Summary and general discussion

Summary of this thesis
In the research described in this thesis, we explored two related questions.
1) What are the molecular properties of the human peripheral nerve lesion, and in particular, why is scarring at the lesion site, or neuroma formation, detrimental to functional recovery?
2) Can we apply lentiviral (LV) vectors to overexpress neurotrophic factors to enhance regeneration after surgical repair?

The first two experimental chapters dealt with the first research question. We studied small segments of human neuroma tissue that were removed during reconstructive surgery and discovered that they contain the chemorepulsive protein semaphorin3A. Staining for semaphorin3A shows that the protein surrounds nerve fibers in a punctate pattern and a functional in vitro test shows an inhibition of the neurite outgrowth of cells from a neuronal cell line that were cultured on slices of human neuroma tissue. Encouraged by this discovery, in the next chapter we performed a genome-wide expression analysis in human neuroma tissue and found that the expression of a significant number of genes involved in scar formation and axon guidance is differentially regulated. Further investigations into the precise role of several of these factors, specifically their potential influence on phenomena such as repulsion, fasciculation and defasciculation of regenerating of axons, will help to improve the understanding of the outgrowth-inhibitory nature of the neuroma. Perhaps more importantly, these investigations could yield novel targets for future therapies aimed at improving regeneration after peripheral nerve injury.

In the following chapters, LV vectors were applied to express neurotrophic factors in the injured peripheral nerve in an attempt to enhance regeneration after surgical repair. First, we developed a protocol to genetically modify cells in cultured segments of human sural nerve using LV vectors expressing the marker gene green fluorescent protein (GFP). With the application of an LV vector encoding nerve growth factor (LV-NGF) long-term production of biologically active NGF could be directed in cultured human nerve segments. This technique was subsequently used to investigate in vivo the effect of LV vector-mediated overexpression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) on the regeneration of rat motoneurons after avulsion and reimplantation of the ventral nerve root. In this model, we found positive effects of LV vector-mediated overexpression of GDNF: a complete reversal of avulsion-induced motoneuron atrophy and an increased density of regenerating axons in the reimplanted roots. However, the strongest effects of LV-
GDNF were negative: high levels of transgenic GDNF expression induced a truly striking neuroma-like ‘oasis’ of coiled axons in the nerve root, and the number of regenerated fibers more distally was, in fact, lower in comparison with control treatments.

In the next chapter, the effect of LV vector-mediated overexpression of NGF and GDNF was investigated in a rat model for peripheral nerve transection and repair. The regeneration of motoneurons does not appear to be affected by the LV vector-mediated overexpression of NGF. However, similar to the root avulsion repair study, LV vector-mediated expression of GDNF in the transected nerve impaired the long-distance regeneration of motoneurons. In the affected nociceptive sensory neurons, LV vector-mediated overexpression of both NGF and GDNF causes profound phenotypic changes that are in line with the notion that these factors play an important role in the pathogenesis of pain. Although both the differential regenerative response of motor and sensory neurons upon application of these factors and the changes in nociceptive sensory neurons could form the starting point for new exciting investigations, these findings also highlight the difficulties in improving nerve regeneration and the genuine possibility of unwanted side-effects associated with LV vector-mediated overexpression of neurotrophic factors.

In this general discussion I will further evaluate the merits of the results described above, as I believe that the experiments in this thesis have yielded both clear “winners” and “losers”. I would regard the discovery of semaphorin3A, as well as several other axon guidance molecules in the human neuroma and the successful long term transgene expression in the peripheral nerve after in vivo application of LV vectors as “winners”, i.e. results that merit additional investigation and may form the basis of future clinical applications. As a “loser”, i.e. a strategy that did not live up to expectations, I would consider the exogenous continuous application of neurotrophic factors as a means to enhance long-distance regeneration. In the sections below, I will elaborate on these qualifications. However, the merits of new therapeutic concepts depend on their ability to advance the current clinical practice of nerve repair. Therefore, I will start by giving a concise overview of the challenges that exist in the management of peripheral nerve injuries.

