Chapter 9

GENERAL DISCUSSION
**The clinical problem**

Peripheral nerve injury is a frequently occurring complication of trauma due to traffic accidents, sports accidents or otherwise. The extremities are often affected and if such injury comprises a disruption of the nerve including the surrounding myelin sheath, surgical intervention is needed. Despite advanced surgical techniques and the application of autologous nerve grafts or artificial nerve guides, functional outcome generally is poor\(^{31;34-36;39;40}\). Research is needed, into optimization of surgical techniques and protocols, into the processes involved in the reinnervation, into new nerve guides which allow long range outgrowth of nerve fibres and into the reactions of the tissues denervated by the transection. Results hopefully may enhance clinical prospects and help to design improved therapeutical strategies. A field of research that has been relatively neglected so far is the effects of a nerve transection and a temporary denervation on muscle morphology and the innervation of muscles. This field is the topic of my thesis.

**The experimental animal, the peripheral nerve**

It was decided to study the effects of a transection of the sciatic nerve in adult rats (in the first part) as well as in young rats (in the second part). The sciatic nerve innervates a variety of muscles, among which, the gastrocnemius, tibialis anterior and soleus muscles, as well as skin areas in the foot region. The tibialis anterior muscle is a hindlimb flexor and the gastrocnemius and soleus muscles are hindlimb extensors and this indicates that the branches of the sciatic nerve innervate antagonist muscles. From an experimental point of view, this enables to study the effects of a transection in muscles with different properties, and this also allows to study behavioral consequences of a transection and reinnervation, by observing walking performance, and neurophysiological consequences by recording EMG patterns. The sciatic nerve in rats, basically is similar in its properties and morphology to peripheral nerves of a mixed character in the human, and, besides, this nerve is easily accessible for surgery, for the implantation of nerve grafts or nerve guides and for histological inspection after experimentation.

In our experiments, we transected and repaired the sciatic nerve on one side (in general the left side) and we concentrated on the effects on muscle morphology of the soleus (SOL), the lateral gastrocnemius (LGC) and the tibial (TA) muscles. Observations and countings at the side of the lesion were compared to those at the contralateral side. We also studied whether parameters of this “control” side had changed in comparison to those in normal rats. The research questions comprise the effects of a transection on the muscles, on the innervation of the muscles, and in the second part we concentrate upon the question whether a sciatic nerve transection at young age has less deleterious effects on muscle morphology, on behavior and other functional aspects than at adult age.
Changes in muscles after a transection at adult age

In the first part of my thesis, we studied the effects of the unilateral removal of a section of 12 mm from the sciatic nerve, and the implantation of this section in a reversed orientation. This lesion was made proximal to the bifurcation of the nerve into its two main branches, the common peroneal nerve and the tibial nerve.

Muscles after denervation, gradually diminish in size and the numbers of muscle fibers decrease over a period of weeks, a phenomenon known as denervation atrophy. This atrophy affects the red (type I) and white (type II) fibers equally. In our experiments we observed in the SOL, the TA and the LGC muscles a decrease in cross sectional areas by about 30% after 7 weeks (chapter 2) and similar results were obtained in other research. The atrophy of the muscle fibers appeared to be reversible during a long period of time and the same has also been demonstrated in human patients. Reinnervation in our research started before 7 weeks after the transection (see, below) and after 21 weeks the cross sectional areas in the TA and LGC had regained the values as observed at the control side, and this held as well for the numbers of muscle fibers (chapter 2). The SOL muscle, however, remained smaller and also the numbers of muscle fibers were decreased in comparison with the control side.

The most important changes we observed after a transection were shifts in the proportions and the regional distributions of the type I and type II muscle fibers in the muscles of the rats’ hindlimb. Normally, the TA contains 2 – 3% of type I muscle fibres, the LGC between 10 and 20%, but the SOL has 80 - 85% of type I muscle fibers. After reinnervation we observed an initial increase in the percentages of type I muscle fibers in the TA and LGC until 15 weeks, but at 21 weeks after the transection these percentages had returned to values approaching normal ranges. In the SOL, on the other hand, the type II muscle fibers steadily increased during this period and at 21 weeks we observed that 80% of the fibers, phenotypically, were of type II. As far as we know, this is the first time that the trends in these three muscles collectively have been described. These changes imply that the nerve transection leads to a more or less common fibre type distribution in all the muscles affected.

