 3 adult female Wistar rats. Three weeks after injection Npn-2 expression was analyzed by in situ hybridization. In DRGs injected with virus expressing the control shRNA 37.2 ± 6.6 % of all GFP positive cells expressed Npn-2 (Fig. 5a,b,c,g). One of the two shRNA sequences was able to reduce the proportion of Npn-2 expressing cells to 16.5% ± 5.0 (p<0.05) while the second shRNA was not effective (Fig. 5d,e,f,g). In contrast to the shRNA induced toxicity and aberrant cell morphology observed in the red nucleus, the DRG neurons appeared unaffected by the expression of shRNAs.

Discussion

The aim of the present study was to develop an RNAi based strategy to knock down the expression of the class-3 semaphorin receptors Npn-1 and Npn-2 in neurons of spinal nerve tracts and to employ this methodology to investigate the proposed involvement of these receptors in the failure of CNS-axons to regenerate. We have successfully developed shRNAs that knock down the expression of both neuropilins in a neuronal cell line. In vivo, AAV5 mediated expression of the most effective Npn-2 shRNA resulted in knockdown of Npn-2 in DRG sensory neurons. Unexpectedly, AAV5-mediated expression of a second shRNA had no effect. AAV1-mediated expression of a control shRNA and a Npn-2-shRNA in the red nucleus resulted in an adverse tissue response, including neuronal cell degeneration. These observations demonstrate that, although this technology would have great potential to interfere with multiple chemorepulsive signaling pathways, unanticipated problems with cytotoxicity currently preclude the routine use of this approach in studies on neural repair in vivo.

Selection of shRNAs for in vivo use

Despite considerable efforts to improve the selection of effective RNAi target sequences, including the development of various algorithms (Reynolds et al., 2004, Ui-Tei et al., 2004, Li et al., 2007) and the use of favourable thermodynamic properties (Khvorova et al., 2003, Schwarz et al., 2003), several shRNA sequences against a particular target mRNA need to be screened to obtain efficient knockdown. We initially developed two shRNAs for Npn-1 and seven for Npn-2 and evaluated their capacity to silence Npn-1 or Npn-2 expression in F11 cells. Both Npn-1 and two out of seven Npn-2 shRNAs exhibit potent gene silencing following lentiviral vector-mediated delivery to the F11 cell line. When using
a standard transfection for shRNA expression, most shRNA sequences are capable of reducing target expression (our own observation, not shown). The decreased effectiveness of lentiviral vector-mediated knockdown could be due to significantly lower shRNA expression levels as compared to an expression level achieved using transfection methods. Standard transfection methods deliver several hundred thousand plasmid molecules to one single cell resulting in fast-onset high level expression of shRNAs. In contrast, when shRNAs are introduced by means of lentiviral delivery, the number of genomic copies per cell is reduced by at least 3 orders of magnitude. This would result in reduced shRNA expression levels and consequent diminished knockdown efficiency of the target mRNA.

**In vivo studies**

The efficacy of the selected shRNAs was tested in two neural systems that are widely used to study axonal regeneration: the sensory neurons of the DRG and the neurons of the red nucleus that form the rubrospinal tract. Work from our laboratory has shown that, as compared to AAV vectors, lentiviral vectors poorly transduce sensory neurons in vivo (Mason et al., 2010) and are suboptimal transducers of rubrospinal neurons (Blits et al., 2010). Therefore the most effective shRNAs were expressed via AAV5 vectors in DRG and via AAV1 vectors in rubrospinal neurons. We chose these two AAV serotypes because we have shown that these are the most efficient AAV serotype vectors for these two neuronal populations (Blits et al., 2010, Mason et al., 2010).

Despite our preselection of the most effective shRNA by lentiviral vector-mediated gene silencing in F11 cells, only one of the two selected shRNAs was capable to significantly reduce the population of Npn-2 expressing DRG neurons. Although the observation that the second shRNA is somewhat less effective in vitro may already be an indication that it would be less capable to knock down Npn-2 in vivo, these observations also demonstrate that a shRNA that is effective in vitro not necessarily works in vivo.

AAV1-mediated shRNA expression in the red nucleus caused an unexpected adverse tissue reaction. Three weeks after AAV1 injection, many of the rubrospinal neurons contain vacuolar structures and have an atrophic appearance. Furthermore, there is considerable cell death as shown by the loss of neurons and the acellular granular structure of the tissue at the site of AAV injection. This phenomenon is unrelated to the shRNA sequence used and is not seen after AAV1-mediated GFP expression. It has been reported that saturating the miRNA machinery by overexpressing shRNAs, inhibits endogenous miRNA processing (Lund et al., 2004, Yi et al., 2005, Grimm et al., 2006, Boudreau et al., 2009a) with concomitant adverse effects on the transduced cells (Grimm et al., 2006, McBride et al., 2008, Boudreau et al., 2009a, Ulusoy et al., 2009). In the nucleus, exogenous shRNAs can saturate the function of exportin-5, a factor required for nuclear export of pre-miRNAs and shRNAs (Yi et al., 2003, Lund et al., 2004, Yi et al., 2005). This saturation can be reversed by overexpression...
of exportin-5 enhancing shRNA and endogenous miRNA activity in vitro (Yi et al., 2005) and in vivo (Grimm et al., 2006). Exportin-5 expression is relatively low in brain tissue (Yi et al., 2005) as compared to other tissues, rendering the brain particularly sensitive to exportin-5 function saturation. Similarly, in the cytoplasm, saturating the endogenous RNA induced silencing complex (RISC) may interfere with endogenous RNAi. The observation that overexpression of the catalytic RISC RNase component argonaut-2 enhances shRNA activity (Diederichs et al., 2008) demonstrates that argonaut-2 is a rate limiting component in RNAi and is therefore prone to saturation. The cytotoxic effects observed here following AAV1-mediated delivery of shRNAs to the red nucleus appears very similar to the toxicity described before (Grimm et al., 2006, McBride et al., 2008, Boudreau et al., 2009a). Saturation of the endogenous miRNA machinery may also underlie these adverse effects since lowering the viral dose, and thus the shRNA expression levels, reduced although not completely curtailed the toxic effects.

Interestingly, no toxicity was observed after AAV5-mediated delivery of shRNA to the DRG. This could be explained by our observation that, in terms of GFP expression level, AAV1-mediated expression in the red nucleus outperforms AAV5-mediated expression in the DRG. If the same holds true for shRNA expression levels, the differential expression levels could explain the difference in toxicity. Controlling the shRNA expression level by means of viral vector dose (Ulusoy et al., 2009), regulatable promoters (van de Wetering et al., 2003), tissue specific promoters (Giering et al., 2008) or the use of miRNAs (Grimm et al., 2006, McBride et al., 2008, Boudreau et al., 2009a), will be important variables for future study and in vivo application of shRNAs.

Conclusions

Our data shows that we were able to generate shRNA sequences that efficiently knock down Npn-1 and Npn-2 expression in a neuronal cell line using a lentiviral vector delivery system. Substantial in vivo reduction of Npn-2 expression was achieved by injection of AAV5-shRNA in the DRG, without clear indication of cellular toxicity. In contrast, AAV1 mediated shRNA expression in the red nucleus triggered an adverse tissue response leading to neuronal degeneration. This cellular toxicity is likely due to high levels of shRNA expression resulting in saturation of the endogenous miRNA machinery, and has to be resolved for this technique to be routinely used in neurobiological studies.
Methods

Cloning and characterization of Npn-1 and Npn-2 shRNA sequences

Oligonucleotides encoding shRNAs directed against Npn-1 and Npn-2 (table 1) were cloned in the pRRLsinPPTTh lentiviral (LV) vector, expressing GFP under the cytomegalovirus (CMV) promoter 3' flanked by a Woodchuck hepatitis posttranscriptional regulatory element (WPRE) and a H1 RNA polymerase promoter expression cassette (Fig. 1a). As a control a shRNA lacking homology to the rat transcriptome, targeting the Arabidopsis Thaliana FUSA 5 gene, was generated (table 1). Viral particles were packaged as described before (Naldini et al., 1996b) using the pMD2.G and pCMVΔR8.74 packaging plasmids. F11 cells were transduced with a multiplicity of infection (MOI) of 100. Four days after transduction, protein and total RNA was isolated and neuropilin expression levels were determined by qPCR analysis and Western Blot. The two most effective shRNA sequences were cloned in an AAV vector plasmid containing AAV2 inverted terminal repeats, a CMV-GFP expression cassette 3’ flanked by a WPRE and a H1 RNA § promoter expression cassette (Fig. 1b).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npn-1*</td>
<td>cttcaacccacatttcgat</td>
</tr>
<tr>
<td>Npn-1b</td>
<td>gatagtaagaggtgcatca</td>
</tr>
<tr>
<td>Npn-2</td>
<td>ggagtatctccaggtggac</td>
</tr>
<tr>
<td>Npn-2</td>
<td>gccagccaggtgaagaat</td>
</tr>
<tr>
<td>Npn-2a</td>
<td>agattgtcctcaaactcaaa</td>
</tr>
<tr>
<td>Npn-2</td>
<td>tggccggattgctaata gag</td>
</tr>
<tr>
<td>Npn-2</td>
<td>cattgagttcaat ac caa</td>
</tr>
<tr>
<td>Npn-2</td>
<td>caagaggtatctccaggttgga</td>
</tr>
<tr>
<td>Npn-2b</td>
<td>caagccagccaggtgaagaat</td>
</tr>
</tbody>
</table>

Table 1. shRNA targeting sequences. Targeting sequences in rat neuropilin 1 (Npn-1) and rat neuropilin 2 (Npn-2) genes used to generate short interfering RNAs (shRNA). (a,b) Sequences that displayed efficient shRNA mediated knockdown of Npn-1 and Npn-2 in F11 cells when delivered by lentiviral vectors. The Arabidopsis Thaliana FUSA 5 gene (Fusa5) targeting sequence was used to create a non functional control shRNA construct.
qPCR analysis

cDNA was synthesized using m-MLV reverse transcriptase (Invitrogen) according to manufacturer's guidelines with 500 ng total RNA and random hexamers. Npn-1 and Npn-2 expression levels were determined by quantitative PCR on an Applied Biosystems 7300 real-time PCR system using SYBR green master mix (Applied Biosystems) and 0.3 mM oligonucleotide (Eurogentec) (table 2). Gapdh, Beta-actin and Ef1-alpha expression levels were used to normalize the data for variations in cDNA input.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acc.No</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
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<tr>
<td>Npn-1</td>
<td>NM_145098</td>
<td>ctgtgcacaaaccacaagcagctagat</td>
<td>gttcttgctgcctttcttct</td>
</tr>
<tr>
<td>Npn-2</td>
<td>NM_030869</td>
<td>tcgggagagatttccatcga</td>
<td>aaagccgagatggttcac</td>
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<tr>
<td>Beta actin</td>
<td>NM_031144</td>
<td>gctccctctagccgaag</td>
<td>cactgtgctggaagtggtca</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_017008</td>
<td>tgcaccaaccaactcttcagc</td>
<td>ggcatgaactgtggcatga</td>
</tr>
<tr>
<td>Ef1 alpha</td>
<td>NM_175838</td>
<td>accccctctccgttccttttg</td>
<td>agtcctgcagctctttgact</td>
</tr>
<tr>
<td>CMV promoter</td>
<td>n.a.</td>
<td>aatgggccggctagccttgta</td>
<td>aggcagatctgacgggtcactaa</td>
</tr>
</tbody>
</table>

Table 2. Primers used for QPCR analysis

Western blot analysis

Cells were lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1 % SDS pH 7.5). Samples were separated on 8% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). To detect endogenous Npn-1 a goat anti-Npn-1 antibody (1:1000, AF566, R&D systems) was used. A mouse anti-actin antibody (1:1000, A5316, Sigma-Aldrich) was used to correct for variation in gel loading. Bands were visualized and quantified using an Odyssey Infrared Imaging Station (LI-COR) using a donkey anti-mouse-IRDye800 antibody, donkey anti-goat IRDye800 (both 1:4000, Rockland Immunochemicals) or donkey anti-mouse-Cy5 (1:400, Jackson ImmunoResearch)

Adeno-associated viral vector preparation

Ten 9.5cm culture dishes (Greiner Bio-One) of HEK293T cells were transfected with 50 µg AAV vector plasmid and 150 µg packaging plasmid (pDG1, pDG2 or pDG5 for AAV serotype 1, 2 or 5 respectively) (Grimm et al., 2003). The cells were harvested in lysis buffer (50 mM Tris-HCl pH 8.5, 150mM NaCl, 2 mM MgCl₂, 1% Triton X-100) 72 hrs after transfection and incubated with 10 µg/ml DNase I (Roche) for 1 hour. Cell lysates were cleared by centrifugation at 3,200 RCF for 15 minutes and centrifuged on a step gradient containing 60, 40, 25 and 15% iodixanol (Axis Shield) for 1h10m at 69,000 RPM in a Ti70 rotor (Beckman...
Coulter). The virus was recovered at the 40-60% interface and concentrated in Dulbecco’s phosphate buffered saline (D-PBS) with 5% sucrose using a 100 kDa MWCO Amicon Ultra-15 centrifugal device (Millipore). To determine the viral titer, viral ssDNA was isolated by digesting the protein capsid with Proteinase K (Roche) and purified using MageneSil Blue beads (Promega) in SV RNA lysis buffer (Promega). Viral titers were determined by qPCR using SYBR green master mix (Applied Biosystems) and 0.3 mM primers directed against the CMV promoter (table 2).

**Experimental animals**

A total of 42 adult female Wistar rats (225-250 g, Harlan) were used in this study. Animals were housed in groups under standard conditions with food and water ad libitum and a 12h:12h light/dark cycle. Experimental procedures were performed in accordance with the committee for laboratory animal welfare and experimentation of the Royal Netherlands Academy of Sciences.

