Muscular reinnervation and differentiation after peripheral nerve transection

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FUNCTIONAL RECOVERY AFTER TRANSECTION OF THE SCIATIC NERVE AT EARLY AGE, A PILOT STUDY IN RATS
SUMMARY

Transections of mixed peripheral nerves, with branches to antagonist muscles and skin areas, in humans or in animal studies usually are followed by poor functional recovery. The aim of this study was to determine whether nerve transections in rats at the 10th postnatal day lead to less impairments in walking performance than similar lesions in adults. The follow-up period was 52 weeks.

Walking movements were recorded on video and performance was qualitatively assessed. In addition, the Stance Factor and the Sciatic Function Index were calculated. Furthermore, abnormalities as automutilation of the toes or flexion contractures were noted. Post mortem inspection aimed at the continuity of the regenerated nerve, and the presence of neuromas.

Nine days after the transection and nerve repair, walking performance in all rats was remarkable fluent and resembled that of normal rats. Only in two rats we observed an exorotation of the hindleg at the operated side. At 20 days after lesioning, however, the walking performance started to deteriorate, and all but one rat showed severely handicapped walking patterns until the end of the observations. Only one out of the nine animals walked relatively undisturbed, with an SFI approaching normal values but with an abnormal Stance Factor.

We conclude that a sciatic nerve transection in rats aged 10 days leads to long-term impairments in walking patterns.
INTRODUCTION

After transection and repair of a peripheral nerve by epineural suturing, by implanting an autologous nerve graft or a nerve guide the nerve may successfully regenerate. Functional recovery after such lesions, however, usually is poor even in cases of optimal operation techniques and rehabilitation strategies. This is because the outgrowing axons are unable to reestablish the original innervation patterns despite having reached the denervated muscles.

Clinical evidence has suggested that lesions or transections at neonatal and young age may lead to less severe impairments than similar lesions at adult age, and similar results have been reported by Almquist et al. in baby monkeys. Others as Anand and Birch, however, have questioned this optimism. Evidence from animal experiments is only scanty. Watanabe and co-workers studied the effects of transection and repair of the posterior tibial nerve at 6 and 22 days in rats. They observed that walking in the animals operated at the 6th day was more impaired (as indicated by walking track analysis) than in rats operated at the 22nd day.

Research in adult animals in which the sciatic nerve was transected has repeatedly demonstrated that motoneuronal axons do have the capacity to grow out towards the distal stump of the severed nerve. However, when arriving at the target area, the outgrowing axons obviously are unable to relocate their muscles of origin and they randomly reinnervate these muscles. Studies in which the motoneurones to these muscles were retrogradely labeled showed different neurons within one and the same motoneuronal pool but innervating antagonistic muscles. This and the drastic effects on the properties of the denervated and reinnervated muscles are the main reasons for maladaptive functioning.

It might be hypothesized that nerve transections at early stages of development have less severe consequences. The outgrowing axons might have maintained the possibilities to locate their genuine target muscle, e.g. because the trophic factors, guiding the outgrowing axons during initial outgrowth are still present, or because the distance from the lesion site to the target organ is shorter (and allowing tropic factors from the muscle to be more effective). Another possibility might be an enhanced compensational capacity of central brain areas at young stages of development. This hypothesis is in line with the clinical notion that nerve transections in babies and very young children seem to show better functional recovery after nerve repair. With this background we decided to study the effects of sciatic nerve transection and repair in rats at the youngest age at which still appreciable numbers of motoneurones survive transection.

The age for transecting was identified by studying motoneuronal cell death after unilateral sciatic nerve transection in rats between their 4th and 40th day. Transections at the 4th and the 7th day led to massive cell death but after transections
at the 10th day survival rates are about 49% in the pool of the tibial muscle and 59% in the pool of the soleus muscle. Transections at later ages led to the survival of increasing numbers of motoneurones in the pools of the anterior tibial and the soleus muscles, but we choose the 10th day as an acceptable compromise16. It is of interest to note that the stage of brain development around the 10th and 13th day in rats, to a certain extent, is similar to that at term age in human babies8;24.

Therefore, the 10th day was chosen to perform a study into the long-term recovery of locomotion after a transection of the sciatic nerve.