In an earlier experiment, we recorded EMG patterns in the LGC and the TA muscles during walking in rats after a sciatic nerve transection. The results showed a marked coactivation of these antagonists and these were interpreted such that after transection, the outgrowing axons aselectively and randomly reinnervate the muscles. This conclusion was supported by experiments in which we retrogradely traced the motoneurones connected to these muscles. Many motoneurones appeared to be far outside the normal territories of the respective motoneuronal pools. Results by Bodine Fowler et al. are in line with our interpretation pointing to an at-random reinnervation.
On the basis of these findings, we hypothesized that the trends towards a common fibre type compositions in the reinnervated muscles is the consequence of the at-random reinnervation of the muscles. Crucial to this hypothesis obviously is, that muscle fibres in adult rats adjust to the properties of the newly innervating motoneurones. The classical experiment on cross-innervation in kittens and adult cats by Buller et al. indeed points to a decisive role of motoneurones in the adjustments in muscle fibers \(^{10}\) (for extensive discussions see, chapter 2, and also chapter 8). Cross uniting the axons originally innervating the “slow” SOL muscle to the “fast” flexor digitorum longus muscle changed this muscle into a slow muscle and vice versa (see also,\(^{27;53;57}\)). Changes in the properties of the muscle fibers, possibly are not only induced by alternative activation patterns of the newly innervating motoneurones but also by neurotrophins. Neurotrophins, released from the nerve ending might gain access to the muscle fiber through endocytosis or by binding to receptors on the muscle fiber membrane where they influence a second messenger system. This second messenger system in turn, controls the expression of muscle protein-genes which may lead e.g. to an increase of myosin heavy chain molecules. Recent research has pointed to NT3 as a possible candidate \(^{48}\).

Although the fiber type compositions in the LGC and TA after a long recovery are more or less within the normal ranges, an abnormality remains that histochemically identical fibers are grouped together in these muscles (chapter 2). This fiber type-grouping indicates previous denervation of the muscle \(^{13;24}\). Rafuse and Gordon explained this clumping of muscle fibers by distal sprouting of motor axons after entering into the muscle, leading to larger groups of muscle fibers with identical histochemical properties \(^{41}\).

**Denervation and reinnervation of the motor endplate**

Recordings of action potentials over the muscle membrane showed that after a sciatic nerve transection in the rat all electrical activity wanes within 24-36 hours \(^{38}\). Soon thereafter, the neuromuscular junctions start to degenerate, the complete disruption of the endplate taking only between 3 and 5 hours \(^{37}\). The specialization in the basal membrane of the muscle fibre, the so-called sole plate of the motor endplate in contrast, remains intact for several weeks or even months, as investigations e.g., in the frog have demonstrated \(^{42;60}\).

In our research on the fate of the motor endplate after denervation, and in which we prevented the axons to grow out, all nerve fibers within the muscle had disappeared by two weeks after transection (chapter 3). At that time, the motor endplates had changed in their morphology, they had decreased in size, were shrunken and sometimes they showed clear signs of degeneration and fragmentation. However, only by 7 weeks all motor endplates had disappeared. These findings illustrate that the motor endplates are much longer resistant to a
denervation than the terminal segments of the motoneuronal axon (chapter 3).

In case of reinnervation, it is generally taken that the former soleplate of the motor endplate is the spot where reinnervating axons contact the muscle fiber\textsuperscript{4,9,28,44}. At 7 weeks, we already observed reinnervated motor endplates in all three muscles and strikingly around 20\% of the muscle fibers in the TA and the LGC and 40\% in the SOL were innervated by more than one axon, a polyneural innervation. Probably, the terminal Schwann cells around the last axonal segment, upon denervation upregulate the genes for GAP-43 production\textsuperscript{43,62}, and they then produce long, axon-like extensions to neighboring endplates. These outgrowths seem to stimulate the new axonal sprouts to contact several endplates\textsuperscript{47}. The percentages of polyneurally innervated endplates had decreased to around 10\% in all muscle fibers at 21 weeks after the nerve transection. This decrease in polyneural innervation might theoretically influence the fiber type distributions of the reinnervated muscles. We indeed observed some changes between 7 and 21 weeks, suggesting that both processes might be related. (chapter 3).