**AAV mediated knockdown of Npn-2 in the red nucleus**

The experimental groups were composed as follows: Six animals were injected with AAV1 expressing Npn-2 shRNA(a) (1.4x10^{12} GC/ml), Npn-2 shRNA(b) (1.1x10^{12} GC/ml) or control shRNA (1.7x10^{12} GC/ml). To further study the dose dependent toxicity, 3 animals were injected with 1 µl AAV1 expressing control shRNA (1.7x10^{12} and 1.7x10^{11} GC/ml) or GFP only (1.7x10^{12} GC/ml). To assess toxicity induced by AAV2-mediated expression of shRNAs, 6 animals were injected with 1 µl AAV2 expressing control shRNA (6.0x10^{11} GC/ml). Stereotaxic injections were performed as described previously (Ruitenberg et al., 2002) under deep anesthesia with an intramuscular injection of Hypnorm (Fentanyl/Fluanisone, 0.08 ml/100 g, Janssen Pharmaceuticals) and Dormicum (Midazolam, 0.02 ml/100 g, Roche). The skull was exposed and a hole was drilled to aid positioning of the needle. A glass needle was lowered into the brain at A/P – 5.4; L+0.7; DV-6.6 from bregma. 1 µl viral vector was infused at a rate of 0.2 µl/min. Three weeks after vector injection animals were euthanized by injecting an overdose of Nembutal (sodium pentobarbital, Sanofi Sante) followed by transcardial perfusion of sequentially ice cold saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer.

**Injection of AAV5 in dorsal root ganglia**

Per virus, three adult female Wistar rats were injected with virus expressing control shRNA (2.2x10^{12} GC/ml), Npn-2 shRNA(a) (1.9x10^{12} GC/ml) or Npn-2 shRNA(b) (3.9x10^{12} GC/ml). Animals were deeply anesthetized by an intramuscular injection of Hypnorm (Fentanyl/Fluanisone, 0.08 ml/100 g, Janssen Pharmaceuticals) and Dormicum (Midazolam, 0.02 ml/100 g, Roche). The muscles overlaying the lumbar vertebral column were retracted and dorsal
root ganglia at L4 and L5 were exposed by a partial laminectomy. 1 µl viral vector was unilaterally injected in the L4 and L5 DRG at a rate of 0.2 µl/min. Muscle layers were sutured and the skin was closed with Michell clips (Fine science tools). Three weeks after vector injection animals were euthanized by injecting an overdose of Nembutal (sodium pentobarbital, Sanofi Sante) followed by transcardial perfusion of ice cold saline followed by 4% PFA in 0.1 M phosphate-buffer.

**Immunohistochemistry and In situ hybridization**

After perfusion and dissection, tissue was post fixated in 4% PFA in P-buffer, incubated in 0.25 M EDTA/PBS and cryoprotected in 25% sucrose/PBS. Tissue was embedded in OCT compound (Sakura) and snap frozen in 2-methylbutane. 20 µm Cryo sections were cut, mounted on Superfrost Plus slides (Fischer scientific) and stored at -80 °C. Immunohistochemistry staining for GFP was performed by blocking sections for one hour in Tris buffered saline (TBS) containing 0.4% Triton X-100 and 5% fetal calf serum (FCS), followed by incubation with rabbit anti-GFP (1:100, AB3080, Chemicon) for 16 hours at 4°C. After 3 washes sections were incubated with donkey anti-rabbit-Biotin (1:400, DAKO) for 3 hours in TBS, 5% FCS and 0.4% Triton X-100 followed by streptavidin-Alexa488 (1:400, Invitrogen). Fluorescent images were captured using a Zeiss LSM510 confocal laser scanning microscope. To visualise general brain histology, sections were counter stained with cresy violet after which bright field images were acquired using a Zeiss Axioplan microscope.

In situ hybridization was performed using digoxigenin (DIG) labelled RNA probes transcribed from rat Npn-2 cDNA as described before (Pasterkamp et al., 1998). Antisense and sense probes were generated by in vitro transcription from linearized cDNA templates using T3 or T7 RNA polymerase (Roche) and alkali-hydrolyzed to an average length of 200 nucleotides. Slides were post-fixed in 4% PFA in PBS, digested for 10 minutes with 10 µg/ml Proteinase K (Boehringer Mannheim) in PBS containing 0.1% Triton X-100, post fixed for 15 minutes in 4% PFA in PBS and acetylated for 10 minutes in 1% triethanolamine containing 0.25% acetic anhydride. Hybridization was performed for 16 hours at 60 °C in 5xDenHarts, 250 µg/ml yeast tRNA, 5xSSC and 50% formamide. After stringency washes (all at 60 °C, 5 minutes 5xSSC, 1 minute 2xSSC and 30 minutes 0.2xSSC, 50% formamide) slides were blocked in 1% blocking reagent (Roche) and incubated for 16 hours at 4°C with alkaline phosphatase conjugated anti-DIG fab fragments (1:3000, Boehringer Mannheim) and rabbit anti-GFP (1:100, AB3080, Chemicon) in 1% blocking agent. Secondary antibody incubations were preformed as described before. The anti-DIG antibody was visualized by incubating with Fast Red (Sigma) following manufacturers guidelines allowing color development for 16 hours at room temperature. Fluorescent images were acquired using a Zeiss Axioplan2 microscope. An algorithm was designed in Image-Pro plus (MediaCybernetics) to outline all GFP positive neurons. Per DRG,
all Npn-2 positive cells were counted within this GFP positive cell population. For each experimental group (n= 6 DRGs), the average percentage of Npn-2 expression cells was calculated.

**Statistical analysis**

All results are expressed as mean ± SEM. Statistical significance was tested with the student's *t*-test. A value of *p* < 0.05 was considered significant.

**Acknowledgements**

The authors would like to thank Joop van Heerikhuize for his excellent technical assistance with image analysis and Jurgen Kleinschmidt for providing the AAV packaging plasmids. These studies were supported by the International Spinal Research Trust (STR 090), the European Commission under the 7th Framework Programme - HEALTH - Collaborative Project Plasticise (223524) and the Netherlands Institute for Neuroscience.
Genetic mutation of the semaphorin 3A receptor component neuropilin-1 in neurons does not enhance corticospinal tract regeneration

Erich M.E. Ehlert, Ruben Eggers and Joost Verhaagen

manuscript in preparation
Abstract

After a spinal cord lesion, a myriad of axon growth inhibitors present in myelin and in the neural scar contribute to the failure of injured axons to regenerate. Class 3 semaphorins are repulsive axon guidance cues that are induced in the meningeal fibroblasts that infiltrate the neural scar. Most if not all injured spinal cord neurons, including neurons that form the corticospinal tract (CST), continue to express the semaphorin receptor components neuropilin (Npn-1 and 2) and plexinAs. This had led to the hypothesis that semaphorin/neuropilin signalling limits axonal regeneration through the scar. To test this hypothesis we have investigated the regeneration of the CST in mice with an Npn-1-deletion specifically targeted to neurons and in littermate control mice. Mice deficient in Npn-1 in neurons do not exhibit enhanced regenerative growth of injured CST axons and do not display improved recovery of motor function as compared to control littermates. We therefore conclude that Npn-1/Sema3A signalling does not have a major impact on the failure of injured CST axons to regenerate. This illustrates that neuron-specific targeting of a single ligand-receptor pair (Sema3A/Npn-1) in the multi-component, divergent semaphorin/neuropilin/plexin signalling pathway is insufficient to enhance regeneration of the CST.

Introduction

Following a spinal cord lesion, axon growth inhibitors present in CNS myelin and in the neural scar are considered to contribute to the failure of injured CNS axons to regenerate. Nogo, Myelin-associated glycoprotein (MAG) and Oligodendrocyte myelin glycoprotein (OMgp) are the prototypical myelin-derived axon growth inhibitors. Chondroitin sulphate proteoglycans (CSPGs) and chemorepulsive axon guidance proteins of at least four gene families (ephrins, slits, RGM and semaphorins) are induced in cells of the neural scar and may act as major impediments to regenerative axon growth (reviewed by Giger 2010, Niclou et al. 2006, Fawcett 2006, Bolsover 2008).

The hypothesis that the failure of axon regeneration in the CNS is, at least to some extent, caused by molecular inhibitors, has been tested extensively. A majority of these studies have focused on the contribution of myelin-derived growth inhibitors. Two independent laboratories demonstrated that mice with a mutated Nogo gene have increased axon regeneration of lesioned spinal cord axons (Kim et al., 2003, Simonen et al., 2003, Dimou et al., 2006, Cafferty et al., 2010). A third laboratory, however, did not observe significant improvements in sprouting or axon regeneration (Zheng et al., 2003, Lee et al., 2009, Lee et al., 2010b). Genetic deletion of MAG or OMgp did not enhance CST regeneration, but had a modest effect on the sprouting of serotonergic fibers (Bartsch et al., 1995, Ji et al., 2008, Lee et al., 2010b). The myelin derived growth inhibitors bind to the Nogo-A receptor (NgR1) and paired Ig-like receptor B (PirB) (Fournier et
al., 2001, Atwal et al., 2008) and signal through co-receptors including p75<sup>NTR</sup>, LINGO-1 and TROY (Wang et al., 2002a, Mi et al., 2004, Park et al., 2005, Shao et al., 2005). NgR1 knockout (KO) animals display a moderately improved regeneration capacity of certain spinal nerve tracts, although regeneration of the corticospinal tract (CST) was not affected (Kim et al., 2004, Zheng et al., 2005). In mice where p75<sup>NTR</sup> or PirB was genetically deleted, regeneration of the CST was also unaffected (Zheng et al., 2005, Nakamura et al., 2011). The first efforts in dealing with the redundancy of these myelin inhibitors and their receptors have led to conflicting results (Cafferty et al., 2010, Lee et al., 2010b).

The inhibitory effect of scar-associated CSPGs on axon regeneration has been overcome by infusing the enzyme chondroitinase (ChABC) into the lesioned spinal cord. This enzyme removes the glycosaminoglycan (GAG) chains from the CSPGs and this produces a more permissive environment for injured axons that encounter the inhibitory neural scar. Following ChABC treatment a small number of transected axons was able to regrow back for several millimetres into and beyond the neural scar (Moon and Fawcett, 2001, Bradbury et al., 2002). These studies have been replicated in multiple injury paradigms and collectively demonstrate the potential of this approach (reviewed by Bradbury and Carter, 2011).

The studies on myelin inhibitors and CSPGs show that interfering with the signalling of neurite inhibitory molecules is a promising approach to promote axon regeneration in the CNS. But it is also clear that after the interventions described above, regeneration is still limited. It is likely that the induction of a number of repulsive axon guidance molecules in scar-associated cells could potentially prevent more profound regeneration (Luo et al., 1993, Fawcett and Asher, 1999, Pasterkamp et al., 1999). Evidence for the contribution of repulsive axon guidance molecules to regenerative failure has recently been demonstrated in mice with genetic deletion of EphA4 (Goldshmit et al., 2004). Additional evidence comes from experiments using neutralizing compounds or inhibitors that functionally interfere with RGMa or semaphorin3a (Hata et al., 2006, Kaneko et al., 2006).

In this article we have investigated the contribution of neuronal neuropilin-1 signaling to the failure of injured CST axons to regenerate. Semaphorins were originally identified as repulsive axon guidance molecules that act during CNS development (Kolodkin et al., 1993, Luo et al., 1993). In the adult nervous system, the secreted, class 3 semaphorins (Sema3s) are re-expressed by meningeal fibroblasts that form the core of the glial scar (Pasterkamp et al., 2001, De Winter et al., 2002b). Cultured meningeal fibroblasts do express high levels of Sema3A and meningeal fibroblasts of Sema3A KO animals are a more permissive substrate than WT cells for sensory axons (Chapter 2, Niclou et al., 2003). Moreover human peripheral nerve scar tissue contains Sema3A and neurons cultured on slices of neuroma tissue display increased neurite outgrowth when the Sema3A receptor expression in these neurons is reduced by short hairpin RNA-mediated knock-down (Tannemaat et al., 2007).
With the exception of Sema3E, all Sema3s act through binding to neuropilin, a protein that is part of a multimeric semaphorin receptor complex (He and Tessier-Lavigne, 1997, Kolodkin et al., 1997). Semaphorin receptors continue to be expressed by injured CST and DRG neurons (Gavazzi et al., 2000, De Winter et al., 2002b). Regenerating axons are therefore well-equipped to respond to these axon guidance cues. Adult sensory neurons retain their sensitivity to Sema3A in vitro and in vivo (Tanelian et al., 1997, Reza et al., 1999, Tang et al., 2004), however, the response of severed descending spinal cord axons to semaphorins is largely unknown. To determine to what extent the direct interaction between Sema3A and axonal Npn-1 contributes to impaired axonal regeneration, we generated knockout mice in which Npn-1 is specifically ablated in neurons just before birth and during early postnatal development. In contrast to constitutive Npn-1 KO mice, which die midway through gestation (Kitsukawa et al., 1997, Kawasaki et al., 1999), the genetic mutation of Npn-1 in late embryonal and early postnatal neurons results in viable mice that survive into adulthood. We show, that following CST lesion, disruption of Npn-1 in these neuron-specific Npn-1 mutants does not improve the outgrowth of CST axons. In addition, we do not observe enhanced recovery of motor function in lesioned conditional Npn-1 KO mice.

Methods

Experimental animals

The Npn-1 conditional knockout mice (Gu et al., 2003) were provided by Dr Alex L Kolodkin and Dr David D Ginty (The Johns Hopkins University School of Medicine, Baltimore, MD). Transgenic animals expressing Cre recombinase under control of the murine Neurofilament-H promoter (B6;129(FVB)-Tg(Nfh-cre)1Kul) were obtained from the Mutant Mouse Regional Resource Centres (MMRRC, Davis, CA). These mouse lines were crossbred to obtain Npn-1<sup>f/f</sup> / NefhCre<sup>+/−</sup> mice, hereafter labeled as Npn-1 KO mice in a C56BL/6 background. Npn-1<sup>f/f</sup> / NefhCre<sup>−/−</sup> littermates, hereafter labeled as wild type mice (WT) were used as control animals. Animals were housed in groups under standard conditions with food and water ad libitum and a 12h:12h light/dark cycle. Experimental procedures and behavioral tests were performed in accordance with the committee for laboratory animal welfare and experimentation of the Royal Netherlands Academy of Sciences.

