CHAPTER 10

General discussion and future directions

Misdirection of regenerating axons

Despite the capacity of the peripheral nervous system to regenerate, functional results after nerve injury and repair are often poor. Several factors can explain the disappointing recovery, including: delay in nerve repair, degree and level of the nerve injury, and age of the patient. In the first part of this thesis we demonstrate that misdirection or misrouting of regenerating axons also plays an important role. Using a sequential retrograde tracing technique in which the first tracer was injected into the peroneal nerve before the injury (DY) and the second tracer 8 weeks after repair (FB) (see illustration Figure 1, **Chapter 4**), we found that after crush injury of the rat sciatic nerve 71% of the peroneal motoneurons were correctly directed towards the peroneal nerve branch, and after transection of the sciatic nerve and direct coaptation repair, only 42%. This difference in percentage of correct direction after transection injury and repair compared with crush injury can be explained by disruption of the continuity of the basal lamina tubes. Apparently, axons that cross the coaptation site disperse and end up in different distal basal lamina tubes. After autograft repair, with two coaptation sites, the percentage correctly directed peroneal motoneurons was even lower: 25%.

Subsequently we studied the impact of misdirection on functional recovery. For this purpose, we developed a novel evaluation method, called *2D-digital video ankle motion analysis*. This method was first validated in normal animals and after sciatic, tibial, and peroneal nerve crush injury (**Chapter 3**). We compared this technique to the sciatic function index, which is the standard assessment technique in the rat sciatic nerve model. Our new method was more sensitive in the detection of functional recovery, especially in showing differences in the recovery of ankle plantar and dorsiflexion (tibial and peroneal nerve function, respectively). *2D-digital video ankle motion analysis* of function after transection injury and repair showed interesting effects with a negative impact on recovery: the ankle angle at midswing (normally the moment of maximum dorsiflexion/ peroneal nerve function) was reduced 1 week after nerve injury and repair, which was expected, but in time the angle decreased even further. This finding can be explained by misdirection of a significant portion of the peroneal motoneuronpool towards the tibial nerve branch. In our sequential tracing experiment only 42% of the peroneal motoneurons were correctly directed towards the peroneal nerve branch. Considering that the number of motoneurons from which axons had regenerated 8 weeks after autograft repair (Chapter 7) was not significantly different from normal, it is likely that the other peroneal motoneurons (remaining 58%) had regenerated towards the tibial nerve branch. This misdirection could thus result in active plantar flexion during the swing phase and therefore a further reduction in dorsiflexion angle. Also of interest is the work by Puigdellivol et al. [1], who used the same model, technique of sequential retrograde tracing and time point of evaluation, but instead investigated correct direction towards the tibial nerve branch. They found that about 1/3 of the motoneurons were labeled only by the second tracer (262.8 out of 877.6,

numbers obtained from Table 1 Puigdellivol et al. [1] multiplied by 4). Considering the sizes of the tibial and peroneal motoneuronpools (around 800 and 400 motoneurons, respectively, see Figure 4 **Chapter 7**), this means that also in their study more than 50% of the peroneal motoneurons (262.8 out of 400) were misdirected towards the tibial nerve branch. They concluded that 'epineurial suture repair leads to a high degree of topographically correct directional axon regeneration', because they found that 88% of the tibial motoneurons were correctly directed to the tibial nerve. One cannot, however, draw this conclusion if one considers the difference in size of the tibial and peroneal nerve branches.

In our experiment on motion analysis we did not see any signs of a mechanism that might correct for misdirection (**Chapter 2**). The further decrease in midswing ankle angle appeared 8 weeks after the nerve injury and repair and lasted for the entire follow-up period of 16 weeks. One might expect that pruning of misdirected axons in favor of correctly directed ones, which has been reported to occur between 3 and 8 weeks in the rat femoral nerve model [2], would already have taken place. Plastic changes, such as for example remodeling of spinal cord circuits, may, however, require more time [3]. Longer follow-up is, therefore, needed to investigate further whether this angle will improve in time. Preferably, this analysis should be combined with simultaneous recordings of compound muscle action potentials in the plantar and dorsiflexion muscles, which might show whether the reduced angle of dorsiflexion is caused by active plantar flexion during the swing phase. Another interesting future experiment would be to analyze the percentages correct direction at multiple time intervals after nerve injury and repair to investigate potential effects of pruning of misdirected collaterals. In the same experiment the organization of differently labeled motoneurons in the anterior horn could also be analyzed to see whether more grouping of motoneurons with the same label occurs in time compared with the disorganized distribution of differently retrogradely labeled motoneurons that we observed in our simultaneous tracing experiments (**Chapter 7 and 9**).

