Misdirection and guidance of regenerating motor axons after experimental nerve injury and repair

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CHAPTER 1

Introduction Aims and Outline

Peripheral nerve injuries and repair

Peripheral nerve injuries are a common type of injury that can be caused by various mechanisms including, for example, sharp transection (as in iatrogenic injury), disruption due to fracture of long bones, and stretch injuries (as in adult traumatic and neonatal brachial plexus palsy). The exact incidence of traumatic nerve injuries in The Netherlands is not known. In Canada, a prevalence rate of 2.8% has been reported in the trauma population [1]. In Sweden, the incidence rate is 13.9 per 100,000 person years [2].

The peripheral nervous system has the capacity to regenerate and, depending on the severity of the nerve lesion, spontaneous recovery can occur. When the continuity of the nerve is lost or when a neuroma-in-continuity has formed, surgical intervention may be indicated. In sharp transection injuries nerve ends can be coapted directly without tension. In blunt or stretch injuries, however, direct suture of the nerve ends is often not possible without tension at the coaptation site. In these cases, a graft is needed to bridge the gap between the proximal and distal stumps. The introduction in the second half of the previous century of the operating microscope and surgical loupes, as well as the development of microsurgical techniques and the use of the autologous nerve graft to reestablish continuity of the injured nerves have considerably improved the outcome following nerve surgery. Despite these developments, however, functional recovery is often incomplete.

Timing of surgery

Several factors can account for the incomplete recovery. First of all, the timing of surgery is an important factor. The best chance of recovery is when nerve repair is performed directly after trauma, because the capacity for regeneration has been shown to decrease with time and because changes occur in the distal nerve and targets due to the prolonged period of denervation [3, 4]. In closed nerve traction or compression lesions, it can be difficult to predict whether the continuity of the nerve has been lost. The decision whether to await spontaneous recovery or perform surgical exploration within days is determined by various factors, for example, the extent of the neurological deficit and type of trauma. Electrophysiological analysis can be helpful in determining the extent of the nerve injury and in detecting early signs of muscle reinnervation.

Unfortunately, even after immediate repair, functional recovery of proximal injuries is limited because of the length axons have to elongate, from the site of the injury to the distal targets. For example, after reconstruction of brachial plexus injuries, it may take years before axons reach the hand (with a regeneration speed of 1-3 mm a day) [5]. Therefore, recovery of hand function is often poor. Novel strategies (e.g. electrical stimulation [6] and gene therapy [7]) have been developed which focus on increasing the speed of axonal regeneration in order to reduce the time between the nerve injury and target reinnervation. Alternatively, *sensory protection* (which is pursued by temporary reinnervation of a denervated muscle by transfer of a sensory branch to the distal nerve stump [8] or via side-to-side nerve grafts [9]) is used to slow the process of degeneration in the distal targets.

Misdirection or misrouting

Another factor, which plays a role in the incomplete recovery after nerve injury and repair, is misdirection or misrouting of regenerating axons. There is always a certain degree of misdirection, even following microsurgical coaptation of the individual fascicles, because the continuity of the endoneurial or basal lamina tubes cannot be restored and axons cross the coaptation site in a random way (**Figure 2, Chapter 2**). This misdirection of axons across the repair site may lead to reinnervation of inappropriate targets. In the repair of mixed nerves, for example, motor axons may be directed towards the skin, and vice versa, sensory axons towards the muscle. Even in the repair of a pure motor nerve innervating different muscles, axons may end up in pathways towards a different muscle, which might function antagonistically. In sensory nerve repair, misdirection may lead to an increased perceptual territory [10].

The first indication that axons are misdirected at the coaptation site dates back to the beginning of the 20th century. In his book on *degeneration and regeneration of the nervous system*, In 1928, Ramon y Cajal demonstrated [11] that a single axon can have multiple projections to different distal targets (**Figure 2, Chapter 2**). Since then, numerous studies have investigated accuracy of regeneration and reinnervation (**for review see Chapter 2**). As yet, however, the extent and impact of misdirection on the level of function is not known.

AIMS AND OUTLINE

The first aim of this thesis was *to quantify the degree of misdirection* and *to determine the impact of misdirection on functional recovery* after different types of nerve injury and repair. A sequential retrograde tracing technique was used to quantify the degree of misdirection. First, a tracer was injected into an intact nerve before the injury to label the original motoneuron pool. Subsequently, a second tracer was used at a specific period of time after the injury and repair to label the motoneurons that had regenerated to the same nerve branch. As a model, the rat sciatic nerve was chosen, not only because it is the most frequently used model in experiments on nerve regeneration, but also, because the nerve divides distally into a tibial and peroneal nerve branch; these have antagonistic functions: ankle plantar and dorsiflexion, respectively (**Figure 1, Chapter 4**). The degree of misdirection to either the peroneal or tibial nerve branch can thus be investigated. For this purpose, a new functional evaluation method was developed in collaboration with the motion analysis laboratory at Mayo Clinic. Equipment for analyzing motion, normally used in patients with neurological disorders, was adapted to assess the recovery of ankle plantar and dorsiflexion in rats after sciatic nerve injury. This new technique of ankle motion analysis was validated and compared to the current gold standard of walking track analysis (**Chapter 3)**. Sequential retrograde tracing and ankle motion analysis were subsequently used to quantify the degree of misdirection after different types of nerve injury and repair, and the impact on the

recovery of function respectively (**Chapter 4**). Different types of nerve injury (crush vs transection injury) and repair (direct coaptation vs autograft repair) were investigated to determine the impact of intact versus interrupted basal lamina tubes on misdirection, and nerve repair with one versus two coaptation sites.