**Long term effects on fiber type distributions and innervation**

After 7, via 15 and until 21 weeks after the nerve transection we observed ongoing changes in fibre type distributions and innervation patterns and the question obviously is, whether further changes occur after longer survival periods (chapter 4). As the most prominent changes had occurred in the SOL muscle we concentrated on that muscle. Even one year after transection, the muscle still was smaller than the homologous muscle at the control side and the affected muscle still contained preponderantly type II muscle fibers. The percentage of the polyneurally innervated motor endplates had increased from a value of 10\% at 21 weeks after the transection, to 20\% at one year, and in parallel to that we counted in these rats (which were close to two years old), 10\% polyneurally innervated motor endplates in the muscle at the control side. Possibly these increases at both sides are phenomena related to a similar aging process.

**The effects of the nerve transection at adult age summarized**

After transection of the sciatic nerve and repair, the axons of the proximal stump are able to sprout and grow back to the target areas via the distal stump of the nerve. The axons, however, are unable to relocate their former muscles and reinnervation of the muscles is a random process. As a consequence of this random reinnervation the affected muscles acquire a common fiber type distribution. This random reinnervation obviously has dramatic consequences for the activation of the muscles by segmental circuits and suprasegmental brain areas. The results of the research referred to above, in combination with results of functional effects after a transection indicate that the altered properties of the muscles by the shifts in fibre type distributions probably is an important factor in the impaired functions
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of the affected extremity.

Nerve lesions at young age

Several reports indicate that extensive plexus lesions, sometimes occurring during the birth of particularly, heavy babies, or after a breech delivery, or nerve transections in young children have a better outcome when compared to similar lesions in adults. Others, however, have questions this notion. Undoubtedly, sensory deficits recover more readily but prospects after lesions of motor nerves particularly in cases of a total rupture are considered to be generally poor. An important factor in functional recovery undoubtedly is an increased compensational capacity of brain areas. It seems, also in central compensational processes, that sensory recovery due to central re-modeling seems to be much better than motor recovery.

Evidence from animal experiments is only scanty. It might be hypothesized that nerve transections at young ages have less severe consequences as tropic factors being effective during initial outgrowth (for review see) might still be present. On the other hand, the CNS including the spinal cord during development is more vulnerable to peripheral injury because of an increased dependency upon signals from the periphery.

We choose to study the effects of a sciatic nerve transection in young rats. Rats are born at an early stage of brain development. Romijn et al., on the basis of parameters of brain development considered a rat of about 13 days after birth to be at the same stage as human babies at term birth, and although Clancy et al. thought this point should be set earlier in time, both studies indicate that a rat in its postnatal period is similar, as far as the development of neuromuscular development is concerned to a human before birth. Obviously, the rats’ birth at an early stage of neural development has important experimental advantages, as interference with early developmental stages can be performed postnatally.

A peripheral nerve transection at neonatal age in rats leads to the upregulation of genes associated with cell death resulting in the death of almost all motoneurones. This is in contrast to such lesions at adult age when almost all motoneurones survive transection. We systematically studied motoneuronal death after peripheral nerve transections and observed that such lesions at the 10th day leaves between 50 and 60% of the motoneurones alive, and that age was chosen as the age for sectioning the sciatic nerve (chapter 5). At earlier ages less motoneurones survive, and strangely also transections at the 15th day lead to increased cell death-counts. We hypothesized that this increase after the 10th day in motoneurones innervating the SOL muscle is due to impressive reorganizations taking place both in the muscle (a transfer from a muscle with mainly type II into a muscle with mainly type I muscle fibres), in the innervation (a regression of polyneural innervation) as well as in the innervating...
motoneurones (a reorganization in the dendrites). This points to the important rule that development does not proceed in a linear fashion\textsuperscript{61}.

**Sciatic nerve transection at the 10\textsuperscript{th} postnatal day, consequences for function**

Walking patterns were severely impaired, from two–three weeks after the transection until one year and we suppose that the deterioration of motor performance started to deteriorate from the point at which the muscles became reinnervated (chapter 6). EMG recordings of the gastrocnemius (GC) and the TA muscles at the side of the transection at P10 we observed a coactivation of the TA and GC muscle both during the stance and the swing phase. This and the badly phased activation of the muscles in relation to the stance and the swing phase indicated an at random reinnervation of these muscles. Retrogradely labeling the muscles by injections into the newly reinnervated muscles demonstrated that the motoneurones often were located outside the former domain of the pools of these muscles (chapter 7). These results supported the hypothesis of an at-random reinnervation after the sciatic nerve transection at P10.