METHOD

Surgical Procedures
Nine black and white hooded rats of the Lister strain were studied. The experimental procedures as described below had been approved by the Ethics Committee of the Medical Faculty of the University Medical Center Groningen (FDC 0791). Two male rats and one female rat were placed in a cage and after an appropriate delay, the male rats were removed and the cage was inspected twice daily for offspring. The day of birth was designated as P1.

At P10, the pups were injected intramuscularly with hypnorm (10 mg/ml fluanisone and 0.2 mg/ml fentanyl: Janssen Pharmaceuticals, Tilburg, The Netherlands); 1 ml/100 g body weight. The left sciatic nerve was exposed by splitting the gluteal muscle. The nerve was transected proximal to the bifurcation into the common peroneal and tibial nerves. After transection, the proximal

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**Figure 1** The set up for qualitative assessment of walking performance using a video camera and recorder and a runway with a mirror in 45 degrees in order to obtain a split screen image.
and distal nerve ends were sutured by 10.0 prolene and the wound was closed. After the operation, the rats were allowed to recover from the anaesthesia and returned to the mother. Surgical procedures were performed under an operation microscope (Zeiss OPMI-6, Weesp, The Netherlands), and a sterile technique was used throughout the procedure. Good laboratory practice (GLP) was maintained, according to the guidelines of the Dutch Ministry (Veterinary Health Inspection), similar to the international rules for animal experimentation (International Guide on Animal Biomedical Research and Ethical Code for Animal Experimentation of the Council for International Organization of Medical Sciences).

Evaluation of Locomotion

At 9, 20 and 34 days after the operation, as well as after 21 and 52 weeks, the rats were tested and they were individually identified by the black and white pattern of their skin. Each rat was placed in a perspex runway (Fig 1, see also30). The lateral view of the animal as well as the ventral view (visualized by means of a mirror under the cage at a 45° angle position) was simultaneously recorded with a digital video camera (GR-DVL9800 JVC camera, recording 50 frames/sec). In this manner a split-screen image was obtained with the lateral view of the rat in the upper half and the ventral view in the lower half (Fig. 1). The runway was illuminated with two 120W concentric light bulbs. Walking movements of each rat were recorded on digital tape, until at least four consecutive and nonhesitant step cycles were collected.

The video-tape was then replayed frame by frame. Three aspects of locomotion were considered. The quality of locomotion was evaluated by means of a scoring list for the walking performance (for further details see23). Two investigators, experienced in analyzing walking performance in rats evaluated the different aspects of walking performance. Previous studies indicated abnormalities in the affected leg with regard to the following aspects and the scoring of abnormalities consequently focused thereupon: (1) toe spread during the stance phase; (2) the nature of ground contact of the foot during stance phase (the plantar side contacting the floor or a variety of abnormalities in placing); (3) eversion of the foot; (4) dragging of the limb and foot; (5) exorotation of the foot during the stance phase; (6) regular and alternating steps during walking; (7) the nature of the leg excursions during the swing phase; (8) the hindfoot placing in relation to the body contour (in the ventral aspect); and (9) the fluency of walking. The scoring system was such that abnormalities or deviations from the normal in each of the 9 aspects were scored by a “1”, this leading to a sum score of “9” in case of maximal abnormality and “0” in case of normal movements.

Secondly, video recordings were analyzed in order to calculate the stance factor (SF), as described by Walker et al.28 The stance factor is the ratio between the durations of the stance phase (or, floor contact) of the left, operated foot, and the
right, non-operated foot during walking (both expressed in the numbers of frames). Floor contact of a foot can easily be discerned from recordings of the ventral aspect of the animal. Normally, this factor approaches 1, but rats after a sciatic nerve transection at the left side usually attain lower values as the stance durations at this side decrease (because of an increased duration of the swing phase) and those at the unoperated side relatively increase.

Thirdly, after 52 weeks we calculated the sciatic function index (SFI) from a selected still of the ventral view of the footprints. For the calculation we used the formula developed by Bain and Mackinnon. The SFI is a quantitative method for analyzing the sciatic nerve function in rats. An SFI of 0 is normal whereas an SFI of -100 means total impairment.