The second aim of this thesis was to improve *guidance of regenerating motor axons* in the rat sciatic nerve model. In **Chapter 2** we reviewed several factors that may be involved in the routing of regenerating axons after nerve injury and repair. In recent years, different strategies have been developed that may guide and direct regenerating axons toward their correct target organ. Most of these guiding strategies have been investigated *in vitro* using neurite outgrowth assays of e.g. explanted dorsal root ganglion (DRG) cells. For example, physical guidance of neurites has been investigated using grooved microsurfaces. This research has shown that neurites orient parallel to the walls of microchannels [12]. Other examples include research on *in vitro* outgrowth of neurites on polymer filaments, with different shapes and coatings [13], and polymer surfaces patterned with gradients of peptides or neurotrophic factors to guide neurites in a certain direction [14]. Only a limited number of studies have investigated the influence on *in vivo* nerve regeneration. In the second part of this thesis we tried to improve the *in vivo* guidance of regenerating axons using two different tools: (1) *mechanical guidance* with *multichannel nerve tubes*, and (2) *biological guidance* with *gene therapy*.

Multichannel nerve tube

Single lumen nerve tubes, guides or conduits have been developed as an alternative for repair with an autologous nerve graft, mainly because of the disadvantages of the autograft, such as donor-site morbidity, limited availability and size mismatch with the injured nerve, necessitating the use of multiple pieces of grafts (so called cable grafts, **figure 1 Chapter 5**). Different single lumen nerve tubes are now available for clinical use (a review of the experimental and clinical data is provided in **Chapter 5**). Unfortunately, single lumen tubes are only effective in the repair of small defects (<3 cm). Furthermore, in larger mixed or motor nerves, repair with a single lumen nerve tube may lead to inappropriate target reinnervation due to the *dispersion of regenerating axons* across the lumen [15]. We have found similar results as shown by a decrease in *type grouping* in reinnervated gastrocnemius and anterior tibialis muscles after repair of a 1-cm sciatic nerve defect with a single lumen nerve tube [16].

Multichannel nerve tubes that have been developed for both experimental peripheral nerve [17-19] and spinal cord repair [20-23] may limit this axonal dispersion by separately guiding groups of regenerating axons inside the channels. To investigate the influence of multichannel structure on regeneration, we developed a single lumen and 7-channel nerve conduit from the polymer, poly(lactic co-glycolic acid) (PLGA). in collaboration with the bio-engineering laboratory at Mayo Clinic. These conduits were first analyzed *in vitro* for different ratios of lactic to

glycolic acid to assess certain nerve tube properties: permeability, flexibility, swelling and degradation (**Chapter 6**). Subsequently, in a pilot study we compared the accuracy of regeneration across a 1-cm gap after autograft repair and repair with single lumen or multichannel nerve tubes using sequential and simultaneous retrograde tracing (**Chapter 7**). The technique of sequential tracing has already been described above. In simultaneous tracing, the same tracers, FB and DY, were used, but were now applied at the same time to the tibial and peroneal nerve, respectively, to label motor axons that had regenerated to either one or both branches. Our hypothesis was that more double labeling (motoneurons with projections to both branches) would be observed after single lumen nerve tube repair compared with autograft repair due to dispersion of axonal branches originating from the same motoneuron, and, that multichannel nerve tube repair would limit this dispersion. In a second study, we additionally analyzed the influence of 2-, 4-channel conduits on regeneration using multichannel nerve tubes made of collagen (**Chapter 8**). Again, simultaneous retrograde tracing was performed to investigate the dispersion of regenerating axons across these conduits. In addition, functional recovery was assessed using ankle motion analysis.

Gene therapy

In addition to mechanical guidance through multichannel conduits, we also investigated the possibility of biological guidance with gene therapy by selective injection of a lentiviral vector encoding for GDNF (glial cell line-derived neurotrophic factor). This neurotrophic factor has been shown to improve motoneuron survival and regeneration after prolonged axotomy [24]. This study was performed at the Netherlands Institute for Neuroscience (NIN). Previous experiments from this institution have shown that the lentiviral vector encoding for GDNF (LV-GDNF), after injection into a nerve can transfect Schwann cells that subsequently produce GDNF [25]. We injected the same viral vector LV-GDNF into the peroneal nerve, after transection and repair of the sciatic nerve just proximal to the tibial-peroneal bifurcation (**Chapter 9**). The directing effect of selective LV-GDNF injection into the peroneal nerve branch was investigated after 4 weeks with the same simultaneous tracing method mentioned above. Our hypothesis was that more motoneurons would be labeled by the tracer applied to the peroneal nerve branch (DY) and fewer by the tracer applied to the tibial nerve branch (FB) after LV-GDNF injection into the peroneal nerve branch, when compared with the control groups (repair without viral vector injection and injection of a control vector encoding for green fluorescent protein).