Animal surgery

CST transection: Animals were deeply anesthetized by an intraperitoneal injection of Hypnorm (0.1 mg/kg Fentanyl citrate/ 3.3 mg/kg Fluanisone HCl, Janssen Pharmaceuticals) and Dormicum (8.3 mg/kg Midazolam, Roche). During surgery, body temperature was maintained at 37°C using a heating pad. The spinal cord
was exposed by laminectomy of the C4 vertebra. The dura matter was opened using Vannas scissors followed by a bilateral lesion of the dorsal column using a micro knife transecting the dorsomedial CST, leaving the dorsolateral CST intact. Muscle layers were sutured and the skin was closed with Michell clips (Fine science tools). Postoperative analgesia was administered by one subcutaneous injection of Metacam (0.4 mg/kg, Boehringer Ingelheim). In animals of the sham procedure group a laminectomy was performed leaving the dura-mater and spinal cord intact.

CST tracing: CST fibers were anterogradely traced four weeks after surgery. To this end animals were anesthetized as described before and 0.8 µl of biotinylated dextran amine (BDA) solution (10% in PBS, MW 10.000, Invitrogen) was infused bilaterally into the primary motorcortex at a flow of 0.2 µl/min (coordinates 0.26, 1; 1.18, 1.25; 1.18, 1.8; 1.5, 1.8 millimeter anterior/posterior, lateral from bregma).

Experimental groups: All animals were 13 to 17 weeks of age on the day of surgery. A total of 14 KO and 12 WT mice received a bilateral CST lesion. The sham group consisted of 9 WT mice

Behavioural testing and evaluation
All animals were tested 3 days before and 3, 7, 10, 14, 17, 21, 24 and 28 days after surgery.

Narrow beam walk: To evaluate regain of proper hind limb placements after CST lesion, a narrow beam test was performed. Animals were pre-trained for one week to cross a 8mm wide, 100cm long and 15 cm elevated beam. The narrow beam was flanked on both sides by a platform from which the animals initiated a run voluntarily. The total number slips and steps of both hind limbs were counted and averaged from 3 successful runs by two observers blinded to the experimental group.

Forelimb grip strength: Forelimb muscle strength was measured using the grip strength meter (TSE-systems). Mice were held by the base of the tail and positioned above the grip-bar. Upon lowering, the animal grabbed the bar after which the animal was gently pulled away from the bar. The maximum force at which the mouse released the bar was averaged for five trials. To correct for variations between animals, the muscle strength was normalised for the maximum strength observed at 3 days before the lesion.

Rotarod: To assess overall motor coordination, animals were paced on the Rotarod (Ugo Basile Biological Research Apparatus) rotating at a constant speed of 5 RPM. The rotation was accelerated to 40 RPM over a period of 5 minutes. The time the mice could remain on the rotating beam was recorded and normalised for the maximum performance at 3 days before surgery.

Catwalk gait analysis: Gait analysis was preformed as described before (Hamers et al., 2001, Hendriks et al., 2006). Briefly, mice were pre-trained for one week to cross a 100 cm long glass plate, confined by Plexiglas walls 35mm apart
in a darkened room. Paw prints were recorded digitally and analysed using the Catwalk software. All analysed parameters were normalised for the performance at 3 days before surgery.

**Tissue preparation**

One week after CST tracing, animals were euthanized by injecting an overdose of Nembutal (sodium pentobarbital, Sanofi Sante) followed by transcardial perfusion of ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer. The spinal cord and brain were dissected and post fixed overnight at 4°C followed by incubation in phosphate buffered saline (PBS) containing 0.25M EDTA and cryopreservation in PBS containing 25% sucrose. Tissue was embedded in OCT compound (Sakura), snap frozen in 2-methylbutane and stored at -80°C until sectioning. 20 µm Thick transversal cryosections at cervical level C1 were thaw mounted on Superfrost Plus slides (Fisher Scientific). The C2-C6 spinal cord segment was cryosectioned sagitally. All sections were dried and stored at -80°C until use.

**Histological analysis and quantification**

To determine the total number of BDA traced CST fibers, transversal sections were incubated in Tris-buffered saline (TBS), 0.2% Triton X-100, 5% bovine serum (block buffer) for 1 hour followed by incubation with streptavidin-Alexa488 (1:400, Invitrogen) in blocking buffer for 3 hours at room temperature. The sections were washed three times in TBS containing 0.1% Triton X-100. Tiled images of the dorsal column were captured using an Axioplan 2 microscope (Zeiss) with a 40x objective. The CST was outlined using Imagepro Plus (MediaCybernetics) and a grid was placed over the outlined area. Fibers were counted in approximately 25% of a systematic randomized selection of the outlined area.

Sagittal sections were blocked as described above and incubated overnight at 4°C with Rabbit-anti-GFAP (1:1000, DAKO) in block buffer. The following day, sections were washed three times in TBS containing 0.1% Triton X-100 and incubated with goat anti-Rabbit-Cy3 (1:400, Jackson Immunoresearch) and streptavidin-Alexa488 (1:400, Invitrogen) for 3 hours at room temperature. The sections were washed 3 times in TBS containing 0.1% Triton X-100 and coverslipped. Tiled images were captured from every second section using an Axioplan 2 microscope (Zeiss) with a 20x objective.

Using the GFAP IHC signal, the ventral, caudal and rostral borders of the lesion site were identified. Using these three reference points, the center of the lesion was determined. In all images containing BDA positive CST fibers and lines were placed in dorsal-ventral orientation in the core of the lesion and 0.25, 0.50, 0.75 and 1.0 mm rostral and caudal from the lesion center using Imagepro Plus.
(MediaCybernetics). The number of CST fibers running through the white matter that crossed these lines was counted.

**Statistics**
All results are expressed as mean ± SEM. For the CST fiber index and narrow beam test, statistical significance was tested with a Kruskal-Wallis test followed by a Mann-Whitney U post hoc test. The rotarod, grip strength and catwalk experimental data were tested with an ANOVA analysis with a Bonferroni post hoc test. A value of p < 0.05 was considered significant.

**Results**

*Characterisation of Npn-1<sup>f/f</sup> / NefhCre<sup>+/−</sup> mice*
Mice that are homozygous for the loxP flanked exon 2 of Npn-1 and carry one allele of NfhCre (Npn-1<sup>f/f</sup> / NefhCre<sup>+/−</sup>) are born at Mendelian distribution and survive into adulthood.

*Histological analysis of injured CST fibers*
To analyse the location of CST fibers, animals were traced by injecting BDA in the motor cortex. One week after tracing, animals were perfused and spinal cord tissue was processed for immunohistochemical analysis. One knock out and one wild type animal died during CST lesion surgery. One knockout animal died during the BDA tracing procedure. Due to tissue processing error, histological analysis was not possible on 2 KO, 2 WT and 3 sham group animals. Therefore complete analysis was carried out on 10 KO, 10 WT and 5 sham animals. In all lesioned animals the dorsal CST lesion was complete. The total number of traced CST fibers was counted in transversal sections on cervical level C1 (Fig. 1b). We observe no significant differences in the C1 fiber counts in lesioned WT and KO animals (WT:1035 ± 150, KO:1276 ± 313). The number of CST fibers at 1, 0.75, 0.5, 0.25 and 0 mm caudal and rostral to the center of the lesion was determined in sagittal sections (Fig. 1a,c). To correct for variation in tracing efficiency, the CST fiber index was calculated by dividing the fiber counts proximal and distal to the lesion by the total number of CST fibers at the C1 cervical level (Fig. 1d). In all animals the CST fiber index observed 1 mm caudal to the lesion was similar to non-injured animals at the corresponding cervical level. In lesioned animals the number of fibers decreased as they advanced into the GFAP positive area around the injury site. In a few animals, a minute fraction of the fibers reached to or beyond the core of the lesion. Overall, we did not observe significant changes in fiber indexes at fixed distanced from the lesion core when comparing WT and KO animals (Fig. 1d).
Figure 1. CST fibers regenerate equally in Npn-1 knockout and wild type animals. Immunohistochemical double staining was used to visualize BDA traced CST fibers (green) and GFAP (red) in sagittal sections in the lesioned area (a, c). The total amount of CST fibers at cervical level C1 (b) was used to correct for tracing variability. Quantification of CST fibers at set intervals of the injury site (d) revealed no differences between wild type and knockout animals. Scale bar: 250 µm (a) and 50 µm (b,c)
Section: Behavioural analysis

**Narrow beam walk**

To analyse the regain of coordinated paw placement after CST injury, we subjected the animals to a narrow beam test. After one week of pre-training, all animals were able to cross the 8 mm wide beam with less than 5% misplacements or slips of the hind limbs (Fig. 2). Three days after the lesion, wild type animals were not able to properly place their hind limbs on the beam in 65.6 ± 8.3% of combined hind limb steps, where knockout animals made 84.2 ± 3.5% slips. At day 10 post injury, wild type animals had recovered to making 16.2 ± 1.8% slips from which point on they slightly recovered to 11.2 ± 1.3% at 28 days post injury. In contrast, knockout animals recovered to 32.8 ± 7.8% slips at 10 days post injury and gradually improved to making 20.2 ± 5.5% slips at 28 days post injury. Overall, upon CST injury, we observed an increase in hind paw slips in knockout as compared to wild type animals. This difference reached significance at 7, 14, 21 and 24 days post injury.

*Figure 2. Narrow beam walk analysis shows decreased coordinated hind paw placement in Npn-1 knock out mice. After CST lesion, Npn-1 KO mice showed 84.2 ± 3.5% of combined hind limb steps, where wild type animals made 65.6 ± 8.3% slips. For the remainder of the experiment the animals displayed a similar recovery profile, maintaining the difference in hind limb function. (* p<0.05 significance between KO and WT)*
Rotarod

Coordinated motor performance was measured using the Rotarod system. Three days after CST lesion, wild type and knockout animals performed at 59.6 ± 5.3% and 46.9 ± 5.9% respectively of their performance recorded before surgery (Fig. 3). Similar to the performance on the narrow beam test, knockout animals showed an increased loss of motor coordination upon CST lesion as compared to wild type animals. This trend was persistent throughout the duration of the experiment, but did not reach significance.

Forelimb grip strength

To measure forelimb grip strength, animals were pre-trained for one week before CST lesion. Three days after lesion wild type and knockout mice had maintained 63.9 ± 5.0% and 59.7 ± 4.7 respectively of their maximum grip strength where sham animals performed at 90.6 ± 11.5% of their initial strength (Fig. 4). The loss of grip strength of lesioned animals did not significantly change throughout the duration of the experiment. Unexpectedly, sham group grip strength started decline from 10 to 17 days post operation to 62.9 ± 4.8%
Automated gait analysis was performed using the catwalk system. We quantitatively analysed several specific gait parameters. The regularity index, the fraction of normally placed step sequences, was unchanged. The base of support of the front limbs decreased significantly to 75.5 ± 3.7% and 81.3 ± 3.4% in lesioned wild type and knockout animals on the third day after CST lesion and recovered completely on day seven for the remainder of the experiment (data not shown). The lesion had no effect on the base of support of the hind limbs. The stride length of the front and hind paws decreased as a response to the lesion (Fig. 5a,b). This decrease was significant for the front paws of knockout animals from 7 until 21 days after injury, and in hind paws of knockout animals at 10 and 21 days after injury. The wild type animals showed a trend towards complete recovery at 24 days after injury. The knockout group stride length recovery is delayed and is significantly different from the wild type injured animals at 20 and 24 days after injury for the front paws and at 24 days after injury for the hind paw measurements. Again, similar to the rotarod and narrow beam test, knockout animals have an increased response to the lesion that seems to persist throughout the duration of the experiment and reaches significance at the later time points due to the lack of recovery.
Discussion

In this study we have examined regeneration of the injured CST in conditional Npn-1 deficient mice by analysing the regrowth of transected CST fibers and functional recovery. We have done so by using an animal model in which the Npn-1 gene is ablated specifically in neurons around the time of birth. Our studies show that knocking out Npn-1 does not promote the regeneration of CST fibers. Conditional Npn-1 knockout animals appear to have an increased loss of function.

Figure 5. Impaired stride length recovery in CST lesioned Npn-1 KO animals. Upon CST lesion WT and Npn-1 KO mice show a decrease in fore (a) and hind limb (b) stride length. This motor impairment recovers completely in WT animals whereas in KO animals this trend of recovery is delayed. (* p<0.05 significance between KO and WT)
upon CST injury. The recovery of motor function in KO and WT mice is similar, but a subtle difference in gait behaviour suggests a delayed recovery of KO animals.

The presence of the chemorepulsive protein Sema3A in the neural scar that forms after injury to the CNS is well established but the relative contribution of Sema3A to the plethora of growth inhibitory molecules encountered by injured CNS axons has not been thoroughly studied. Studying spinal cord regeneration in mice lacking the Sema3A or Npn-1 gene would be a bona fide approach to determine the contribution of Sema3A-Npn-1 signalling to the inhibition of axonal regeneration. However, mice that lack the Sema3A receptor Npn-1 do not survive beyond developmental stage E12.5 as a result of extensive neural and vascular defects (Kitsukawa et al., 1997, Kawasaki et al., 1999). Similar nervous system defects have been reported for mice that lack the Sema3A gene (Behar et al., 1996, Taniguchi et al., 1997). Although most Sema3A KO mice die a few days after birth (Behar et al., 1996), the few mice that survive could be used for regeneration studies although these mice may have developmental abnormalities of the nervous system. However, besides the possible behavioural changes due to the developmental abnormalities of the nervous system, the constitutive knockout of Sema3A could have an effect on regeneration by directly affecting migration of cells that form the neural scar, by influencing neovascularization (Joyal et al., 2011) or oligodendrocyte precursor cell migration (Spassky et al., 2002, Williams et al., 2007, Syed et al., 2011). To study the repulsive effects of Sema3A on injured neurons only, we have generated a mouse model in which Npn-1 is mutated in neurons starting at embryonal stage E18.5 by using neurofilament-H promoter driven Cre recombination in a conditional Npn-1 KO mouse. The Nefh-cre expression is initiated around birth (Hirasawa et al., 2001), at which time the majority of the nervous system has fully developed. Although the CST continues to develop during the first two weeks after birth, the majority of CST fibers have grown past the thoracic level T1 at postnatal day P1 (Gribnau et al., 1986). While the anatomy of the CST at the cervical level of the spinal cord and overall motor performance of non-lesioned KO animals were not different from WT animals, we cannot rule out that other parts of the nervous system are affected by knocking out Npn-1 perinatally.