**Challenges in nerve repair – opportunities for novel therapies**

In this section I will briefly discuss the present state of the art in peripheral nerve repair. This is by no means intended as an exhaustive review of the entire body of knowledge on peripheral nerve regeneration, but instead, I will focus on those issues that are relevant to the new therapeutic strategies that have been explored in this thesis. In contrast to the injured spinal cord, in which the regenerative process is thwarted by the abundance of growth-inhibitory factors, the lesioned peripheral nerve is capable of regeneration. However, regeneration, and subsequent functional recovery, depends on the degree of nerve injury.
Clinically, the most important distinction is between axonotmetic injuries, in which axons are severed but the integrity of nerve fascicles is preserved and neurotmetic injuries, in which the physical continuity of the entire nerve (including axons and nerve fascicles) is lost. Although a regenerative response does occur in neurotmetic lesions, axons are unable to bridge the gap that forms between proximal and distal nerve stumps and surgical reconstruction is therefore usually indicated. In contrast, axonotmetic injuries tend to regenerate completely, although this does not always result in full functional recovery. At a rate of regeneration of 1-5 mm/day, it may take several months for regenerating axons to reinnervate their distal targets and during this time these targets may have atrophied, resulting in a poor restoration of function. This slow rate of regeneration and associated atrophy of denervated target organs is therefore a clinical problem and a potential target for therapeutic intervention.

In cases of acute neurotmetic injuries (e.g., the complete transection of the ulnar nerve in a stab wound), the clinical strategy to treat the injured nerve is relatively straightforward and consists of immediate repair surgery, either through the direct coaptation of proximal and distal nerve stumps, or through the application of a nerve grafts that acts as a scaffold for regenerating axons.

However, the treatment strategy is not always this clear-cut, especially if a lesion is characterised by extensive intraneural fibrosis. In this so-called neuroma in continuity, both axonotmetic and neurotmetic injury types can be present, and it is therefore difficult to assess the likelihood of spontaneous recovery. The absence of a reliable method to determine the degree of injury at an early time point means that most surgeons recommend surgical exploration when clinical examination does not show functional recovery of the associated muscle after a certain waiting period. However, the regenerative potential of axotomised motoneurons diminishes over time and therefore, this approach is in effect a trade-off between not waiting long enough (running the risk of performing surgery on injuries that would have recovered spontaneously) and waiting too long (impairing the outcome of reconstructive surgery for severe lesions). This is a second clinical challenge, one that could be addressed by either improving the ability to assess the degree of injury at an early time point or by enhancing the regenerative capacity of chronically axotomised neurons.

After nerve repair, both motor and sensory neurons are able to regenerate and re-establish functional connections, but there are differences in their regenerative response. Motoneurons preferentially reinnervate muscles, a phenomenon that is the result of both interaction between the motoneuron and the distal endoneurial tubes at the coaptation site and of the “pruning” of motoneuron axons that have inaccurately entered endoneurial tubes that lead to sensory organs. However, this increased likelihood that motoneurons reinnervate muscles, called “preferential motor reinnervation,” does not mean that these motoneurons are capable of selectively find-
ing their original targets. Instead, the outgrowth of motor axons is a random process that frequently results in the reinnervation of inappropriate, antagonistic muscles or even reinnervation of two muscles by branches from one motoneuron. This leads to the cocontraction of antagonizing muscles and subsequently a failure to recover functionally. This “misrouting” phenomenon is the third challenge that provides an opportunity for future therapies.

Finally, the regeneration of motoneurons through grafts derived from purely motor nerves is better than through grafts from sensory nerves and vice versa. Whereas some researchers have attributed this fact to the differential expression of several neurotrophic factors in motor and sensory nerve grafts, others claim that physical differences play an important role. Axons of sensory neurons generally have a smaller diameter, and the endoneurial tubes in grafts derived from sensory nerves may not be ideally suited to accommodate the larger-diameter axons of motoneurons. The most commonly used graft in human reconstructive surgery is derived from the sural nerve, because this sensory nerve can be missed without causing major deficiencies. The putative drawback of using sensory nerve grafts to support the regeneration of motoneurons is the fourth and final problem in the clinical practice of nerve repair that I would like to mention here.

