Survival of soleus and tibial motoneurones after transection of the sciatic nerve at different ages in the young and adult rat.
SUMMARY

Clinical investigations into the effects of peripheral nerve injuries in babies and young children often report less impairments than after similar lesions in adults. We decided to investigate this notion in a series of systematic investigations in young rats into the consequences of peripheral nerve transections.

It is well known that peripheral nerve lesions at neonatal age lead to massive motoneuronal death, but at adult age the motoneurones generally survive axotomy, if at some distance from the cell body. In the present study we investigated the earliest stage of development at which still a substantial proportion of the motoneurones remain alive after nerve transection. To that, we analysed the numbers of motoneurones innervating the soleus and tibialis anterior muscles after a unilateral sciatic nerve transection between the 4th and the 40th postnatal day. Around 50 days after the transection, the motoneurones innervating these muscles were retrogradely labeled by injections of unconjugated CTB into the respective muscles, and numbers at the operated side were compared to those at the side of the transection.

Results indicate that motoneuronal death after transections at the 4th and 7th day was high indeed. Transections at the 10th day left 49% of the motoneurones in the anterior tibial muscle and 59% of the motoneurones in the soleus muscle. Remarkably, after transections at the 15th day, the percentages of remaining motoneurones of both muscles had decreased. Thereafter they increased again and reached values of around 100% after operations at the 40th day. These results indicate that the 10th postnatal day would be the youngest age at which still a reasonable number of motoneurones survive axotomy. Consequently this age was chosen for transections in studies to follow the present investigation.
INTRODUCTION

Peripheral nerve injuries in adult humans, even if treated by optimal techniques such as the implantation of nerve conduits, still, in most cases lead to poor functional recovery\textsuperscript{23;24}. Several reports suggest, however, that nerve transections or even extensive plexus lesions in babies and young children have a better outcome\textsuperscript{2;7;14;15;26;35;37}. Babies, after an obstetric brachial plexus lesion (OBPL), leading to an initial paralysis of the shoulder and elbow muscles, recover in 90\% of the cases within weeks to a few months\textsuperscript{37}. In contrast, brachial plexus injuries with subsequent paralysis in adults never spontaneously recover. Surgical reconstruction in such cases generally is required but despite that, the functional outcome is poor\textsuperscript{36}. Similarly, the improvement of sensibility after surgical intervention to reconstruct the median nerve after lesions at the wrist-area is better in young children than in adults\textsuperscript{26;32}.

The reasons behind an improved recovery in babies and young children are badly understood. One of the possibilities is that the distance from the transection to the target area generally is shorter in young individuals. From experiments in adult animals, it is well known that the proximal stump of the injured peripheral nerve is capable of sprouting and these axons randomly reinnervate muscles\textsuperscript{4;9} and skin areas\textsuperscript{5}. Transecting the soleus nerve close to the soleus muscle results in a near to normal fiber type distribution whereas a highly abnormal distribution is observed when the nerve is transected at the knee or more proximal levels\textsuperscript{33}. On this basis it seems that a smaller distance to the target area, to be bridged by the outgrowing axons enhances the accuracy of the reinnervation, and this in turn might lead to a better functional recovery. Another possibility is that at early stages of development, an increased availability of tropic factors or an increased responsiveness of neurons to the effects of such tropic and trophic factors improve the pathfinding capacity of axons\textsuperscript{30}. Still another factor might be a greater plasticity of the CNS in young individuals leading to a central compensation after a peripheral nerve lesion with an at-random reinnervation of target areas\textsuperscript{26;39}. Vredeveld and coworkers\textsuperscript{39} have suggested that the biceps brachii and deltoid muscles in the human initially are innervated by the C5 and C6 ventral roots and also by fibres in C7. The fibres in the C7 root disappear by apoptosis during normal development, but in OBPL the motoneurones and their fibres survive, playing a role in an alternative reinnervation, which might explain the recovery in these infants. Lundborg\textsuperscript{25} suggests that after deafferentation a rapid cortical synaptic remodelling takes place, resulting in a changed cortical representation of the deafferented area. Such remodelling also occurs at adult age\textsuperscript{28}, but this relearning process might be more pronounced at younger ages.

We decided to analyse the effects after peripheral nerve lesions at young age. If such effects include less severe impairments then, elucidating the factors
explaining this improved functional outcome could help to design more effective treatments after such lesions in young individual and also in adults. The effects were studied in young rats and we compared the data with earlier results obtained in adult animals\textsuperscript{9;16-18;27}.

Data in the literature indicate that transection of a peripheral nerve in the neonatal period in rodents leads to massive death of the motoneurones\textsuperscript{13;20;34;40}. Schmalbruch\textsuperscript{34} found after transecting the sciatic nerve in newborn rats at a proximal level, that almost all the motoneurones had vanished. At the 7th postnatal day about half the motoneurones died, but at 4 weeks after birth no noticeable cell death occurred. Similar results have been observed after transecting the tibial nerve at the 6\textsuperscript{th} or the 22\textsuperscript{nd} day, after which 55\% and 95\% respectively of the motoneurones survived\textsuperscript{40}. On the other hand, Kashihara and coworkers found that 77\% of the motoneurones survived after transecting the medial gastrocnemius nerve at the 4\textsuperscript{th} day after birth\textsuperscript{20}.