In conclusion, misdirection is an important factor that contributes to reduced functional recovery after nerve injury and repair, especially when we consider that other conditions in our experiment were optimal: immediate repair with reconstruction of the original fascicular orientation. In clinical nerve repair results may be poorer, because there is often a delay in repair and because the original fascicular orientation cannot be reconstructed. Another experiment that might be interesting for further study is the role of misdirection in delayed nerve repair, where the capacity of regeneration is decreased; this mimics the clinical situation more closely. Finally, functional analysis may be combined with other methods that also evaluate sensory recovery.

GUIDANCE OF REGENERATING AXONS

In the second part of this thesis, we investigated the use of two different strategies to guide nerve regeneration after injury and repair. The first strategy was *physical guidance* using a multichannel conduit. The second strategy was *biological guidance* using gene therapy. The results and future perspectives of both these tools will be discussed separately below.

MULTICHANNEL NERVE TUBE

Our hypothesis for axonal guidance through a multichannel nerve tube was that the internal structure would limit the axonal dispersion that is seen after single lumen nerve tube repair (Figure 1B and C). Multichannel conduits bear a closer resemblance to the structure of an autograft containing multiple basal lamina tubes (Figure 1A). To test this hypothesis, we developed a novel multichannel nerve tube made from poly(lactic co-glycolic acid) (PLGA) using a modified injection-molding technique (Figure 1, **Chapter 6**). The end caps that were used to align the wires inside the mold were provided with an extra top-layer to create a sleeve for insertion of the nerve stumps. The same injection-molding technique was used to fabricate single lumen nerve tubes with a single wire. Before *in vivo* implantation, single lumen PLGA nerve tubes made from different copolymer ratios (50:50, 75:25, and 85:15 PLGA) were first tested *in vitro* to determine optimal flexibility, swelling and degradation characteristics (important properties of nerve tubes for peripheral nerve repair). The middle ratio (75:25 PLGA) was chosen to fabricate multichannel nerve tubes, because of greater flexibility than the higher ratio (85:15) and less swelling than the lower ratio (50:50) as well as a slower rate of degradation. These 75:25 PLGA multichannel nerve tubes were also tested *in vitro* and the results were compared to the results for single lumen nerve tubes made from the same ratio of PLGA. In addition to the flexibility, swelling and degradation properties mentioned above, a new method was developed to compare *in vitro* permeability properties of the single lumen and multichannel nerve tubes. The results of this *in vitro* study showed that the multichannel structure did not negatively influence the permeability and flexibility properties of the conduit: 75:25 PLGA multichannel nerve tubes were even more permeable and flexible than the 75:25 PLGA single lumen ones. For these conduits, however, this can be explained by the injection-molding evaporation technique that leads to more interconnection of the pores in the wall in the multichannel nerve tubes compared with the single lumen ones (Figure 2, **Chapter 6**). Theoretically, one would expect multichannel tubes to be less permeable and less flexible. This unexpected finding demonstrates the importance of *in vitro* analysis of novel conduits before *in vivo* implantation, because adding internal guiding structures may not only have an impact on guidance of regenerating axons, but may also have an effect on regeneration by changing other physi-