In the light of the issues described above, I will now continue to discuss the merits of the work described in this thesis, starting with the “losing strategy” of applying neurotrophic factors to enhance regeneration.

**Exogenous neurotrophic factors: useful to prevent atrophy, but probably not to promote outgrowth**

Since the discovery that NGF can promote neurite outgrowth by Rita Levi-Montalcini in 1952, neurotrophic factors have arguably been the most widely studied proteins in the field of neuroregeneration. Interestingly, in the treatment of several neurodegenerative diseases (e.g., Alzheimer’s disease and Parkinson’s disease), the application of neurotrophic factors has been quite promising. Clinical trials with neurotrophic factors for these diseases are currently underway and encouraging results have been reported. In contrast, the application of neurotrophic factors to enhance neuroregeneration has proven to be far more difficult. One explanation for this difference must be that unlike survival, successful regeneration depends on precise time- and location-dependent expression of these factors to create an ever-shifting gradient towards which axons continue to extend, sometimes over periods of several months. The “candy-store” effect, which was first described in the spinal cord and also observed after local application of LV-GDNF in Chapters 5 and 6 shows that in this case, more is not necessarily better. Furthermore, we have recently obtained unpublished data on the concentration of NGF, Neurotrophin-3, BDNF and GDNF in avulsed nerve
roots that clearly show the subtle temporal changes in the expression of these factors. In other words, the endogenous expression of these factors in the peripheral nerve already appears to be optimised to support continuous regeneration and it is hard to envision how their exogenous application will have any other effect than disrupting this delicate balance.

Another striking observation in this respect is that in each paradigm studied in this thesis, the peripheral nerve clearly “wants to regenerate”, probably stimulated by endogenously elevated levels of neurotrophic factors distal from the lesion site. This is exemplified by three observations: (i) the many regenerating axons in the human peripheral nerve scar (Chapter 3), (ii) the numerous motor axons crossing spinal cord white matter and entering the implanted nerve root (Chapter 5) and (iii) the high number of neurites that did grow into the nerve stump distal to a peripheral nerve lesion (Chapter 6). The robust regeneration in rat models for peripheral nerve injury (ii and iii) do not necessarily translate to the human clinical situation, where the distance to be bridged from lesion site to target organ is usually much longer \(^9\). Nonetheless, the injured human peripheral nerve is also capable of regenerating over long distances \(^1,4\), suggesting the presence of a mechanism that shifts the elevated endogenous production of neurotrophic factors ahead of regenerating axons. Exogenously and locally increasing the amount of neurotrophic factors in the nerve therefore seems neither needed nor helpful. This is disappointing, because the transduction with lentiviral vectors to overexpress a combination of motoneuron-specific neurotrophic factors \(^12\) could be an interesting strategy to render sensory sural nerve grafts more supportive of the regeneration of motoneurons. As described in Chapter 6, the regenerative response to the overexpression of GDNF of sensory neurons differs from motoneurons and such findings could theoretically be exploited to create grafts that are specifically permissive for the regeneration of either sensory or motoneurons. However, unless it becomes possible to create a time- and location-dependent gradient of these factors in the nerve, this approach is not likely to enhance the long distance regeneration through nerve grafts.

In summary, the exogenous application of neurotrophic factors still faces significant challenges if the goal is to stimulate peripheral nerve regeneration. However, by exploiting the survival-enhancing properties of trophic factors, there may be several possibilities to enhance the functional outcome of nerve repair.

Firstly, as described in Chapter 5 of this thesis, the viral vector-mediated application of GDNF could be a promising approach to prevent motoneuron atrophy after nerve root avulsion, “keeping them in shape” prior to reconstructive surgery. This is of particular importance as the diminished regenerative capacity of chronically axotomised motoneurons \(^21\) can be boosted by the temporary application of GDNF \(^21\). This could therefore be a way to compensate for the deleterious effect of the waiting period that is currently part of the surgical decision-making process.
Secondly, there may be a role for neurotrophic factors in the prevention of target muscle atrophy during the period of denervation. Ciliary neurotrophic factor (CNTF) has a strong myotrophic effect on denervated skeletal muscle \(^{220,221}\), but systemic application of CNTF causes unwanted side-effects such as severe weight loss \(^{222}\). Local, LV vector-mediated expression in the muscle \(^{46}\) of CNTF may be a method to prevent muscle atrophy without causing significant side effects, thereby addressing the clinical issue of chronic denervation.