Four weeks after transection of the CST, we did not observe differences in CST fiber regeneration in WT and KO mice. Furthermore, Npn-1 KO animals do not show an improved recovery using various motor skill tasks. We did however observe an increased deficit in KO animals directly after CST injury. The acute nature of this phenotype indicates an underlying developmental difference between KO and WT animals that is only uncovered after injury. From these experiments it is clear that eliminating Npn-1 signalling alone does not influence the functional and histological outcome of a CST lesion. A number of factors may account for the absence of an effect on regeneration in Npn-1 KO animals. First, besides Npn-1, corticospinal neurons also express neuropilin-2 (Npn-2), the receptor for Sema3B, 3C and 3F, rendering the regenerating CST axons sensitive to the other members of the class 3 Semas that are expressed in the scar (De
Winter et al., 2002b). Secondly, the presence of other growth inhibitory molecules may mask the contribution of, or be dominant over the effects of Sema3A. This idea finds some support in the observations showing that it is possible to improve neurite outgrowth by interfering with Sema signalling in relatively simple tissue culture based models. Primary neurons on a monolayer of Sema3A KO meningeal cells display improved neurite outgrowth, and inhibiting Npn-2 signalling using blocking antibodies allowed neurites to cross the astrocyte-meningeal cell boundary in vitro (Shearer et al., 2003, Niclou et al., 2006). In contrast to the situation in vivo, myelin-derived inhibitors do not play a role in these simplified in vitro regeneration assays, while semaphorins are more prominent constituents of in these in vitro models.

The redundancy of inhibitory molecules that play a role in the failure of spinal cord regeneration is currently an important point of discussion in the field. Eliminating multiple inhibitory pathways could provide a way to move forward and opening possibilities for improving spinal cord repair. Two studies have now reported the effects of deleting multiple myelin inhibitors with varying success. Studies by the Strittmatter laboratory have reported that double knockout of myelin and OMgp does not improve CST fiber regeneration and functional behaviour, but does have a synergistic effect on both parameters in the absence of nogo (Cafferty et al., 2010). Studies by the Zheng laboratory, however, do not confirm these results in similar experiments (Lee et al., 2010b). The same group recently reported a second study in which two different classes of inhibitory molecules are targeted to promote axonal regeneration by knocking out NgR and plexinA3/A4, the two co-receptors for class 3 semaphorins (Lee et al., 2010a). They showed that interfering with one class of molecules, or both at the same time, did not enhance regeneration of injured axons. Currently there is extensive debate about how mouse strain, approach of genetic mutation, location and method of injury application and technical details in the procedure of the histological analysis play a role in the conflicting results that are reported (Dimou et al., 2006, Cafferty et al., 2007, Steward et al., 2007, Lee et al., 2009, Lee et al., 2010a, Lee et al., 2010b, Schwab, 2010).

Interfering with growth inhibitory molecules using pharmacological inhibitors, blocking antibodies and peptides appears to have been more successful than genetic perturbation studies. In particular the class of myelin inhibitors and their receptors have been rewarding targets. Using the neutralizing antibody IN-1, the soluble NgR-ecto receptor domain, and neutralizing peptide NEP1-20, has proven to be successful in a variety of regeneration paradigms (Schnell and Schwab, 1990, GrandPre et al., 2002, Fischer et al., 2004, Li et al., 2004). The reason why neutralising agents are more effective than genetic mutation of the myelin inhibitors or its receptors is continuously under debate. One of the possible explanations is that the effects of the blocking reagents may extend beyond target neutralization. Also there may be roles of Nogo and NgR beyond growth inhibition. The unimpressive regenerative capacities of the various mutant mice as compared to acute intervention with blocking agents, could also be explained
by compensatory mechanisms that can occur in mutant animals (reviewed by Teng and Tang, 2005, Schwab, 2010).

Inhibition of non-myelin inhibitors also led to enhanced regeneration. The use of EphA4 blocking peptide or a RGMA blocking antibody has shown to have a significant effect on CST axon regeneration and functional improvement (Hata et al., 2006, Fabes et al., 2007). Antibodies blocking Sema3A function have been described but have not been used in spinal cord injury research (Shirvan et al., 2002). The Sema3A inhibitor SM-216289 or xanthofulvin has been found to be beneficial to olfactory nerve and spinal cord injury (Kikuchi et al., 2003, Kaneko et al., 2006). Administration of SM-216289 after a spinal cord transection improves raphespinal axon but not CST regeneration. The inhibitor also increases Schwann cell migration into the lesion area, which leads to myelination of regenerating axons, contributing to the positive effects of SM-216289 administration. Finally, the inhibitor has beneficial effects on angiogenesis and motoneurons survival. This inhibitor is not entirely selective for Sema3A as it does bind to matrix metalloprotease-2 and the epidermal growth factor receptor, and thereby possibly modulates the extracellular matrix (ECM) and cell adhesion, migration and proliferation. Therefore, it is evident that the inhibitor acts on a multitude of regeneration related processes.

The precise mechanism behind the inhibitory effects of proteoglycans is not well understood, but enzymatic degradation of the glycosaminoglycan side chains of CSPGs that are part of the ECM of the neural scar improves the outgrowth of injured axons, and facilitates functional recovery (Bradbury et al., 2002). Besides that proteoglycans themselves are inhibitory, the ECM could be a scaffold that binds Sema3A (De Wit et al., 2005) or other inhibitory proteins (reviewed in De Wit and Verhaagen 2007). Consequently, degradation of the ECM should also relieve the hypothesized contribution of the Sema3A to the inhibitory environment of the neural scar.

Interfering with down stream signalling of inhibitors such as Rho and PKC or stimulating the intrinsic growth capacity by elevating cAMP level is a promising method to deal with the redundancy in inhibitory mechanisms by neutralizing a broad spectrum of inhibitors (Dergham et al., 2002, Neumann et al., 2002, Qiu et al., 2002, Sivasankaran et al., 2004).

It is important to realize that regeneration of the central nervous system fails for multiple reasons. The idea that inhibitory molecules in myelin and in the neural scar are major factors is well established and progress has been made in finding ways to neutralize some of these molecules. However, in addition to inhibitory control at least two other components of the failure of repair mechanisms deserve attention: the lack of positive influences at a lesion site and the inadequate intrinsic response of CNS neurons to axon injury. A logical combination therapy would include a neurotrophic treatment (e.g. a neurotrophic and/or protective factor), a treatment that promotes axon growth from within an injured neuron (e.g. cAMP) and an anti-inhibitory treatment that overcomes inhibition by myelin and scar-derived inhibitors.
Genetic mutation of the class-3 semaphorin receptor component Npn-2 does not enhance rubrospinal tract regeneration

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manuscript in preparation
Rubrospinal tract regeneration in Npn-2 deficient mice

Abstract

After spinal cord injury, axon outgrowth inhibitors present in myelin and in the neural scar are considered to contribute to the failure of injured axons to re-establish functional connections. Class-3 semaphorins are expressed by the meningeal cells that infiltrate the glial scar after injury and signal by binding to neuropilin-1 (Npn-1) or neuropilin-2 (Npn-2). Since neurons of the red nucleus express only Npn-2, rubrospinal axons of Npn-2 knock out (KO) animals should be insensitive to class 3 semaphorins. To examine the effect of genetic deletion of Npn-2 on the inability of rubrospinal tract (RST) axons to regenerate, we have analysed RST regeneration in Npn-2 KO and wild type littermates. In this study we report that Npn-2 deficient mice do not exhibit improved RST axon outgrowth and do not show enhanced recovery of motor function.

Introduction

Following spinal cord injury (SCI), most injured neurons fail to regenerate and do not re-establish functional synaptic connections. The virtual lack of a growth response in vivo is, at least in part, caused by the presence of growth inhibitory molecules in the spinal cord and at the lesion site. These growth inhibitory molecules include myelin-associated inhibitors and scar-associated molecules such as chondroitin sulphate proteoglycans and chemorepulsive axon guidance molecules (reviewed by Giger 2010, Niclou et al. 2006, Fawcett 2006, Bolsover 2008).

Semaphorins are chemorepulsive guidance molecules originally identified as repulsive cues that act during development of the nervous system (Kolodkin et al., 1993, Luo et al., 1993). The semaphorin family is comprised of a large number of membrane-bound and secreted proteins, subdivided in eight classes of invertebrate semaphorins (class 1 and 2), vertebrate semaphorins (class 3-7) and viral semaphorins (class V) (reviewed by Pasterkamp and Giger). The secreted class 3 semaphorins, with the exception of Sema3E, bind to the neuropilin (Npn) receptor and signal through a plexin class-A (PlxA) signal transducing subunit (Yaron et al., 2005). Two Npn receptors have been identified (Chen et al., 1997, He and Tessier-Lavigne, 1997, Kolodkin et al., 1997). Sema3A exclusively binds to Npn-1 while Sema3C binds predominantly and 3F exclusively to Npn-2 (He and Tessier-Lavigne, 1997, Kitsukawa et al., 1997, Kolodkin et al., 1997, Chen et al., 1998, Giger et al., 1998b, Renzi et al., 1999).

After SCI, class 3 semaphorins are (re-)expressed at the site of injury. In the lesioned spinal cord they can have at least two possible functions. First, following SCI, the meningeal cells that invade the core of the lesion site, express the secreted class 3 semaphorins Sema3A, 3B, 3C, 3E and 3F (Pasterkamp et al., 1999a, Pasterkamp et al., 2001, De Winter et al., 2002b). Most injured spinal cord neurons continue to express class 3 semaphorin receptor components (De Winter et al,
The presence of semaphorins is likely to contribute to the growth inhibitory properties of the neural scar. Moreover, oligodendrocytes express the membrane-associated Sema4D and 5A (Cohen et al., 2003, Moreau-Fauvarque et al., 2003). The semaphorins produced by oligodendrocytes could act in concert with the classical myelin-associated inhibitors to inhibit axon regeneration. Second, semaphorins may affect the formation of the neural scar by influencing scar-associated cell migration, proliferation and neovascularisation (Chedotal, 2007, Joyal et al., 2011).

The presence of class 3 semaphorins in the neural scar and their potential contribution to the inhibition of axonal regeneration, suggest that interfering with semaphorin signalling could be beneficial to spinal cord regeneration. In chapter 5 we have investigated the role of Sema3A during corticospinal tract (CST) regeneration by selective mutation of Npn-1 in neurons. This study showed that knocking out Npn-1 did not improve CST fiber regeneration and did not improve motor function. In chapter 5 we discuss several factors that may underlie the lack of improved recovery in these conditional Npn-1 animals. One of the possible explanations is that corticospinal neurons express both Npn-1 and Npn-2 (De Winter et al., 2002b). Knocking out Npn-1 alone does therefore not abrogate all scar derived repulsive Sema3 signals. Since neurons of the red nucleus express only Npn-2 (De Winter et al., 2002b), rubrospinal axons of Npn-2 KO animals should be insensitive to all class 3 semaphorins. To study the effect of genetic deletion of Npn-2 on the failure of injured RST axons to regenerate, we have analysed rubrospinal tract (RST) regeneration and functional motor behaviour after unilateral lesion of the RST in Npn-2 KO mice. We show that after an RST lesion, disruption of Npn-2 signalling does not improve outgrowth of RST axons and does not lead to enhanced recovery of motor function.

**Methods**

**Experimental animals**

The Npn-2 knockout mice were provided by Dr Roman Giger (University of Michigan, MI, USA). The animals were maintained as Npn-2+/− heterozygous animals in a C56BL/6 background. Npn-2−/− KO animals were obtained by heterozygous Npn-2+/− intercrossings. Wild type (WT) littersmates were used as control animals. Animals were housed in groups under standard conditions with food and water ad libitum and a 12h:12h light/dark cycle. Experimental procedures and behavioural tests were performed in accordance with the committee for laboratory animal welfare and experimentation of the Royal Netherlands Academy of Sciences.
Animal surgery

RST transection: Animals were deeply anesthetized by an intraperitoneal injection of Hypnorm (0.1 mg/kg Fentanyl citrate/ 3.3 mg/kg Fluanisone HCl, Janssen Pharmaceuticals) and Dormicum (8.3 mg/kg Midazolam, Roche). During surgery, body temperature was maintained at 37°C using a heating pad. The spinal cord was exposed by partial laminectomy of the C4 vertebra. The dura mater was opened using Vannas scissors followed by a unilateral lesion of the left dorsal horn using a micro knife transecting the RST. Muscle layers were sutured and the skin was closed with Michell clips (Fine science tools). Postoperative analgesia was administered by one single subcutaneous injection of Metacam (0.4 mg/kg, Boehringer Ingelheim). In animals of the sham procedure group a laminectomy was performed leaving the dura matter and spinal cord intact.

RST tracing: RST fibers were anterogradely traced four weeks after surgery. To this end animals were anesthetized as described before and 0.8 µl of biotinylated dextran amine (BDA) solution (10% in PBS, MW 10.000, Invitrogen) was infused into the red nucleus (coordinates; AP: -3.5 mm, L: -0.5 mm from lambda, DV: -3.5 mm from dura) at a flow of 0.2 µl/min.

Experimental groups: All animals were 13 to 17 weeks of age on the day of surgery. A total of 12 KO and 14 WT mice received a unilateral RST lesion. The sham group consisted of 4 WT mice.

Behavioural testing and evaluation

All animals were tested 3 days before and 3, 7, 10, 14, 17, 21, 24 and 28 days after surgery.

Narrow beam walk: To evaluate recovery of proper hind limb placements after RST lesion, a narrow beam test was performed. Animals were pre-trained for one week to cross an 8 mm wide, 100 cm long and 15 cm elevated beam. The narrow beam was flanked on both sides by a platform from which the animals initiated their run voluntarily. The total number of slips and steps of the left hind limb were counted and averaged from 3 successful runs by two observers blinded to the experimental group.

Rotarod: To assess overall motor coordination, animals were placed on the Rotarod (Ugo Basile Biological Research Apparatus) rotating at a constant speed of 5 rpm. The rotation was accelerated to 40 rpm over a period of 5 minutes. The time the mice could remain on the rotating beam was recorded and normalised for the maximum performance at 3 days before surgery.

Cylinder test: We analysed forelimb motor behaviour by making use of the natural exploratory behaviour of the animals using the cylinder test (Liu et al., 1999). After an animal was placed in a 6 cm diameter glass cylinder, the animals spontaneously started exploring the vertical surface by rearing to a standing position using one or both of its forepaws for support. For the duration of 5 minutes or 20 events we scored the placement of right, left or both forepaws. An event started when the animal reared to a standing position, bearing body
weight with its hind limbs, and supporting the upright position with a weight bearing contact of one or two fore paws with the vertical surface. The event was ended when the animal landed on either one or both fore paws. A single front paw placement was defined as the weight bearing use of the right or left front paw for support in the upright position. The use of both front paws was scored when the animal used both paws consecutive or simultaneously for weight bearing support within one event.