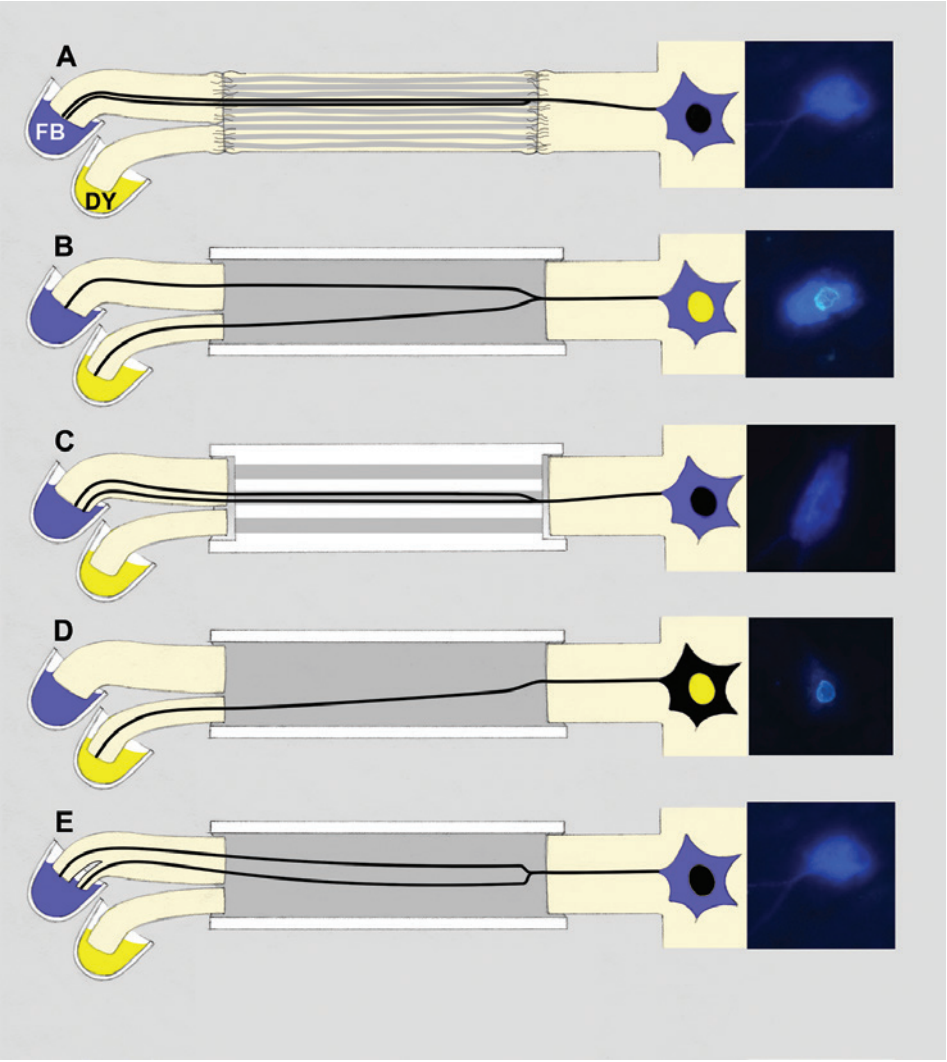

Figure 1

Concepts for the dispersion of regenerating motor axons after (A) autograft, (B) single lumen, and (C) multichannel nerve tube repair, and the technique of simultaneous tracing with fast blue (FB) and diamidino yellow (DY) tracers being applied to the tibial and peroneal nerve branches, respectively, 8 weeks after implantation. FB is transported retrograde to the cell body of the motoneuron and DY to the nucleus.

A: After autograft repair, regenerating axons originating from the same motoneuron are contained by the basal lamina tubes, and both end up in the same (tibial) nerve branch.

B: After single lumen nerve tube repair, axons originating from the same motoneuron disperse and end up separately in the tibial and peroneal nerve branches.

C: After multi-channel nerve tube repair, axons originating from the same motoneuron are contained on the inside of a channel and end up in the same (tibial) nerve branch.

D and E: other examples of dispersion across the single lumen nerve tube, which do not cause double labeling, such as dispersion of a single projection from a motoneuron (D), and double projections to the same branch but towards different fascicles inside this branch (E).

cal properties of the conduit (as for example permeability, flexibility, swelling and degradation characteristics). In addition, these properties are also important for potential future clinical application. The methods and results presented in **Chapter 6** can be used as a basis for the development of novel conduits with more complex internal structures.