Future directions

Finally, the last Chapter of this thesis (**Chapter 10 General discussion and future directions**), summarizes the results and discusses future directions of both mechanical and biological guidance.

REFERENCES

1. Noble, J., et al., *Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries.* The Journal of Trauma: Injury, Infection, and Critical Care, 1998. **45**(1): p. 116-122.
2. Asplund, M., et al., *Incidence of traumatic peripheral nerve injuries and amputations in Sweden between 1998 and 2006.* Neuroepidemiology, 2009. **32**: p. 217-228.
3. Fu, S.Y. and T. Gordon, *Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation.* J Neurosci, 1995. **15**(5 Pt 2): p. 3886-95.
4. Fu, S.Y. and T. Gordon, *Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy.* J Neurosci, 1995. **15**(5 Pt 2): p. 3876-85.
5. Guttmann, E., et al., *The rate of regeneration of nerve.* J Exp Biol, 1942. **19**: p. 14-44.
6. Al-Majed, A.A., et al., *Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration.* Journal of Neuroscience, 2000. **20**(7): p. 2602-08.
7. Tannemaat, M.R., et al., *From microsurgery to nanosurgery: how viral vectors may help repair the peripheral nerve.* Prog Brain Res, 2009. **175**: p. 173-86.
8. Elsohemy, A., et al., *Sensory protection of rat muscle spindles following peripheral nerve injury and reinnervation.* Plastic and Reconstructive surgery, 2009. **124**(6): p. 18601868.
9. Ladak, A., et al., *Side-to-side nerve grafts sustain chronically denervated peripheral nerve pathways during axons regeneration and result in improved functional reinnervation.* Neurosurgery, 2011. **68**(6): p. 1654-1666.
10. Dyck, P.J., et al., *Assessment of nerve regeneration and adaptation after median nerve reconnection and digital neurovascular flap transfer.* Neurology, 1988. **38**(10): p. 1586-91.
11. Cajal, S., *Degeneration and regeneration of the nervous system.* London: Oxford University Press, 1928.
12. Mahoney, M.J., et al., *The influence of microchannels on neurite growth and architecture.* Biomaterials, 2005. **26**: p. 771-778.
13. Ribeiro-Resende, V.T., et al., *Strategies for inducing the formation of bands of Büngner in peripheral nerve regeneration.* Biomaterials, 2009. **2009**(30): p. 5251-5259.
14. Yu, L.M.Y., J.H. Wosnick, and M.S. Shoichet, *Miniaturized system of neurotrophin patterning for guided regeneration.* J Neurosci Methods, 2008. **171**(253-263).
15. Brushart, T.M., et al., *Joseph H. Boyes Award. Dispersion of regenerating axons across enclosed neural gaps.* J Hand Surg [Am], 1995. **20**(4): p. 557-64.
16. Vleggeert-Lankamp, C.L., et al., *Type grouping in skeletal muscles after experimental reinnervation: another explanation.* Eur J Neurosci, 2005. **21**(5): p. 1249-56.
17. Bender, M.D., et al., *Multi-channeled biodegradable polymer/CultiSpher composite nerve guides.* Biomaterials, 2004. **25**(7-8): p. 1269-78.
18. Sundback, C., et al., *Manufacture of porous polymer nerve conduits by a*

novel low-pressure injection molding process. Biomaterials, 2003. **24**(5): p. 819-30.
19. Yang, Y., et al., *Neurotrophin releasing single and multiple lumen nerve conduits.* J Control Release, 2005. **104**(3): p. 433-46.
20. Friedman, J.A., et al., *Biodegradable polymer grafts for surgical repair of the injured spinal cord.* Neurosurgery, 2002. **51**(3): p. 742-51; discussion 751-2.
21. Moore, M.J., et al., *Multiple-channel scaffolds to promote spinal cord axon regeneration.* Biomaterials, 2006. **27**(3): p. 419-29.
22. Stokols, S. and M.H. Tuszynski, *The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury.* Biomaterials, 2004. **25**(27): p. 5839-46.
23. Chen, B.K., et al., *Axon regeneration through scaffold into distal spinal cord after transection.* J Neurotrauma, 2009. **26**(10): p. 1759-71.
24. Boyd, J.G. and T. Gordon, *Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo.* Exp Neurol, 2003. **183**(2): p. 610-9.
25. Tannemaat, M.R., et al., *Differential effects of lentiviral vector-mediated overexpression of nerve growth factor and glial cell line-derived neurotrophic factor on regenerating sensory and motor axons in the transected peripheral nerve.* Eur J Neurosci, 2008. **28**(8): p. 1467-79.