Apart from practical issues involved in applying viral vectors to, for instance, axotomised motoneurons in the spinal cord, the success of these strategies will depend on two factors: the ability to tightly regulate the amount of neurotrophic factors produced and the ability to switch off transgene expression completely when it is no longer helpful. This is theoretically possible with viral vectors with regulatable gene expression. As discussed in Chapter 1, there are still unresolved issues regarding the safety and clinical applicability of vectors that direct regulatable transgene expression. It will therefore require several years of additional research before these strategies are ready to be tested in a clinical setting.

**Failed functional recovery: not the engine is missing, but the steering wheel**

As proposed in the previous paragraph, in most cases regeneration of the peripheral nerve does not seem to need stimulation by the exogenous application of factors like NGF and GDNF as they are already tightly and autonomously regulated to optimise continuous outgrowth. Why then, is functional recovery of the peripheral nerve after reconstructive surgery often not complete? If this is not the result of an insufficient regenerative response, there must be another reason why, for instance, patients with a brachial plexus lesion often fail to regain function of distal targets like the hand after reconstructive surgery \(^{85}\).

There is an increasing body of evidence suggesting that the misrouting problem is a strong contributing factor to the lack of functional recovery after nerve repair surgery \(^{115,116,223}\). Younger patients may be able to partially compensate for misrouted axons due to the plasticity of their still maturing central nervous system \(^{224,225}\), but this ability of the brain to adapt to the newly formed peripheral connections diminishes with aging. To eliminate the cocontraction of antagonising muscles, Botulinum toxin type A has been injected in the triceps muscle of patients with brachial plexus injuries \(^{116}\). The purpose of this approach is to facilitate motor learning by temporarily inducing the relaxation of antagonist muscles and allowing increased activity in the reinnervated biceps muscle. Other than this, there are no pharmacological options to treat misrouting and the problem is usually addressed with intensive physical therapy \(^{226}\).

In research, the routing problem has mainly been addressed mechanistically, for instance by applying artificial scaffolds with a three-dimensional structure to enhance
the physical guidance of regenerating axons\textsuperscript{227-230}. It is true that the physical properties of the distal endoneurial tubes determine the fate of regenerating axons and thus strongly influence the degree of functional recovery\textsuperscript{7}. However, surgical reconstruction of severe neurotmetic nerve injuries is inherently accompanied by a loss of continuity of nerve fascicles, impairing the ability of the nerve to physically guide regenerating axons. In addition, the sural nerve graft that is most commonly used for reconstructive surgery (see above) already contains thousands of aligned Schwann cells in longitudinally oriented endoneurial tubes, so it is difficult to imagine how this could be improved by artificial guides. Theoretically, one could envision a refinement of surgical techniques up to a level where each individual axon in the proximal stump is matched to one endoneurial tube in carefully prepared (artificial or sural) nerve grafts, but even then it is hard to imagine how the appropriate distal targets for these axons could be identified or how branching at the coaptation site could be prevented. Antibodies against neurotrophic factors have been applied in a rat model for peripheral nerve transection to reduce the branching of regenerating axons and thus improve the quality of regeneration\textsuperscript{231}, but this approach carries the risk of interfering with the neurotrophic factor signalling that is needed for successful regeneration.

Another approach could be to allow regeneration to take place after repair as usual, but then enhance the ability to use the newly formed connections by increasing plasticity at the level of the spinal cord\textsuperscript{120} or brain. Although this approach will not be able to compensate for synkinesias caused by the innervation of antagonising muscles by branches from the same motoneuron\textsuperscript{115}, it shows the importance of including the central nervous system, perhaps in a multi-level approach, to achieve functional recovery after peripheral nerve injury.