After the *in vitro* analysis, the 75:25 PLGA single lumen and multichannel nerve tubes were implanted in a 1-cm gap in the rat sciatic nerve model to investigate the accuracy of regeneration across these conduits using simultaneous and sequential tracing techniques (**Chapter 7**). The results of this study showed that single lumen nerve tube repair indeed leads to a higher percentage of dispersion, with 21.4% double projecting motoneurons to both the tibial and peroneal nerve branch compared with 5.9% after autograft repair. Multichannel nerve tube repair showed a trend towards reducing this dispersion, although the percentage double projecting motoneurons (16.9%) was not significantly different from that after single lumen nerve tube repair. This can be explained by the finding that only 3 out of 7 channels were filled with myelinated axons. In addition, overall success rate in this study was small: regeneration across the conduits was found in 53% of the cases after single lumen nerve tube repair and 43% of the cases after multichannel nerve tube repair. A possible explanation for these disappointing results may be extensive swelling of the nerve tubes due to the accumulation of small degradation products that had increased the osmotic value of the tube (**Chapter 6**).

Following the experiment with the multichannel 75:25 PLGA nerve tubes, a novel series of multichannel nerve tubes (1-, 2-, 4- and 7-channel) was developed with different physical properties. The natural material collagen (type I) was used and a multiple step-molding technique was applied [4] (see Figure 1, page 131, **Chapter 8**). The results of this study showed that multichannel structure can limit axonal dispersion with 2.7% double projecting motoneurons after 2-channel and 2.4% after 4-channel collagen conduit repair compared with 7.1% after single channel collagen conduit repair. Although this reduction might appear to be small, it is important to realize that this percentage only indicates part of the axonal dispersion that occurs during regeneration across the conduit. In addition, single unbranched axons might disperse (Figure 1D). This cannot be detected with simultaneous tracing. Also, axonal branches originating from the same motoneuron might end up in the same nerve branch, but within different fascicles innervating different target muscles, for example the gastrocnemius and soleus muscles in the case of the tibial nerve (Figure 1E). Further, in the analysis of the results, it is important to consider the time-point of evaluation. The percentages double projections might have decreased in time due to pruning of misdirected collaterals. In our first study (**Chapter 7**), in which the observation period was 8 weeks instead of 16 weeks, we also found much higher percentages of double projecting motoneurons (16.9% after multichannel nerve tube repair and 21.4% after single lumen nerve tube)

compared with the percentages found in the second study, although the results of these two studies cannot be compared, because in the second study conduits with four channels were used (instead of seven, as in the first study) and conduits were made of different biomaterials using different techniques of fabrication. More research is, therefore, needed to investigate whether there is a decrease of double-projections with time.

Another interesting result of our second *in vivo* study on multichannel conduit repair was that the quantitative results of regeneration (for the numbers of regenerated myelinated axons and retrogradely labeled profiles) were again (as in the first study) not significantly decreased compared with single lumen tubes, despite the reduction in the total cross-sectional area for axons to grow into. Also, successful regeneration was found in 39 out of 40 cases of conduit repair and almost all channels contained fascicles with myelinated fibers. The first phase of successful regeneration across a single lumen nerve tube is the formation a fibrin matrix between the two nerve ends (Figure 2, **Chapter 5**). Possibly the formation of multiple fibrin cables to guide regeneration is also more advantageous. More research is needed to further investigate what happens in the initial stages of regeneration across multichannel nerve tubes.

Finally, as in the first study, quantitative results of regeneration were still superior after autograft repair. Although this did not result in a better functional outcome, it is important to realize that an ideal alternative for the autograft should perform better than the autograft.