Catwalk gait analysis: Gait analysis was performed as described before (Hamers et al., 2001). Briefly, mice were pre-trained for one week to cross a 100 cm long glass plate, confined by Plexiglas walls 35 mm apart in a darkened room. Paw prints were recorded digitally and analysed using the Catwalk software. All analysed parameters were normalised for the performance at 3 days before surgery.

Tissue preparation

One week after RST tracing, animals were euthanized by injecting an overdose of Nembutal (sodium pentobarbital, Sanofi Sante) followed by transcardial perfusion of ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer. The spinal cord and brain were dissected and post fixed overnight at 4°C followed by incubation in phosphate buffered saline (PBS) containing 0.25M EDTA and cryopreservation in PBS containing 25% sucrose. Tissue was embedded in OCT compound (Sakura), snap frozen in 2-methylbutane and stored at -80°C until sectioning. Twenty µm thick transversal cryosections at cervical level C1 were thaw mounted on Superfrost Plus slides (Fisher Scientific). The C2-C6 spinal cord segment was cryosectioned sagitally. All sections were dried and stored at -80°C until use.

Histological analysis and quantification

To determine the total number of BDA traced RST fibers, transversal sections were incubated in Tris-buffered saline (TBS), 0.2% Triton X-100, 5% bovine serum (block buffer) for 1 hour followed by incubation with streptavidin-Alexa488 (1:400, Invitrogen) in blocking buffer for 3 hours at room temperature. The sections were washed three times in TBS containing 0.1% Triton X-100. Tiled images of the dorsal column were captured using an Axioplan 2 microscope (Zeiss) with a 40x objective. The RST was outlined using Imagepro Plus (Media Cybernetics) and a grid was placed over the outlined area. Fibers were counted in approximately 25% of a systematic randomized selection of the outlined area.

To quantify the RST fibers rostral en caudal to the lesion site, we prepared sagittal sections of the cervical C2-C6 region of the spinal cord. The sections were blocked as described above and incubated overnight at 4°C with Rabbit-anti-GFAP (1:1000, DAKO) in block buffer. The following day, sections were
washed three times in TBS containing 0.1% Triton X-100 and incubated with goat anti-Rabbit-Cy3 (1:400, Jackson Immunoresearch) and streptavidin-Alexa488 (1:400, Invitrogen) for 3 hours at room temperature. The sections were washed 3 times in TBS containing 0.1% Triton X-100 and coverslipped. Tiled images were captured from every second section using an Axioplan 2 microscope (Zeiss) with a 20x objective. Using the GFAP IHC signal, the ventral, caudal and rostral borders of the lesion site were identified. Using these three reference points, the center of the lesion was determined. In all images containing BDA positive RST fibers and lines were placed in dorsal-ventral orientation in the core of the lesion and 0.25, 0.50, 0.75 and 1.0 mm rostral and caudal from the lesion center using Imagepro Plus (MediaCybernetics). The number of RST fibers running through the white matter that crossed these lines was counted. The fiber index was calculated by dividing the fiber counts at the set intervals in the C2-C6 lesion area by the total number of fibers at C1 level.

**Statistics**

All results are expressed as mean ± SEM. For the RST fiber index, narrow beam and cylinder test, statistical significance was tested with a Kruskal-Wallis test followed by a Mann-Whitney U post hoc test. The rotarod and catwalk experimental data were tested with an ANOVA analysis with a Bonferroni post hoc test. A value of p < 0.05 was considered significant.

**Results**

**Animal breeding and peroperative mortality of Npn-2 knockout mice**

Npn-2 KO mice were obtained using a heterozygous breeding scheme. In our colony of 325 animals that survived into adulthood, 5.8% were of the Npn-2 +/- genotype, 48.5% and 35.7% were Npn-2 +/- and Npn-2 +/- respectively. As reported previously, this clearly indicates a phenotypic effect on the survival rate of neonatal KO mice (Giger et al., 2000). The homozygous KO animals that survived into adulthood had an average decreased body weight of 16 % as compared to their heterozygous littermate as reported previously. To analyse RST regeneration, we performed a unilateral RST lesion at cervical level C4. During this surgical procedure, 5 out of 12 knock out animals (42%) died, where in the wild type group 2 out of 12 (17%) died perioperative.
Figure 1. RST fibers regeneration is not enhanced in Npn-2 knockout animals. Immunohistochemical double staining was used to visualize BDA traced RST fibers (green) and GFAP (red) in sagittal sections in the lesioned area (a, b). The total amount of RST fibers at cervical level C1 (c) was used to correct for tracing variability. Quantification of RST fibers at set intervals of the injury site (d) revealed no differences between wild type and knockout animals. Scale bar: 250 µm (a) and 50 µm (b,c).
Histological analysis of RST fibers

To analyse the regenerative response of the rubrospinal fibers, we traced the RST by injection of BDA in the lesioned red nucleus. The animals were sacrificed one week after tracer injection, and tissue was processed for immunohistochemical analysis. Due to tissue handling and cryosectioning procedure error, full histological analysis was possible on 5 KO, 9 WT and 3 sham lesioned animals. The number of RST fibers at 0, 0.25, 0.5, 0.75 and 1 mm rostral and caudal to the lesion site were counted (Fig. 1a,b). To correct for variation of tracing efficiency, the fiber index (Fig. 1d) was calculated by dividing the fiber counts by the total number of traced RST fibers at cervical level C1 (Fig. 1c). The fiber index at 1 mm rostral to the lesion was similar for KO and WT animal. The KO animals show a small trend of increased RST fibers growing towards the lesion. At 0.5 mm rostral to lesion the difference between the regenerating fiber index reaches a maximum of 0.40 ± 0.03 and 0.28 ± 0.05 in KO and WT animals respectively. However, this trend of an increased number of regenerating axons did not reach statistical significance.

Behavioural analysis

The rubrospinal tract plays an important role in voluntary movement of front and hind limbs. A unilateral lesion of the RST at cervical level C4 results in significant motor impairment of the ipsilateral paws. To analyse the loss and regain of motor function in RST lesioned KO and WT mice, we conducted several functional tests.

Narrow beam walk

We examined coordinated hind paw placement of Npn-2 KO and WT animals using the narrow beam walk. After one week of pre-training, all animals crossed the 8 mm wide beam making less than 2% slips or misplacements of the left hind limb. Lesioning the left RST resulted in 82.0 ± 7.6% and 83.4 ± 10.5 incorrect left hind paw placement at 3 days after surgery for KO and WT animals respectively (Fig. 2). Seven days after RST lesion, the incorrect foot placement in the WT animal group decreased to 75.1 ± 7.3% while the KO group increased to 91.3 ± 8.3 % reflecting a significantly difference between the WT and KO group. The WT animals continued to recover to 61.4 ± 8.7% at 14 days after injury and maintained this level of performance throughout the remainder of the experiment. The knockout animals recovered to only 74.0 ± 11.5% at 10 days post injury. From seven days post injury onwards, an average difference of 12% in paw placement error rate of KO animals compared to the WT group persisted throughout the experiment, but did not reach statistical significance.
Rotarod

Coordinated limb movement was analysed using the Rotarod system (Fig. 3). Three days after RST injury, all animals showed a decreased performance as compared to sham-operated animal. Unexpectedly, KO animals showed a significantly larger deficit (43.8 ± 3.9%) than WT animals (64.9 ± 4.2%). Over the following 3 days, WT animals gradually recovered, reaching a plateau at 14 days post injury (88.7 ± 5.6 %). KO animals showed a slow continuous recovery to 80.6 ± 8.8 % at 28 days post injury, without showing a clear plateau within the timespan of this experiment.

Cylinder test

We analysed forelimb function using the cylinder test. Intact animals voluntarily explore the vertical wall using both forepaws in more than 90% of all rearing events. After lesioning the left RST, animals exhibited a strong preference in using their right paw only. On day 3 after injury, we observed an increased right paw usage of 38.4 ± 7.2 % and 55.2 ±12.9 % in WT and KO animals respectively (Fig. 4). WT animals recovered to 22.5 ±7.2 % on day 10, while in KO animals the deficit essentially remained the same until 14 days after injury. From day 21 after injury all animals performed equally without further improvement in paw usage (KO: 32.4 ± 8.1, WT: 32.4 ± 8.9). Similar to the rotarod test, as a result of a
lesion of the RST we unexpectedly found that KO animals showed an increased dysfunction as compared to WT animals.

**Catwalk**

Using the catwalk system, we quantitatively analysed several specific gait parameters: The stride length of both front and hind limbs was unaffected by the RST lesion. Intact and injured KO animals showed a significantly smaller front and hind limb stride length (Fig. 5a, b). The stand time, the duration a certain paw has contact with glass plate, was unaffected after injury to the RST. Although this parameter was not indicative for deficits in motor function that were caused by the lesion, it did show to be significantly increased in uninjured KO animals as compared to WT littermates (Fig. 6). The swing time, the duration between paw placements, was measured for the individual paws (Fig. 7a, b, c, d). Three days after injury, the swing time of the left forelimb was significantly increased in WT and KO animals as compared to sham-operated animals. This increase did not recover throughout the duration of the experiment (Fig. 7a). The swing time of the left hind limb was significantly increased in KO animals as compared to WT littermates 7 days after injury (WT: 119 ± 9 ms, KO: 184 ± 24 ms) (Fig. 7c). KO animals continued to show a trend of increased swing time as compared to WT animals until day 24, and recovered to the level of injured WT animals 28 days after injury.
Discussion

In this study we have examined the regenerative response of Npn-2 KO mice after injury of the RST. Deletion of the Npn-2 gene has no effect on the regeneration of injured RST fibers. After RST injury, Npn-2 KO animals do not show an enhanced recovery of motor function. Similar to the Npn-1 conditional KO animals studied in chapter 5, Npn-2 KO mice show an increased loss of motor function as compared to control animals. Non-lesioned, intact Npn-2 KO animals have a smaller stride length and an increased stand time on the Catwalk gait analysis. The decreased stride length is likely due to their smaller size. Additional phenotypic differences in motor function between WT and Npn-2 KO mice are revealed after RST injury as shown by the decreased performance in the other functional tests used here. The precise mechanism underlying these phenotypic effects is unknown, but could be attributed to the observation that KO animals have developmental defects in cranial and spinal nerves. (Chen et al., 2000, Giger et al., 2000). In addition to its role in axonal guidance in the developing CNS, Npn-2 plays a role in spine density and synaptic activity in the adult CNS (Sahay et al., 2005, Tran et al., 2009). Therefore normal neuronal function may be affected in Npn-2 KO animals.

With the exception of Sema3E, the other four class 3 semaphorins that are expressed in the neural scar signal by binding to Npn-1 or Npn-2. In WT animals, neurons of the red nucleus express only Npn-2. As a result, the RST fibers in Npn-
rubrospinal tract regeneration in npn-2 deficient mice

2 KO animals are considered to be unresponsive to most class 3 semaphorins. By studying RST fiber regeneration in the Npn-2 KO mouse it is possible to determine the combined contribution of the majority of class 3 semaphorins to the inability of the RST to regenerate. Unexpectedly, both RST fiber regeneration and motor function was not enhanced in RST lesioned Npn-2 KO animals.

There are at least 3 possible reasons by which these negative results could be explained. First, the presence of receptors for other growth inhibitory proteins, including receptors for myelin inhibitors, ephrins, slits and RGMa may continue to suppress regenerative growth of injured RST fibers. A first attempt to simultaneously compromise signalling by two classes of inhibitors has recently been reported in a study by Lee et al. In PlxA4/PlxA3/Nogo receptor-1 triple knockout mice, raphe spinal and corticospinal fibers did not show improved axon regeneration after complete spinal cord transection (Lee et al., 2010a). Thus,

Figure 5. The stride length is unaffected by RST lesion. Intact, uninjured Npn-2 KO animals show a decreased stride length in front (a) and hind limbs (b) as compared to WT littermates. The stride length did not change after RST lesion. (* p<0.05 significance between KO and WT)
the simultaneous attenuation of the semaphorin and nogo signalling pathways by the constitutive genetic mutation of specific components of the multimeric receptor did not result in a measurable regenerative response. However, even in this triple transgenic animal the signalling pathways of three other repulsive protein families (ephrin, slit, RGM) are still intact.

Second, Npn-2 is a receptor for splice forms of VEGF (Gluzman-Poltorak et al., 2000) and some forms of VEGF also have neurotrophic activity (Sondell et al., 1999, Matsuzaki et al., 2001, Yasuhara et al., 2004). Constitutive KO of Npn-2 in neurons may render these cells insensitive to two factors with opposing effects, namely lack of a neurotrophic or neuroprotective influence (VEGF) and a chemorepulsive effect (semaphorins). In this scenario, neutralisation of semaphorins in the scar would be effective, since it would selectively remove the repulsive component and would leave the beneficial signal (VEGF) for the Npn-2 receptor intact. Third, constitutive knockout of Npn-2 in other cells than neurons (including blood vessel, lymph vessel and scar cells) may have effects on scar formation and/or wound healing. In Npn-2 knockout animals, new blood vessel formation in the retina was suppressed after an ischemic lesion (Shen et al., 2004). Similar effects may have occurred after a spinal cord lesion and this

Figure 6. The stand time is unaffected by RST lesion. Injury to the left RST does not significantly affect the stand time of the individual paws (a, b, c, d) of KO and WT animals. Uninjured KO animals show an increased stand time as compared to WT animals. (* p<0.05 significance between KO and WT)
Rubrospinal tract regeneration in npn-2 deficient mice may “mask” the role of neuronal Npn-2 in the repulsion of injured RST axons per se.

In the PNS axonal regeneration through a sciatic nerve crush site was delayed in Npn-2 knockout mice (Bannerman et al., 2008). This indicates that Npn-2 expression has a beneficial effect on axon regeneration following a lesion that does not result in scar formation and allow regeneration of axons along a pathway of growth promoting Schwann cells. Studies on the role of Npn-2 in injured CNS and PNS neurons and in the various cell types in a non-permissive spinal cord scar and a permissive denervated sciatic nerve will have to be executed to further our understanding of the role of Npn-2 in neuroregeneration.