Lastly, we recorded abnormalities as mutilation of the toes or flexion contractures. These phenomena regularly are observed after operations at adult age.

**Post Mortem analysis**

The rats were sacrificed after 52 weeks. At post mortem analysis, the nerves at the affected side were studied for continuity, the presence of neuromas, and histological changes.

**RESULTS**

At post mortem analysis, in all rats the sciatic nerve had regrown and we did not observe neuromas at the site of the lesion or at any other localization along the nerve.

**Qualitative Assessment of Walking Performance**

Normal rats at P19 walk fluently and swiftly, with a regular and alternating foot-fall pattern. Exorotation during the stance phase, which normally occurs until P15 or P16 has waned by this age and the placing of the feet is within the body contours (when viewed from a dorsal or ventral view); (for further details see). Rats in the present study, nine days after surgery, only in two cases showed a mild exorotation of the foot at the affected side, but apart from this, none of the rats showed gross abnormalities. Walking was reasonably fluent. Only in a few cases, the affected hindleg was somewhat slower during the swing phase and consequently, the foot-fall pattern sometimes was mildly irregular. But apart from this locomotion in all respects was similar to that in normal rats of that age. No auto-mutilation, flexion contractures or dragging were observed during walking. This had changed dramatically 20 days after the transection of the sciatic nerve, when all but two rats showed dragging of the left hindpaw. This was accompanied by abnormal ground contact of the feet, often by eversion and an irregular walking pattern. From this age onwards flexion contractures often were observed.
At 34 days after the operation, and at 21 and 52 weeks only one rat showed a relatively undisturbed walking pattern with a near to normal foot placing, without exorotation of the foot, and no dragging. Abnormalities in the other rats mainly showed by eversion of the left foot and walking on the heels (Fig. 2A), by dragging of the hindpaw (Fig. 2B), and by flexion contractures of the toes with or without exorotation of the foot (Fig. 2C). Furthermore, the majority of the rats had problems with placing the hindfoot within the body contour. Three rats showed auto-mutilation.

**Stance Factor (SF)**

The stance factor at nine days after the operation attained a value of 0.81, indicating a shorter stance phase duration at the affected side. This value slightly decreased on the following post-operative intervals to 0.74 at 34 days after the operation. Thereafter no further decrease or increase was noticed (Fig. 3).

**Sciatic Function Index (SFI)**

At 52 weeks, five rats had flexion contractures of the toes and three rats showed auto-mutilation. These conditions made that dimensions which normally are used in order to calculate the SFI could not be measured. Only one rat showed a relatively normal walking pattern without auto-mutilation and flexion contractures. The SFI in this rat was -13.6 (normal: SFI = 0, total impairment: SFI = -100).

*Figure 2* Posture of the foot at the affected side, 34 days after transection of the sciatic nerve and repair. Three different walking patterns could be observed in all but one rat; eversion of the foot and walking on the heels (A), dragging of the hindpaw (B), and flexion contractures of the toes (C).
In conclusion, this pilot study into the walking patterns in P10 rats after a sciatic nerve transection and repair at a proximal level indicated that nine days after the operation all rats still walked close to normal. Only in two rats we observed an exorotation of the foot at the operated side. From 20 days after the operation, however, the walking performance deteriorated dramatically, and all but one rat showed severely handicapped walking patterns and this lasted until they reached the age of one year. Only one rat out of nine walked relatively undisturbed, with an SFI approaching normal values but with an abnormal Stance Factor. We could not detect any possible explanation for this performance, deviant from what was found in the other 8 rats.

DISCUSSION

A common clinical notion is that lesions to the nervous system, be it the central nervous system or the peripheral nervous system, lead to less impairments than such lesions at adult age. Lesions of the brachial plexus (also, “obstetric brachial palsy”, or, “Erb’s paralysis”) occurring during birth and most often as the consequence of a heavy birth weight may lead to palsy but, particularly if the C7 root remains intact the prognosis is relatively favorable. This probably is due to fibers in this root which normally disappear during development but which now remain functional and these innervate the areas denervated by the fibers in the C5 and C6 roots. The same has been found in case of an avulsion of the C7 and C8 roots but with the more proximal roots still intact. Another factor, and this specifically applies to human babies, children and young primates might be that after transections, and an at-random reinnervation, reorganization processes

Figure 3 Trends in the Stance Factor with time after the operation.
or, relearning processes in the cerebral cortex at young age might be enhanced in comparison to compensational processes at older age. Lundborg in a recent review suggested this possibility although the evidence so far seems to limit to sensory functions\textsuperscript{21}.