**Addressing the routing problem at a molecular level**

The reason why I declared the discovery of the expression of semaphorin3A and a number of other axon guidance molecules in human neuroma tissue a “winner” is that these findings show for the first time that there are molecules present within the human nerve scar itself that help determine the fate of regenerating axons. In other words, the routing of regenerating axons is not only influenced mechanically by the physical alignment of regenerating axons in endoneurial tubes, but also by lesion-induced expression of specific genes in the glial cells of the peripheral nerve. This finding may provide several new inroads to address the misrouting problem.

Firstly, the chemorepulsive protein semaphorin3A (Chapter 2, and other possible inhibitory molecules described in Chapter 3) present in the human neuroma could have contributed directly to the observed trapping and disorganisation of axons in the human nerve scar. Secondly, in Chapter 3 we describe that the expression of several axon guidance molecules is differentially regulated in human neuroma tissue.
Although this data is still preliminary, previous literature suggests that some of the newly discovered guidance molecules are likely to play a role in the branching, fasciculation (the tendency of regenerating axons to grow in the same direction) and/or defasciculation of regenerating axons. All these phenomena are highly relevant for the misrouting problem. For instance, if some of the newly discovered proteins stimulate neurite sprouting, viral vector-mediated expression of short interfering RNAs to knock down the expression of such proteins could limit the number of regenerative branches extending from one transected motoneuron. In this way an attempt can be made to diminish the likelihood of double innervation of antagonising muscles and subsequently the development of unwanted cocontractions. As described in Chapter 4, the expression of these genes in a sural nerve graft could be manipulated with the use of an LV vector.

Furthermore, some of these newly discovered axon guidance molecules may play a role in the phenomenon of “preferential motor reinnervation”, which is in part caused by an interaction of axotomised motoneurons and endoneurial tubes at the site of axotomy. Influencing the expression of these genes could preferentially stimulate the regeneration of motoneurons, stimulate or reduce motoneuron sprouting or perhaps even stimulate the pruning of redundant axons of regenerated motoneurons, once again increasing the likelihood of accurate reinnervation of target muscles.

Perhaps an even more promising approach to address the routing problem would be to use newly discovered axon guidance cues (including semaphorin3A) to actually guide regenerating axons towards their original targets by mimicking the patterning process that takes place during development. In the developing brachial plexus of the chick embryo, axonal outgrowth indeed leads to successful target finding due to an elaborate interplay between motoneurons and the differential expression of the repulsive guidance cues semaphorin3A and semaphorin3F in the ventral and dorsal parts of the developing limb. Provided that these motoneurons continue to express their respective receptors in the same pattern in the adult human nervous system, it may be possible to use semaphorin3A and 3F to help guide them towards their original targets after a brachial plexus injury. It is currently not known whether such patterning cues are expressed during regeneration in the same way as during development. A relatively simple first step to investigate this in humans would be to perform a comparative gene expression analysis of the parts of the brachial plexus distal to the neuroma. Small segments of the anterior and posterior divisions of the superior trunk are sometimes removed during reconstructive surgery of patients with a neuroma of the superior trunk. Analogous to the experiments performed in Chapter 3, it may be possible to identify axon guidance molecules that are differentially expressed in these respective distal trunks. Influencing the expression of such guidance cues in these trunks will perhaps provide a truly novel way to help regenerating axons find the
right target organ. Naturally, the success of this strategy relies on the assumption that the receptors to axon guidance molecules are differentially expressed by subsets of regenerating adult motoneurons (see above) which will be harder to study in human material and needs to be investigated in animal models \(^\text{64}\). A study comparing gene expression patterns during development and regeneration of the rat sciatic nerve has already shown that approximately half of the regeneration-associated genes were also significantly regulated in development, suggesting that regeneration is indeed partly a recapitulation of development \(^\text{55}\).