Figure 7 – Increased swing time in Npn-2 KO mice after RST injury. Three days after injury of the left RST, the swing time of the left forelimb of injured WT and KO animals is significantly increased as compared to sham operated animals, which did not recover throughout the duration of the experiment (fig 7a). The swing time of the left hind limb was significantly increased in KO animals as compared to WT littermates 7 days after injury (WT: $119 \pm 9$ ms, KO: $184 \pm 24$ ms) (fig 7c). KO animals continued to show an increased swing time as compared to WT animals until day 24. (* p<0.05 significance between KO and WT)
Summary and general discussion

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1. Summary

A major reason for the permanent and devastating functional deficits after brain and spinal cord injuries is the failure of injured neurons to regenerate their axons and re-establish functional connections. One of the challenges in regenerative neuroscience is to develop strategies that can efficiently restore the function of damaged neural circuits by promoting axon regeneration in the central nervous system (CNS). The lack of a regenerative response in the CNS is, at least in part, attributed to the presence of inhibitory molecules that are expressed in the injured spinal cord including myelin-associated proteins (NoGo, MAG, OMPg) and scar-associated proteins such as chondroitin sulphate proteoglycans and repulsive axon guidance molecules (semaphorins, slits, ephrins and RGMa). Together, these inhibitory molecules constitute a multi-molecular barrier that inhibits axon regeneration and are molecular targets for the design of therapeutic interventions (reviewed by Giger 2010, Niclou et al. 2006, Fawcett 2006, Bolsover 2008).

Semaphorins were originally discovered as repulsive axon guidance cues that have an important role in guiding axons to their appropriate target cells during development of the nervous system. Interestingly, Class-3 semaphorins are re-expressed by meningeal fibroblasts that infiltrate the scar that forms after spinal cord injury. Injured neurons continue to express the semaphorin receptor components, neuropilins and plexins, rendering them potentially sensitive to these chemorepulsive axon guidance cues after an injury. These observations have led to the hypothesis that semaphorins have a negative impact on CNS regeneration (reviewed in chapter 1, Bolsover et al., 2008 and Harel and Strittmater, 2006).

The aim of the work described in this thesis was to develop methods to interfere with the chemorepulsive activity of secreted, class 3, semaphorins and to understand their role in central nervous system regeneration (Fig. 1). In this chapter, we will first briefly summarize the findings reported in this thesis. Subsequently we will discuss the most important results and provide ideas for future research.
In chapter 2 we demonstrate that meningeal cell-derived semaphorin3A (Sema3A) contributes to the inhibitory milieu imposed by the spinal cord scar. Protein extracts from cultured meningeal cells induce the collapse of embryonic dorsal root ganglion (DRG) growth cones. This collapsing activity can be partially blocked by neuropilin-1 (Npn-1) antibodies and is absent in meningeal cells from Sema3A knockout (KO) mice. Furthermore, the impaired DRG neurite outgrowth on a monolayer of meningeal cells is partially alleviated when DRG neurites are cultured on meningeal cells from Sema3A KO mice. These results show that Sema3A expressed by meningeal fibroblast is a potent inhibitor of neurite growth that is likely to contribute to the inhibitory properties of the neural scar.

We have explored two strategies to interfere with the inhibitory activity of semaphorins in animal models for spinal cord injury. First we developed viral vectors expressing short hairpin RNAs (shRNAs) designed to selectively knock down expression of the class-3 semaphorin receptors Npn-1 and Npn-2 in rubrospinal neurons or in DRG neurons. To ensure optimal delivery of the shRNA, we tested a panel of seven adeno associated viral vectors (serotypes AAV1 to 6 and AAV8) and lentiviral (LV) vectors for their efficacy to transduce DRG neurons (Blits et al., 2010). In chapter 3 we show that AAV5 was the most effective in DRG neuron transduction and outperformed all other tested serotypes: AAV5 transduced more neurons and directed the highest GFP-expression level per neuron as compared to the other serotypes. In chapter 4 we report our findings on AAV-mediated expression of shRNA in the red nucleus and the DRG. Unexpectedly, AAV1-mediated expression of Npn-2 shRNAs and control shRNAs caused an adverse tissue response and neuronal degeneration when delivered to the red nucleus. Three weeks after AAV1 injection, many of the rubrospinal neurons contain vacuolar structures and have an atrophic appearance. Furthermore, there is considerable cell death as shown by the loss of neurons and the acellular granular structure of the tissue at the site of AAV injection. This adverse tissue response was dose dependent and was not observed with AAV1 vectors expressing only GFP. In contrast, with one of the hairpins we developed it was possible to knock down expression of Npn-2 in DRG neurons using AAV5 without a clear tissue response. However, based on these results and studies of a number of other laboratories that showed adverse effects of viral vector-mediated expression of shRNA in the brain (see more discussion below), we concluded that overexpression of shRNAs can lead to saturation of the endogenous microRNA machinery. This can severely affect neural cell function and survival. We will further discuss the use of AAV vectors and their use to knock down genes in the nervous system in section 2.2 below.

In the second approach to neutralize semaphorin signalling we used two mouse models in which the neuropilin genes were genetically mutated. In chapter 5 we analysed regeneration of the corticospinal tract (CST) after dorsal column lesion in mice in which the Npn-1 gene was genetically ablated in neurons. Neurons that form the CST express both Npn-1 and Npn-2. We have found that mice deficient in Npn-1 do not exhibit enhanced growth of injured CST axons and do not show
improved recovery of motor function as compared to control littermates. This demonstrates that neuron-specific removal of a single inhibitory signal (Sema3a/Npn-1) from the multi-component class-3 semaphorin signalling pathway is insufficient to enhance regeneration of the CST.

Neurons of the red nucleus express only the neuropilin-2 semaphorin-binding receptor component and do not express Npn-1. In chapter 6 we studied the regeneration of the rubrospinal tract in Npn-2 KO-mice. Since rubrospinal axons do not express Npn-1 and rubrospinal neurons in Npn-2 KO mice have lost their capacity to detect other members of the class-3 semaphorin (except for Sema3E, which signals neuropilin-independent) we postulated that rubrospinal neurons would display an enhanced regenerative response following transection of their axons in the spinal cord. Unexpectedly, in Npn-2 knockout mice RST axon regeneration and motor function was not enhanced after unilateral RST lesion as compared to wild type littermates. From these studies on spinal cord lesions in mice, we conclude that abolishing class-3 semaphorin signalling by deleting neuropilins is not sufficient to induce a regenerative response in RST and CST neurons after a spinal cord lesion.

There are at least 3 factors which may have contributed to these negative results. First, the sheer abundance of inhibitory molecules (see Fig. 1, chapter 1) indicates that a diverse repertoire of inhibitory forces in the lesioned spinal cord is at work to inhibit recovery after injury. Thus removal of class-3 semaphorin signalling alone is simply not enough to alleviate the inhibition of outgrowth of injured axons. Second, the constitutive knock out of Npn-2 in all tissue types may have effects on scar formation, wound healing and/or on normal neuronal function, which may counter-balance the possible positive effect of the absent neuronal semaphorin-response. Also, in the knock-out mice used in this study compensatory mechanisms that counteract the effect of the deletion of Npn-1 or Npn-2 may have effectively masked the effect of the mutation. Third, Npn-1 and Npn-2 are receptors for splice forms for VEGF (Soker et al., 1998, Gluzman-Poltorak et al., 2000), which can have neurotrophic or neuroprotective effects (Matsuzaki et al., 2001, Yasuhara et al., 2004). The advantage of removing the inhibitory semaphorin/neuropilin signalling may potentially be counteracted by the removal of beneficial VEGF signalling.

2. General discussion

The aim of the work described in this thesis was to develop methods to interfere with the chemorepulsive activity of class 3 semaphorins and to understand their role in central nervous system regeneration. In this section we will first discuss our efforts to develop a method based on the use of viral vector-mediated RNAi to silence semaphorin signalling in vivo. In the second part we will discuss our findings on the role of semaphorins in spinal cord regeneration based on studies using (conditional) knockout mice.
2.1 Genetic tools to modulate gene expression in the CNS

The ability to answer questions about the role of a given protein in a neurobiological process relies on the availability of tools that interfere with the function of that protein in vivo. Ideally, a method to interfere with the function of a protein would allow for spatial and temporal control so that it is possible to target a specific anatomical structure and/or cell type and to determine the onset of the intervention. The use of genetically modified mice that either lack or overexpress a gene of interest is the most common and best-established strategy to study protein function in vivo. It is possible to use cell-specific promoter sequences to target the expression of a specific gene to a defined population of cells. A gene can be knocked-out conditionally by generating mice in which the gene of interest is flanked by recombinase recognition sequences. These mice can then be crossed with a transgenic animal that expresses the recombinase under a specific promoter. The choice of promoter that directs the expression of the recombinase determines the cell type in which the gene is selectively knocked out, as well as the onset of this gene deletion.

RNA interference

A relatively new method to control gene expression is RNA interference (RNAi). RNAi makes use of a natural process of selective gene silencing that occurs in each cell. Inspired by the observation that introducing antisense RNA in plants and C. elegans can inhibit gene expression, Fire and co-workers (Fire et al., 1998) were the first to describe the use of double-stranded RNAs to induce RNAi. By injecting purified RNA molecules in C. elegans, they showed that dsRNAs were more potent than single-stranded antisense RNAs in their ability to silence the unc-22 gene. This seminal discovery was quickly followed by the identification of ~21 base pair dsRNAs called small interfering RNAs (siRNA) and their subsequent use to silence genes in mammalian cells (Elbashir et al., 2001). The gene silencing effect (usually referred to as knockdown) in mammalian cells is transient, and prolonged knockdown requires repeated administration of siRNAs. With the generation of a plasmid vector system that drives expression of a short hairpin RNA (shRNA) under a RNA polymerase III promoter, it became possible to induce sustained shRNA-mediated knock down in mammalian cells (Brummelkamp et al., 2002, Paul et al., 2002, Sui et al., 2002). After transfecting cells with these plasmid vectors, the shRNAs are expressed, transported from the nucleus to the cytoplasm by the exportin-5 protein, and processed into the catalytic siRNAs by the endoribonuclease dicer. The antisense ‘guide’ strand of the siRNA is loaded into the RNA-induced silencing complex (RISC) which, in turn, catalyses the degradation of the target mRNA. These processes are part of the cell-endogenous micro-RNA processing machinery, a natural occurring mechanism by which cells control protein expression levels.

RNAi has several properties that would make it a powerful tool to study the complex molecular mechanisms that inhibit regeneration in the CNS.
First, RNAi can be applied in adult animals following a lesion in a spatially and temporally defined way. This would circumvent some of the problems observed in conventional knockout mice, including developmental effects and long-term compensatory mechanisms. Second, this approach can be applied in the rat, the experimental animal most used in spinal cord research. Finally, by introducing a number of distinct shRNA molecules directed against individual genes that encode inhibitory proteins it would be possible to simultaneously target multiple inhibitory molecules or their receptors. This would make RNAi an ideal intervention strategy to investigate the relative contribution of individual inhibitory proteins and of combinations of inhibitory proteins. The latter property of RNAi is particularly attractive in view of the fact that multiple gene families contribute to the inhibition of reparative processes after a CNS-lesion.

There are several methods to deliver genetic material, including shRNAs, to cells in vivo. Viral vectors have proven to be highly effective for delivery of genes to the nervous system. In chapter 3 and 4 we describe our efforts to develop this technology to efficiently deliver shRNAs to the nervous system using viral vectors. In the following section, therefore, we will discuss the delivery of shRNA to the nervous system by means of adeno-associated viral vectors.

2.2 Adeno associated viral vector mediated expression of shRNAs in the nervous system

Adeno associated virus

AAV is a naturally occurring replication deficient single stranded DNA virus. In order to replicate it needs adeno- or herpes virus as a helper virus. To date, over 100 capsid variants have been identified and phylogenetically classified into six clades. Since the development of AAV serotype-2 (AAV2) as a viral vector and its successful application in the CNS (Kaplitt et al., 1994), over 25 AAV variants have been used to create viral vectors (Gao et al., 2002, Grimm, 2002, Gao et al., 2004, Cearley et al., 2008) that display a differential tropism towards CNS cell types. AAV particles have low immunogenic properties. This is a unique feature of AAV vectors that ensures excellent tolerability and long-term transgene expression.

AAV vectors can be used to deliver their payload to localised areas in the brain by means of stereotaxic injection. Alternatively, large populations of cells can be transduced by delivery of the AAV vector in the cerebrospinal fluid, usually in early postnatal animals (Passini and Wolfe, 2001, Passini et al., 2003, Broekman et al., 2006). The ability of AAV9 to cross the blood brain barrier allows for transgene expression in the CNS by intravenous administration (Foust et al., 2009). By cleverly combining AAV serotype, a cell-specific promoter and the route of delivery, AAV vectors can be used to target basically any population of neurons or glia cells in the brain or spinal cord (Mamber et al., 2010, Mason et al., 2011).
We demonstrated that AAV5 is the most effective AAV serotype to transduce sensory neurons of the DRG in vivo (chapter 4) and AAV1 is the optimal serotype to transduce neurons of the red nucleus (Blits et al., 2010). When shRNAs were delivered to the DRG using AAV5, we were able to knock down Npn-2 expression with one of the two shRNAs that had proven to be successful in vitro. Surprisingly, AAV1-mediated expression of shRNAs in the red nucleus resulted in cellular toxicity. This adverse response was observed with the two Npn-2 targeting sequences and with a non-targeting shRNA control sequence. This indicates that the adverse effect was sequence-independent. The adverse tissue response was partially alleviated by injecting a 10 fold lower viral titer suggesting that the toxicity was shRNA dose-dependent. In the following section we discuss these findings in the context of the current understanding of the use of shRNAs in vivo.

shRNAs in the nervous system
Since the initial proof of principle of viral vector-mediated expression of shRNA in the CNS by Xia and co-workers (Xia et al., 2002), AAV-mediated knockdown of gene expression in the CNS has been widely used in various rodent models, including models for polyglutamine repeat diseases (Xia et al., 2004, Harper et al., 2005, Rodriguez-Lebron et al., 2005, Machida et al., 2006, Franich et al., 2008), amyotrophic lateral sclerosis (Miller et al., 2006, Towne et al., 2008, Towne et al., 2011), Parkinson’s disease (Gorbaytuk et al., 2010, Khodr et al., 2011), neuroprotection (Kalev-Zylinska et al., 2009, Bevers et al., 2010) and endocrine function (Garza et al., 2008, Lebesgue et al., 2009, de Backer et al., 2010, Hayes et al., 2010, Spiteri et al., 2010). Notwithstanding the numerous positive reports, evidence is emerging that overexpression of shRNAs in the CNS can lead to cellular toxicity (Boudreau et al., 2008, McBride et al., 2008, Boudreau et al., 2009a, Boudreau et al., 2009b, Khodr et al., 2011). shRNA-induced toxicity appears to be dose dependent and is also observed with control shRNAs that do not target a specific cellular RNA (McBride et al., 2008, Ulusoy et al., 2009 and Chapter 4). After the first report of lethality due to AAV8 driven expression of shRNAs in the liver (Grimm et al., 2006), a recent study reported lethality in mice due to AAV1-directed shRNA expression in the striatum (Martin et al., 2011).