**Fibre type composition of the soleus muscle after early transection**

The SOL muscle still is developing in many respects after the 10\textsuperscript{th} postnatal day. At the age of about one year after the transection, the muscle appeared to contain mainly type II fibres. The fiber type composition, and also other aspects of its morphology (the decreased size of the muscle, the occurrence of groups with type I fibers) are very similar to that what we described in this muscle after a transection of the sciatic nerve at adult age. Obviously, the mechanisms and processes involved in directing the outgrowing axons towards their muscles (effective during initial development) are not effective anymore after the 10\textsuperscript{th} postnatal day. It is interesting to note that such tropic mechanisms still are active within muscles. Wang investigated the “regionalization” of type I muscle fibers in the gastrocnemius muscle after sectioning the nerve close to its entry into this muscle. In some rats the proximal end of the nerve was reconnected to the distal part, but in others the nerve was connected at a more medial localization on the muscle. These results demonstrated that the regionalization was maintained after recovery, indicating that the ingrowing fibres within the muscle are able to relocate their predilectional region\textsuperscript{58}.

**Summary of the findings after a transection at the 10\textsuperscript{th} day**

Sciatic nerve lesion at the 10\textsuperscript{th} postnatal day in rats resulted in an at-random reinnervation of the muscles, severely impaired motor performance and as far as the SOL muscle is concerned in a strongly abnormal fibre type distribution. These results are very similar to those which we obtained after a sciatic nerve transection
in adult rats. They indicate that at the 10th postnatal day in the rat the processes of pathfinding and routing are not effective anymore and that compensational processes are not sufficient to alleviate functional impairments due to aberrant neuromuscular connections.

Possible directions for future research
The major finding in my thesis is that after a transection of a peripheral nerve containing axons to antagonist muscles, these are randomly reinnervated. This occurs both after transections at adult age but also at young stages of development. This mismatch leads to altered fiber type compositions and severely impaired functions.

Functional recovery, after a disruption of peripheral nerves is dependent upon two important factors (apart, obviously, from the cell bodies of the severed axons vigorously supporting regrowth). These are, that the denervated tissue remains receptive for the reinnervating axons, and secondly, that the axons are accurately guided towards their former target area. In case of regrowth and reinnervation in a reasonable period of time muscles and skin areas remain receptive. The second factor provides the greater problems.

It is well known that the degenerating distal nerve stump after a transection attracts the regrowing axons and it is generally agreed that the Schwann cells are the most important source of these factors (such as NCAM, N-cadherin12;32 as well as NGF, IGF and BDNF7;16;54). Successful regrowth towards the target generally occurs if the basal lamina and the collagenous sheath around the nerve fibers still is intact. In such cases, the axons grow along a well defined path and tropic factors to direct the fibers are not implied. However, when the endoneurium is disrupted, which is the case in transactions, then, the axons generally fail to relocate their target. The identity is not known of factors that specifically might direct the axons towards their own muscle or fiber group within a muscle. During early development, probably an intricate interplay between on the one hand, the timing of cell proliferation, neurite outgrowth and maturational stage of the target cells, and on the other hand, tropic and trophic factors on the cell membrane are decisive for optimal connectivity. It is know for a considerable time22, that in embryonic development of the extremities, a simple topographical relation exists between the location of the motoneurones in the dorsolateral part of the ventral horn, the outgrowth of their axons towards the mesenchymal cells in the dorsal plate in the limb bud, the cells from which later, the flexor muscle develop. Similarly, and somewhat earlier, the axons from the motoneurones in the more ventral part of the ventral horn grow in the ventral ramus of the ventral root towards the mesenchymal cells in the ventral plate, which later develop in the extensor muscles14;22. Identifying the factors which are specific in the guiding of this outgrowth (which might be effective next to factors such as timing of
outgrowth, and mechanical factors) might provide opportunities to adequately sort outgrowing axons after the transection of a peripheral nerve with axons to extensor and flexor muscles. Most probably, in-vitro experiments are needed to identify and isolate these factors, and subsequently these might be tested in in-vivo preparations.
REFERENCES


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