**Viral vectors: highly suitable tools to study peripheral nerve regeneration**

Much has already been said on the maturity and clinical potential of viral vectors in Chapter 1 of this thesis. Indeed, the experiments described in this thesis were motivated in part by recent advances made in the field of gene transfer. Therefore, I will be brief on the advantages of the application of viral vectors, but I would like to reiterate here their tremendous suitability to study peripheral nerve regeneration. The experiments in this thesis and others from our group \(^\text{150}\) show that they can be used to transduce Schwann cells of the rat peripheral nerve consistently, durably, in significant numbers in a well-defined area, without interfering with reconstructive surgery or impairing its functional outcome. The relative ease with which new, potentially interesting genes can be cloned into these vectors means that the *in vivo* effect on regeneration of many proteins can now be studied in far more efficient ways than previously possible. This is the first tangible benefit of these vectors and I expect that this will have a remarkable impact on the future direction of peripheral nerve research.

Therapies aimed at influencing peripheral nerve regeneration are, inherently, only required temporarily. Therefore, it is not unreasonable to question whether it is clinically realistic to inject vectors that will permanently insert a therapeutic gene in cells, when the need for a particular protein is only short-term. In addition, there are viral vector-related issues that need to be resolved: (i) the presently available tetracycline-controlled transactivator necessary for regulatable gene expression is of bacterial origin \(^\text{51}\) and thus immunogenic \(^\text{221}\), (ii) LV vectors may cause side effects in transduced cells, and the possibility of insertional mutagenesis is repeatedly mentioned in the literature but still not reported \(^\text{233-234}\), while this may be prevented with the application of non-integrating LV vectors \(^\text{235}\) and (iii) safer vectors, like adeno-associated viral (AAV) vectors, have so far been unable to transduce Schwann cells. Furthermore, the experiments described in Chapter 4 highlight another potential pitfall of translating results from animal research to the human situation. Whereas the application of LV vector results in the efficient transduction of Schwann cells in the rat peripheral nerve, fibroblasts are the predominantly transduced cell type in human nerve segments.
Therefore, at least in the near future, the most likely role for viral vectors in the peripheral nerve will remain as powerful research tools. It will take several years to definitively establish the beneficial effect of viral vector-mediated expression of a protein in animal models for peripheral nerve injury. During this time, the safety of viral vectors for human application will have been established in the clinical trials that are currently underway and solutions will probably emerge for the issues described above. Depending on the progress that is made in the field of gene therapy, it may be possible to translate positive effects of viral vectors in animal peripheral nerve injury models directly to a gene therapy approach in humans, or alternatively the therapeutic protein could be delivered by a more conventional method such as biodegradable slow-release capsules. Either way, viral vectors will have a great impact on the development of new therapeutic strategies to enhance the results of peripheral nerve repair, and this is why I consider the results obtained with LV vectors in this thesis to be “winners”.

**Future perspective: the continuing miniaturization of surgical and diagnostic tools**

As described in the introduction of this thesis, the field of peripheral nerve surgery has steadily evolved since World War II. The introduction of the operating microscope and improved microsuturing techniques has had a great impact on the outcome of surgery. Essentially, the work described in this thesis forms a logical extension of this path of continuous miniaturization by identifying new molecular targets in the human peripheral nerve scar and by developing a viral vector-based strategy to influence the expression of these targets. In this final section, I would also like to mention another form of miniaturization that will likely have a great impact on the clinical practice of nerve repair.

A significant challenge is the absence of a reliable method to determine the degree of injury (and the corresponding need to intervene surgically) in neuroma in continuity lesions. New diagnostic tools are much needed as they would enable the early repair of the most severe nerve injuries. I believe that it will not be long before novel imaging techniques like diffusion-tensor imaging and diffusion-direction-dependent imaging will make it possible to assess the integrity of individual nerve fascicles in the lesioned peripheral nerve, and that this will greatly advance the clinical practice and timing of nerve repair. Another intriguing option stems from the recent development of radio-labelled probes that can be used to detect and quantify collagen in humans. As shown in Chapter 3, the expression of several types of collagen is increased in the peripheral nerve scar at 5 months post-injury; indicating that formation of a fibrotic scar is a process that continues for at least several months. An early assessment of the degree of fibrosis in the peripheral nerve scar may constitute a highly reliable