Results on median and ulnar nerve transections in young children point in a similar direction\textsuperscript{4;9;15;25}. Recovery during infancy of such lesions is more complete than similar lesions at adult age. Here, the explanation might be a faster outgrowth of the axonal sprouts at a young age and a relatively simple and straightforward (re)innervation of denervated target areas, without the involvement of sets of antagonistic muscles.

We decided to study the problem of functional recovery after sciatic nerve transections in rats aged 10 days. In a previous study, we demonstrated that at this age, the numbers of motoneurones of the soleus and the tibalis anterior muscle amounted to 59\% and 49\% respectively, and this age appeared to be the youngest age at which a reasonable amount of motoneurones survive nerve transection\textsuperscript{16}. Watanabe et al. at the sixth day observed significantly lower scores in the SFI-index (or, walking track analysis) than in rats which were operated at 22 days after rat tibial nerve transection and repair\textsuperscript{29}. Also the conduction velocity in the nerve was lower in the rats operated at six days and this held as well for the mass of the gastrocnemius muscles (innervated by this tibial nerve).

In the present study we observed relatively undisturbed walking patterns at nine days after the transection at P10 but this had deteriorated at P30 and even further at P44, 20 and 34 days after the operation. We interpret these findings by hypothesizing that reinnervation starts between P19 and P30 and that as soon as these nerve connections become functional this then leads to impaired walking patterns. If so, this would imply that young rats without interference of the aberrantly innervating sciatic nerve walk better than slightly older rats with this nerve having established neuromuscular connections. Further research is needed to prove this hypothesis. Firstly, a time series of the reinnervation after transection at the 10\textsuperscript{th} day should be analyzed and the question would be if, indeed, reinnervation takes place between nine and 20 days after the transection at P10. The other experiment would be to investigate whether a secondary transection of the sciatic nerve e.g. at P30 or P40, in rats in which the sciatic nerve was transected at P10, would ameliorate the impaired walking pattern. A study (still in progress, into the expression of agrin at the endplate region), sheds some light on this last possibility. In rats lesioned at P10, we allowed reinnervation of the transected nerve by suturing, while, in another group reinnervation was avoided by suturing the proximal nerve end into a loop. Those rats in which reinnervation had occurred, recovered to some extent. However, 10 to 14 days after the operation walking performance deteriorated again. In contrast, in the rats of the second group, walking performance remained slightly disturbed and without gross...
abnormalities until the end of the observation period, at 42 days.

In companion studies to the present study we found that the histochemical profile of the soleus muscle at the age of one year, was as disturbed as it was after nerve transections at young adult age\textsuperscript{20}. This muscle had changed into a muscle with predominantly type II muscle fibers and this result was similar to the effects after a lesion at adult age\textsuperscript{18;19}. Also the EMG patterns recorded from the tibial muscle and from the gastrocnemius muscle during walking, 7 weeks after the operation at P10 showed cocontractions of the flexor and extensor muscle indicating an at-random reinnervation of the sciatic nerve and this conclusion was supported by data from retrogradely labeling the motoneurones from the respective muscles\textsuperscript{12;13}.

In summary, nerve transections at early postnatal ages do not lead to an enhanced functional recovery, but instead, they lead to seriously impaired walking patterns, very similar to that after sciatic nerve transections at young adult age\textsuperscript{22;23}. Obviously, the factors which guided the outgrowing motor axons towards their target muscles at initial stages of development, reorganizations of neural circuits at spinal levels or compensations (or, “relearning”) at central levels which might adapt to alternative reinnervation patterns are not effective anymore at this age in rats. Clinical evidence has indicated that in cases of lesions to roots, containing fibers to antagonistic muscles, undamaged nerves in the same plexus might take over the functions of the severed nerves. This question, and also the problem whether this compensation after partial lesions limits to young ages is an intriguing challenge for future research.
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