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 and removal of Sema signalling will result in improved regeneration (reviewed in chapter 1, Bolsover et al., 2008 and Harel and Strittmater, 2006).

Figure 1. Workflow of this thesis

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.