Mechanisms that underlie shRNA-induced toxicity
Saturation of the endogenous miRNA machinery by the shRNA has been implicated in the cause of the adverse effects on the transduced cells. High cellular levels of a foreign shRNA appear to sequester components of the miRNA-processing pathway. It has been shown that the nuclear transport factor exportin-5 and RISC component argonaute-2 are potential rate-limiting components of the miRNA machinery (Grimm et al., 2006, Diederichs et al., 2008). Saturation of exportin-5 also down-regulates expression of DICER, a key component in the miRNA gene-
silencing machinery (Bennasser et al., 2011). Taken together, over-expression of shRNAs can affect three key steps that are required for normal miRNA function: nuclear export by dicer, cleavage of pre-miRNA by DICER and target degradation by RISC. Recently, Martin et al. reported that the genetic background of a mouse influences shRNA-inflicted toxicity. They found that in a mouse line that was more sensitive to the toxic effects of shRNA, the exportin-5 expression levels were lower than that in mice that were relatively less sensitive to shRNA over-expression (Martin et al., 2011). The same may hold true at the cellular level since certain neuronal cell types are more sensitive to shRNA over-expression (Khodr et al. 2010 and chapter 4). This may be caused by the fact that certain populations of cells express relatively low levels of miRNA machinery components, leading to a more rapid saturation with the foreign shRNA.

Taken together there are two interacting factors that determine toxicity of an shRNAs: The shRNA expression level which is determined by the choice of AAV-serotype, viral titer and the promoter driving shRNA-expression; and the availability of miRNA processing components that is variable between cell types and mouse strains. The delicate balance between these two factors may explain that some studies have reported successful shRNA knockdown, while other studies (including our own, chapter 4) have reported cellular toxicity.

How can we control for shRNA-induced toxicity in vivo?

Relatively few studies have carefully controlled for the absence or presence of toxic effects of viral vector-mediated shRNA expression in vivo. The adverse effects can be easily overlooked. To be able to detect shRNA induced adverse responses we recommend the following set of controls to be included in shRNA-knockdown experiments:

1. **Demonstration of successful knockdown at the single cells level in vivo.**
   It is essential to quantify gene expression at the single cell level in vivo by in-situ hybridisation or, even better, immunohistochemistry for the target protein. Analysis of the averaged target gene expression in transduced tissue could mistakenly lead to the conclusion that knockdown occurred while this is in fact due to death of cells that express the shRNA. If the viral vector that harbours the shRNA expression cassette also expresses a reporter gene, gene knockdown efficiency in transduced cells (labelled by the reported gene) can be easily assessed. Moreover, the reporter gene can also be used to assess cell-survival and overall tissue morphology.

2. **Demonstration that gene silencing is not due to saturation of the miRNA machinery.** Over-expression of shRNAs can lead to a malfunction in endogenous miRNA processing and therefore affect overall gene expression. The effects on gene expression by over-expressing a non-targeting control shRNA and a reporter gene should be analysed to prove that the knockdown is sequence specific.
3. **Demonstration of the effects on cell viability.** Both knockdown of certain target genes and the over-expression of shRNAs can have an effect on cell viability. If this occurs, it is important to analyse the viability of cells that are transduced by the targeting-shRNA and a control-shRNA to pinpoint whether this effect was related to gene function or shRNA-overexpression per se.

It is important to realise that shRNAs (partially) silence gene expression from transcripts that share a certain degree of homology with the siRNA sequence (reviewed by Jackson and Linsley, 2010). These off-target effects could also have an effect on cell viability or could even affect target-gene expression levels indirectly. A number of generally accepted controls (Editorial, 2003) should be applied to control for off-target effects of which some are included in the controls described above.

**Recent developments in the use of RNAi-mediated gene silencing**

The currently on-going development of methods for gene silencing in the CNS will benefit from strategies that control the adverse effects of overloading the miRNA machinery and close monitoring thereof in experimental setups (reviewed in Borner and Grimm 2010). One way to overcome the toxic effects is to titrate the viral dose to a level where the adverse effects are absent while maintaining a certain degree of knockdown (Ulusoy et al., 2009). However, the success of this approach is likely to depend on shRNA efficacy, as some shRNAs do not exert effective knockdown at non-toxic expression levels (McBride et al., 2008). Therefore, the dose response curve should be analysed for each individual shRNA sequence in-vivo, and should be re-assessed when changes are made in viral vector, target cell or animal model. This makes in-vivo shRNA-mediated gene silencing a laborious technique.

A promising alternative approach is to use artificial miRNAs to knock down gene expression. miRNAs are expressed at lower levels but are processed more efficiently than shRNAs (Boden et al., 2004, Silva et al., 2005, Boudreau et al., 2008). This reduces the saturation-effect on the miRNA machinery. The first attempts to achieve sustained silencing in the CNS with artificial miRNAs have shown to be effective and free of adverse effects (McBride et al., 2008, Boudreau et al., 2009a, Boudreau et al., 2009b, Nielsen et al., 2009, Liu et al., 2010). An additional benefit of the artificial miRNAs is that they can be transcribed using RNA polymerase II promoters. This paves the way for the use of cell specific promoters and consequently refined spatiotemporal control of expression of miRNAs (Giering et al., 2008, Nielsen et al., 2009, Liu et al., 2010). Thus artificial miRNAs are emerging as a promising new strategy to safely knock down gene expression and may prove to be a favourable method for in vivo application.
2.3 *Semaphorins in spinal cord regeneration*

The hypothesis, that in the injured spinal cord, class-3 semaphorins are inhibitors of axonal regeneration, is based on the following observations (Fig. 1):

1. Semaphorins are chemorepulsive axon guidance molecules that can repel axons and cause collapse of the growth cone.
2. Sema3s expression is upregulated in meningeal fibroblasts that are located in the core of a CNS lesion that forms after injury, and injured neurons continue to express semaphorin receptors.
3. Meningeal cell derived Sema3a inhibits axonal outgrowth and causes growth cone collapse in vitro (chapter 2).

To test this hypothesis, two approaches to remove semaphorin signalling and to improve axonal regeneration following spinal cord injury have been used: pharmacological intervention with semaphorin signalling and molecular genetic intervention. In the following section we discuss the current understanding of the role of semaphorins during spinal cord regeneration based on the results obtained using these two intervention strategies.

**Pharmacological inhibition of Sema3a**

In previous studies two types of inhibitors have been used to investigate the role of Sema3a during spinal cord injury. Kikuchi and colleagues (Kikuchi *et al.*, 2003) identified SM-216289 (or xanthantofulvin), a small molecule isolated from a fungal fermentation broth, that inhibits Sema3a induced growth cone collapse and DRG-axon repulsion by binding to Sema3a. In vivo, this compound accelerates olfactory nerve regeneration (Kikuchi *et al.*, 2003) and has several beneficial effects after complete transection of the spinal cord (Kaneko *et al.*, 2006). In the latter model, SM-216289 positively influenced axon regeneration and preservation, remyelination and Schwann cell migration, angiogenesis, apoptosis and functional recovery.

The second approach interferes with semaphorin signalling by targeting the function of the immunoglobulin-like cell adhesion glycoprotein L1 that is part of the semaphorin receptor complex. L1 is required for Sema3a-mediated repulsion and soluble L1-FC protein or a 6-amino acid L1-peptide can revert repulsion into attraction in vitro. Furthermore, soluble L1-FC protein and the L1-peptide can block Sema3a-induced growth cone collapse and inhibition of neurite growth in vitro (Castellani *et al.*, 2000, Castellani *et al.*, 2002). Intrathecal delivery of L1-FC protein after spinal contusion in rats resulted in improved motor function and CST fiber regeneration (Roonprapunt *et al.*, 2003) while L1-peptide in mice after spinal contusion did not promote axon growth and recovery of motor function after spinal contusion in mice (Mire *et al.*, 2008). The property of L1 to convert sema3a repulsion into attraction is confined to short peptide sequence in the Ig domain of L1. The L1-FC protein also increases neurite growth in vitro when it is
used as a substrate for growing axons (Roonprapunt et al., 2003). The differences in the effects of L1-FC and L1-peptide in vivo could be explained by the growth supporting property of L1-FC that is independent of the Sema3a-modulating activity.

**Genetic approaches to study class-3 semaphorins in spinal cord injury**

To date, three studies on spinal cord injury have used a genetic approach that interferes with Sema-signalling. The first study compared axonal regeneration and functional recovery after spinal cord contusion in mice that lack L1 with wild type littermates. Since L1 is required for Sema3a-mediated inhibition of cortical neuron outgrowth in vitro, L1 knockout mice are in principle insensitive to scar derived Sema3a. Jakeman and co-workers found that there is no enhanced improvement of motor function or axon growth at the site of the lesion in L1 knockout mice, but did observe increased CST axon sprouting into the grey matter caudal to the lesion site (Jakeman et al., 2006).

The second approach analysed regeneration of serotonergic and CST axons following complete transection of the spinal cord at thoracic level 8 in mice that lack PlxA3 and PlxA4, two key receptor components for class-3 semaphorin receptor signalling. Mice lacking these receptors do not show improved regeneration of serotonergic or CST axons (Lee et al., 2010a).

Finally, in this thesis we report our studies on spinal cord regeneration in two mouse models. First we examined regeneration of the injured CST in conditional Npn-1 deficient mice. In these animals, the Sema3A receptor component Npn-1 is abliterated in neurons around the time of birth. We show that knocking out Npn-1 does not enhance regeneration of injured CST fibers and does not result in improved recovery of motor function (chapter 5). Next we studied the effect of genetic deletion of Npn-2 on the failure of RST axons to regenerate. Neurons of the red nucleus express only Npn-2 (De Winter et al., 2002b), hence rubrospinal axons of Npn-2 KO mice should be insensitive to all class-3 semaphorins (except sema3E which signals independent of Npn-1/Npn-2). However, as reported in chapter 6 disruption of Npn-2 signalling does not improve outgrowth of RST axons and does not improve recovery of motor function after RST lesion.

**Inherent differences between pharmacological and genetic interventions.**

From the results of these experiments it is evident that pharmacological interventions that interfere with semaphorin signalling are effective while the results of the genetic approaches are mostly unsuccessful. There are several possible explanations that may account for these differences.

**Small molecules can have effects on multiple scar-associated cells**

Class3 semaphorins are expressed in the core of the spinal cord lesion and we hypothesise that this is where they inhibit the outgrowth of injured axons. The
primary goal of neutralising semaphorin signalling is to improve outgrowth of injured axons that encounter this chemorepulsive cellular barrier. However, small molecules will not only modulate sema-signaling between scar-derived semaphorins and the axons of injured neurons but will also influence sema-signalling between other cells at a spinal cord injury site. These cellular targets may have equally important roles in the success or failure of spinal cord axons to regenerate. For instance, SM-216289 did also have an effect on migration of Schwann cell from the injured dorsal roots into the spinal cord injury site, on the volume of cavities associated with the neural scar, and on angiogenesis and motor neuron survival (Kaneko et al., 2006). Therefore the enhanced 5-HT axon growth observed by Kaneko and colleagues is most likely not solely due to its ability to relieve the inhibition of axonal growth but may be the result of effects on multiple cellular interactions at the site of a lesion.

**Molecular specificity of small molecules.**

Another important factor that has to be taken into account is that small molecules bind to other molecular targets that are not directly involved in semaphorin signalling. SM-216289 also has a high affinity for matrix metalloprotease-2 and the epidermal growth factor receptor (Kaneko et al., 2006). Binding of SM-216289 to these targets may modulate cell proliferation, migration, the extracellular matrix and cell adhesion, which all can have additional effects on scar formation and axonal regeneration. Likewise, similar aspecific effects may occur by interfering with L1CAM signalling. Soluble L1-FC and L1 peptide increases axonal outgrowth by reverting the Sema3A-mediated repulsion of injured axons in to attraction in vitro by binding to neuropilin-1. However, L1 also interacts with Ig CAMs, laminin and CSPGs and is involved in myelination (Barbin et al., 2004) and cell migration (Lavdas et al., 2010). Therefore the beneficial effects of L1-FC on axonal regeneration could partially be the result of interference with Sema3A-unrelated effects.

**Compensation for loss of function in genetic models**

Small molecules are delivered in adult animals during a defined time-window. Therefore their effects are acute and do not influence the anatomical and physiological development of the animal. This is a clear advantage of this approach and may explain at least in part why SM-216289 has a beneficial effect on spinal cord repair. In the above discussed constitutive knockout mouse models, PlxA3/4, L1 and Npn-2 are absent throughout embryonic development, after birth and during adulthood. These animals have several anatomical abnormalities (Chen et al., 2000, Giger et al., 2000, Cheng et al., 2001, Yaron et al., 2005, Jakeman et al., 2006, Lee et al., 2010a) yet the animals that survive into adulthood somehow cope with the loss of gene function and possibly developed compensatory mechanisms to survive and develop seemingly normal behaviour. This is further demonstrated by the poor survival rate of Npn-2 KO animals,
of which only approximately 6% survive into adulthood (Giger et al., 2000, chapter 6). Apparently the lack of Npn-2 decreases their viability but some mice appear to be able to compensate for the deficits that cause the death of their genotypically identical littermates. Such a compensatory mechanism would have consequences for our studies on the role of sema-Npn-2 signalling after a spinal cord lesion because we assume that WT and KO animals only differ in expression of the targeted gene. However, knocking out one gene may affect the expression or function of various other molecules. For instance, one could speculate that KO animals compensated the lack of Npn-2 by increasing their sensitivity for other axon guidance molecules for instance by activating other receptors involved in repulsive signalling to take over the function of the lost Npn-2 receptor.