FUTURE DIRECTION OF RESEARCH ON MULTICHANNEL NERVE TUBES

As demonstrated in this thesis, multichannel nerve tubes limit the dispersion of regenerating axons that occurs across single lumen nerve tubes, as they more closely resemble the structure of an autograft. Although the number of channels is now still limited by the size of the wires and space needed between the wires, novel fabrication techniques in the future may increase the number of channels that can be fitted into the tube. An ideal design would be a conduit with an internal honeycomb structure to reduce the amount of space between the channels and to increase the total cross-sectional surface area available for regeneration. Another future aim could be to build multichannel scaffolds with a 3D printing technique to resemble more closely the original architecture of the nerve that often (especially more proximally) consists of an intraneural plexus instead of parallel-aligned channels.

Besides multichannel nerve tube structure, other modifications to the common hollow or single nerve tube have been developed that may also be applied to guide regenerating axons, including the addition of collagen gels, filaments, supportive cells, and growth factors (see Table 3, Chapter 5). These modifications can be used in combination with the multichannel conduit structure. An additional advantage

of such a multichannel structure is that it provides a greater internal luminal surface area for cell attachment, and, the internal framework can be used for the controlled release of growth factors through microspheres. Adding these microspheres to the internal structure rather than to the lumen would provide the potential for more controlled release during the degradation process of the conduit to overcome the initial burst release that occurs with an 'in-lumen' delivery system [5]. In addition, growth factors could be used to attract regenerating axons into the distal pathways to prevent the dispersion/wandering of axons at the coaptation sites and/or preferentially attract different types of axons to regenerate into separate channels by the expression of different growth factors.

GENE THERAPY

In last part of this thesis we investigated the use of lentiviral vectors (LV) to selectively guide regenerating motor axons. The basis for this project was the observation in **Chapter 4** that peroneal motoneurons were misdirected due to the smaller size of this branch in comparison to the larger tibial nerve branch, which led to impaired recovery of peroneal nerve function. The aim of this project was, therefore, to increase the number of motoneurons regenerating to the peroneal nerve branch by selective injection of a lentiviral vector encoding for the neurotrophic factors GDNF (LV-GDNF). First, in a pilot study, we analyzed the distribution or spread of viral vector into the peroneal nerve after injection of LV-GDNF 0.5cm from the tibial-peroneal bifurcation (in intact animals and after transection and repair just proximal to this bifurcation). The expression levels of GDNF in 0.5cm segments of nerve were analyzed using an ELISA. Results showed that, with this technique, high concentrations of GDNF can be obtained selectively in the peroneal nerve distal to the bifurcation site, without increased levels in the tibial or sciatic nerve, although the levels of GDNF varied from animal to animal. Subsequently, in a larger study, the effect of LV-GDNF injection into the peroneal nerve after transection injury and repair of the sciatic nerve at the tibial-peroneal bifurcation was investigated with simultaneous retrograde tracing four weeks after sciatic nerve transection and repair. This study showed an increased number of motoneurons from which axons had regenerated towards the peroneal nerve branch after LV-GDNF injection compared with injection of a control vector encoding for green fluorescent protein (LV-sGFP). This effect was, however, not statistically significant. In two of the eight animals, the normal ratio of the motoneuronpoolsize, which is tibial: peroneal, 2:1, was completely reversed (1:2). In addition, ChAT immunohistochemistry showed an increased number of motor axons in the peroneal nerve after LV-GDNF injection. Although these results thus provided a first indication that it may be possible to guide regenerating motor axons with gene therapy, more research is needed. This should be focused on optimization of the injection technique to get constant levels of growth factor concentration. Besides, regulatable vectors are being developed

to turn off the elevated growth factor expression once the axons have reached the designated target branch to prevent trapping of axons at the site of highest concentration (phenomenon called the *candy store*). Future application of selective injection of a lentiviral vector encoding for GDNF could be to guide regenerating motor axons to a motor branch, for example after median nerve repair at the wrist. In addition, other potential future applications of gene therapy in peripheral nerve regeneration are to upgrade the autograft by selective overexpression of specific growth factors or to increase the speed of regeneration in more proximal nerve injuries to improve the recovery of, for example, hand function in brachial plexus repair [6]. Finally, combinations of the techniques mentioned above (for example, filling of multichannel nerve tubes with genetically modified Schwann cells) may ultimately be applied to improve the results of nerve repair.

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