**Redundancy of inhibitory signals**

In our studies we have investigated the relative contribution of class-3 semaphorins to the axon growth inhibitory nature of the injured spinal cord. We have found that interfering with semaphorin signalling in knockout animals did not enhance axonal regeneration or improve motor function after CST and RST damage. Does this mean that semaphorins have no impact on the recovery after spinal cord injury at all? The answer to this question is: at least not in a biological context where a multitude of other growth-inhibitory molecules are still present and active.

As discussed in chapter 1, 5 and 6, a plethora of inhibitors of axonal outgrowth is present in the injured spinal cord. It is likely that these inhibitory molecules act in concert and eliminating multiple inhibitory pathways is necessary to improve spinal cord repair. Some evidence that supports this idea comes from studies that interfere with the classical myelin inhibitors MAG, OMgp and Nogo. Mice that lack both MAG and OMgp did not show improved axonal regeneration or enhanced recovery of motor function after spinal cord injury. In a mouse that lacks Nogo-A, CST fibers show some improved regeneration as compared to WT littermates. When Nogo-A, MAG and OMgp we knocked simultaneously, there was a synergistic effect on top of the regenerative response of the Nogo-A knockout mouse (Cafferty et al., 2010). This shows that some inhibitors that do not seem to influence axonal regeneration on their own, can have an effect when an additional inhibitor is removed. The first study, in which myelin and class-3 semaphorin inhibitory signaling were simultaneously targeted, used an NgR/plexinA3/A4 triple knockout mouse (Lee et al., 2010a). These triple knockout animals did not show enhanced regeneration of injured axons. Perhaps the lack of a regenerative response in these animals is due to a redundancy in receptors for MAG, OMgp and Nogo since these myelin inhibitors not only signal through binding to NgR but can also bind to PirB and NgR2 (Venkatesh et al., 2005, Atwal et al., 2008).
Our findings, combined with those of others, are providing a window on the complexity of the signals that inhibit recovery after spinal cord injury. Furthermore, it is important to realize that there are multiple reasons for the failure of the CNS to regenerate after injury. Apart from inhibitory molecules in myelin and the neural scar, the limited intrinsic capability of injured neurons to regenerate and the lack of growth supportive molecules contribute to the poor regenerative response. A combinational approach to address these three hurdles is the next complex but logical step towards improved functional recovery after CNS injury.


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Nederlandse samenvatting

Zenuwcellen in de hersenen (neuronen) geven via lange uitlopers (axonen) hun instructies door aan het lichaam. Deze axonen vormen onder andere de zenuwbijen van het ruggenmerg waarlangs signalen van en naar de hersenen worden gestuurd. Zo worden er bijvoorbeeld vanuit de hersenen motorische informatie ontvangen, en wordt er sensorische informatie ontvangen over de omgeving. De banen van het zenuwstelsel worden tijdens de ontwikkeling gevormd met behulp van moleculen die de axonen aantrekken of afstoten (axon guidance moleculen). Deze moleculen zijn ervoor verantwoordelijk dat de groeiende axonen naar de juiste plaats worden gestuurd en deze moleculen zorgen er ook voor dat neuronen de juiste functionele contacten maken.

Door een dwarslaesie worden er zenuwbijen in het ruggenmerg onderbroken met het verlies van motorische en sensorische functie tot gevolg. De beschadigde zenuwbijen van het ruggenmerg kunnen niet regenereren, onder ander omdat er op de plaats van de laesie littekenweefsel wordt gevormd. Om het herstel na dwarslaesie te verbeteren, is het ontwikkelen van nieuwe strategieën die de groei van beschadigde zenuwbijen stimuleren noodzakelijk.

Het gebrek aan een regeneratieve response van het zenuwstelsel wordt voor een deel veroorzaakt door axon-groei remmende moleculen die aanwezig zijn in het beschadigde ruggenmerg: myeline-geassocieerde remmers (bekende voorbeelden zijn Nogo, MAG, OMgp), littekenweefsel geassocieerde remmers (chondroitine sulfaat proteoglycanen) en repulsieve axon guidance moleculen (slit, ephrins, RGMa, semaphorines, beschreven in hoofdstuk 1). Samen vormen deze moleculen een multi-moleculaire barrière voor uitgroeiende axonen en zijn mogelijke aangrijpingspunten voor nieuwe therapeutische strategieën ter verbetering van herstel na schade aan het ruggenmerg.

Klasse-3 semaphorines zijn gesecreteerde axon guidance eiwitten die tijdens de ontwikkeling de uitgroeiende axonen de weg wijzen om met de juiste cellen contact te maken. Deze semaphorinen worden na schade aan het ruggenmerg opnieuw tot expressie gebracht door de meningeale fibroblasten die zich bevinden in het centrum van het gevormde littekenweefsel. De beschadigde zenuwvezels zijn gevoelig voor deze repulsieve semaphorine signalen omdat ze de hiervoor benodigde receptoren (neuropilinen en plexinen) nog steeds tot expressie brengen. Deze observaties hebben geleid tot de hypothesen dat semaphorinen een negatieve invloed hebben op de regeneratie van beschadigde ruggenmergvezels.

Het doel van het onderzoek beschreven in dit proefschrift is om methoden te ontwikkelen die semaphorine signalen blokkeren om zo de rol van semaphorinen tijdens het herstel na schade aan het zenuwstelsel te onderzoeken.
In hoofdstuk 2 tonen we aan dat semaphorine 3A (Sema3A), afkomstig van meningeale fibroblasten, bijdraagt aan de remmende werking van het littekenweefsel op de regeneratie van axonen. In experimenten met eiwitextracten van geweekte meningeale fibroblasten laten we eerst zien dat deze extracten in staat zijn om de groeikonen aan het eind van een sensorisch axon te laten collaberen. In aanwezigheid van een blokkerend antilichaam gericht tegen het Sema3A receptorcomponent neuropiline-1 (Npn-1), vind deze groeikoon-collaps niet meer plaats. Eiwitextracten van meningeale fibroblasten van Sema3A knock out (KO) muizen waarbij het gen voor Sema3A genetisch is uitgeschakeld zijn veel minder in staat om groeikonen te laten collaberen. Tenslotte is de uitgroei van sensorische axonen op een monolaag van gekweekte meningeale fibroblasten van Sema3A KO muizen verbeterd ten opzichte van de uitgroei op wild-type cellen. Deze resultaten laten zien dat Sema3A, dat tot expressie wordt gebracht door meningeale fibroblasten, een krachtige remmer is van axonale uitgroei in vitro.

Vervolgens hebben we twee strategieën ontwikkeld om de groei remmende semaphorine signalen in dwarlaesie-diermodellen te kunnen blokkeren. De eerste strategie is gebaseerd op het gebruik van zogenaamde short hairpin RNA’s (shRNAs) om de expressie van neuropilines, en daarmee de gevoeligheid voor semaphorinen, te verminderen. De tweede strategie maakt gebruik van genetisch gemodificeerde muizen waarbij het Npn-1 gen selectief in neuronen, of het Npn-2 gen in het gehele dier is uitgeschakeld.

shRNAs kunnen in zenuwstelsel tot expressie worden gebracht met behulp van virale vectoren; gemodificeerde virussen die genetisch materiaal in een cel kunnen afleveren (transduceren). In hoofdstuk 3 hebben we eerst onderzocht hoe we DRG neuronen het meest efficiënt kunnen transduceren met virale vectoren. Uit onze experimenten bleek dat, van de acht geteste virale vectoren, adeno associated virus type-5 (AAV5) het effectiefst DRG neuronen transduceert. Uit een eerder vergelijkbaar onderzoek in ons laboratorium is gebleken dat rubrospinal neuronen het efficiëntst worden getransduced door AAV1.

Vervolgens brengen we in hoofdstuk 4 met deze virale vector technologie shRNAs tot expressie in de rubrospinal- en DRG neuronen. De expressie van shRNAs veroorzaakte onverwachts atrofie en celdood van rubrosinal neuronen. Deze toxische reactie kan worden toegeschreven aan de shRNA expressie, en niet aan de virale vector transductie zelf, omdat virusdeeltjes die alleen het groen fluorescente eiwit (GFP) tot expressie brengen deze nadelige effecten niet veroorzaken. Uit onze studie en uit recente studies van andere groepen blijkt dat de toxische gevolgen van shRNAs expressie afhankelijk is van de shRNA-dosis. Een grote hoeveelheid aan shRNAs veroorzaakt overbelasting van de endogene micro-RNA (miRNA) machinerie, een cellulair proces dat noodzakelijk is voor het normaal functioneren van een cel. Expressie van shRNAs in DRG neuronen veroorzaakte geen cellulaire toxiciteit. Hierdoor was het mogelijk om in DRGs met behulp van shRNAs de het aantal neuronen dat Npn-2 tot expressie brengt te verminderen.
Bij de tweede strategie om semaphorine signalen in diermodellen te kunnen blokkeren maken we gebruik van muizen waarbij één van de twee neuropiline genen is uitgeschakeld. In hoofdstuk 5 hebben we de regeneratie van de gelaedeerde corticospinale baan (CST) geanalyseerd in Npn-1 KO muizen waarbij het Npn-1 gen alleen in neuronen is uitgeschakeld. We hebben gevonden dat bij deze Npn-1 KO muizen, in vergelijking met normale (wild type) muizen, er geen verbeterde uitgroei is van beschadigde CST zenuwvezels. Ook laten vier verschillende motorische testen zien dat er geen verbeterd herstel van motorfunctie is in Npn-1 KO muizen. Npn-1 is de receptor voor Sema3a, echter CST neuronen brengen zowel Npn-1 als Npn-2 tot expressie. Onze experimenten laten zien dat het uitschakelen van één van de inhibitory semahorine klasse-3 signalen (Npn-1/Sema3a) niet voldoende is om regeneratie van de CST te stimuleren.

In hoofdstuk 6 onderzoeken we de regeneratie van de rubrospinaalbaan (RST), die bestaat uit de axonen van de rubrospinal neuronen, in Npn-2 KO muizen. Omdat RST neuronen geen Npn-1 tot expressie brengen, bevatten deze neuronen van Npn-2 KO muizen geen neuropiline receptoren. Hierdoor zijn rubrospinaal axonen ongevoelig voor alle klasse-3 semaphorinen (behalve Sema3E, welke onafhankelijk van neuropiline zijn signaal overbrengt) en verwachtten we verbeterde regeneratieve response van beschadigde axonen in Npn-2 KO muizen. Echter, na unilaterale RST laesie was de RST regeneratie en motorfunctie van Npn-2 KO muizen niet verbeterd vergeleken met wild type muizen. Uit deze studies blijkt dat het uitschakelen van klasse-3 semaphorine signalen alleen, niet voldoende is om een regeneratieve response te induceren na CST en RST laesie.

Er zijn tenminste 3 mogelijk factoren die de bevindingen beschreven in hoofdstukken 3 tot en met 5 zouden kunnen verklaren. Ten eerste is er, zoals bediscussieerd in hoofdstuk 1, een grote diversiteit aan groei remmende moleculen die samen bijdragen aan het beperkte herstel van het beschadigde ruggenmerg. Het uitschakelen van alleen de klasse-3 semaphorine signalen is simpelweg niet genoeg om de groeierrmmende barrière dermate te verminderen zodat axonale regeneratie mogelijk wordt. Ten tweede kan het verwijderen van Npn-2 invloed hebben op de ontwikkeling van littekenweefsel, wondhealing en algemene neuronale functie. Deze effecten kunnen onbedoeld een eventueel positief effect tenietdoen. Ook kunnen mechanismen die compenseren voor het verlies van Npn-1 of Npn-2 het beoogde positieve effect hebben geneutraliseerd. Tot slot, Npn-1 en Npn-2 zijn niet alleen receptoren voor semaphorinen, maar ook voor splice-varianten van VEGF. VEGF kan neurotrofe- en protectivie effecten hebben. Het wegvallen van deze mogelijk positieve VEGF-signalen in Npn-1 en Npn-2 KO muizen kan hierdoor averechte gevolgen hebben.

Onze bevindingen tezamen met die van collega-onderzoekers onderstrepen de complexiteit van het arsenal van remmende signalen die het herstel van schade aan het ruggenmerg bemoeilijken. Bovendien is het belangrijk ons te realiseren dat zijn er additionele factoren zijn die bijdragen aan de slechte
regeneratie na schade aan het zenuwstelsel. Naast de aanwezigheid van een verscheidenheid aan groei remmende signalen, hebben volwassen neuronen een beperkte intrinsieke capaciteit om opnieuw uit te groeien en is er een gebrek aan groei-ondersteunende moleculen. Een gecombineerde aanpak waarbij meerde obstakels tegelijk worden geëlimineerd, is een complexe maar logische volgende stap om te proberen het herstel na schade aan het zenuwstelsel te verbeteren. Om tot een succesvolle gecombineerde aanpak te komen is meer fundamenteel moleculair biologisch onderzoek nodig naar de factoren die een sleutelrol vervullen in het afremmen en bevorderen van neurale regeneratie processen.
Curriculum vitae


In 2002 keerde Erich terug naar Nederland en kreeg een aanstelling bij het toemalige Nederlands Instituut voor Hersenonderzoek, nu bekend als het Nederlands Institut voor Neurowetenschappen. Hier begon hij in de vakgroep Neuregeneratie van prof.dr. J. Verhaagen onder leiding van dr. S.P. Niclou als research analist aan het project dat later de basis zou vormen voor zijn promotieonderzoek. In 2003 werd hij in staat gesteld om naast zijn taken als analist en laboratoriumbeheerder, zijn onderzoek verder uit te bouwen als promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift.

Momenteel is Erich werkzaam bij dezelfde vakgroep waar hij binnen een europees samenwerkingsproject de rol van semaphorinen bij plasticiteit van het centrale zenuwstelsel onderzocht.

Erich woont in Gouda, samen met zijn vrouw Marianne en zijn dochters Eva en Anne.
List of publications


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