When studying the effects of nerve transections at early stages in rats it obviously is important to choose the youngest age at which still a reasonable number of motoneurones survive. The identification of this age is the topic of the present study. To that, we transected the sciatic nerve proximal to the bifurcation in the tibial and common peroneal nerve at the 4\textsuperscript{th} postnatal day (P4), P7, P10, P15, P20 and P40. About 50 - 60 days after the initial operation the soleus muscle (SOL) or the anterior tibial muscle (TA) were injected with a retrogradely transported tracer and we counted the number of labeled motoneurones. Numbers were compared with numbers of motoneurones at the contralateral, control side.

**MATERIALS AND METHODS**

A total of 52 rats of the Black Hooded Lister strain of either sex were used in this study. Ethical approval to conduct the study was obtained from the Ethics Committee on Animal Experimentation, University of Groningen. Rats were premedicated with 0.01 mg/kg buprenorfine (Temgesic) and anaesthetized with 3% isoflurane and O\textsubscript{2}/ N\textsubscript{2}O. The sciatic nerve was transected in rats at postnatal day 4 (P4), P7, P10, P15, P20 and P40 and most age groups consisted of 8 animals but the P10 group of 12 animals.

The left sciatic nerve was exposed at a mid-femoral level by splitting the gluteal muscle. Proximal to the bifurcation into the tibial and the common peroneal muscle the sciatic nerve was transected. In the animals until P15 no retraction of the nerve is observed, therefore the nerve stumps could be carefully aligned and positioned in the surrounding muscle tissue, without suturing the nerve stumps. In rats operated at P20 and P40 the nerve stumps retract upon transection and therefore, the distal and proximal nerve stumps were sutured epineurally with 10-0 nylon. Animals were inspected at least daily and auto-mutilation which
often occurs after sciatic nerve transections was prevented with “nailwatch” (PW Products, UK), a non toxic substance to avoid nail biting in humans.

Fifty – 60 days after the transections the animals were anaesthetized (see above) and 10-15 μl of 0.1% unconjugated cholera toxin subunit B (CTB; List Biological Lab.) was injected into the muscle belly of the SOL or TA muscles of the left and right leg, by means of a pulled glass pipette connected to an injection system. In half of the animals in each age-group we injected the SOL muscle and in the other half the TA muscle. During the injections we took care to avoid spilling of the tracer outside the muscle, and after injection, we carefully rinsed and dried the operation wound in order to minimize leakage and absorption of the tracer by neighbouring muscles. After a survival time of 3 days the animals were deeply anaesthetized and perfused transcardially. We used 0.8% NaCl, 0.8% sucrose, 0.4% D-glucose in 0.05M phosphate buffer (Ph=7.5 ). Thereafter the animals were perfused with a fixative containing 4% paraformaldehyde 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH=7.5). The spinal cord was removed with the right side marked, and the cord was immersed overnight in 30% sucrose in 0.05M phosphate buffer in order to avoid freezing artifacts. Longitudinal sections were cut at 40μm on a freezing microtome. Sections were collected in vials containing 0.05 M phosphate buffer and successively incubated with goat anti CTB (1: 10000; List Biological Lab), rabbit anti goat (1:100; DAKO Z228/ DakoCytomation Z0454) and goat PAP (1:100; DAKO B157). Diaminobenzidin (DAB, Sigma) was used as a chromagen. Between these steps, the sections were rinsed in Tris buffered saline. After immunochemical processing, the sections were mounted with chrome-alum on glass slides, dried, dehydrated and cover slipped.

All intensely labeled motoneurones in the left and right sides of the spinal cord were counted (10x obj). In order to avoid counting the same motoneuron twice in adjacent sections, we applied the correction method of Abercrombie1. Abercrombie’s correction factor is t/(t+d), in which t =section thickness and d = diameter. Diameters were calculated as follows. The outlines of a representative number of the motoneurones (on the average, N = 37) were plotted at a magnification of 20x obj (Nikon microscope with drawing device) and these plots were scanned with a Quantimed 520 + (Cambridge Instruments). The cross sectional areas of these motoneurones were calculated as well as the equivalent diameters.

Data obtained from the affected and the control side of the spinal cord were compared. Statistical significance was calculated using repeated measures ANOVA, the level being set at 0.05.
RESULTS

Injection of CTB in the muscles and successive immunological staining of the retrogradely transported CTB in the motoneurones resulted in a dense labeling of the motoneurones. As the homologous muscles were injected at the control side and the left side within one animal, the numbers of motoneurones at both sides could be compared.

The motoneuronal pools of the TA and the SOL appeared as elongated cell aggregates. The pools at the left side in the animals, however, were less densely packed with motoneurones than the pools at the control side.

The average number of motoneurones to the SOL muscle at the control side in the spinal cord was 115.1 (range 40.1-225.5). The average numbers of SOL motoneurones at the left side of the spinal cord, however, varied strongly with the age at transection. At P4 we counted an average of 17 motoneurones (range 1.1–53.8). When calculating this as a percentage of the motoneurones at the control side in these rats, this resulted in a value of 14% (range 1.0–37.4%); (fig 1). At P7 we counted an average value of 43% (range 24.0–74.6) and at P10, 59% (range 35.1–83.7). At P15 this value had decreased to 23% (range 12.9–29.3) but at P20 the number of soleus motoneurones again had increased to 68% (range 55.1–82.4%) of the levels as found at the contralateral side. At P40 we even

![Figure 1: Average numbers of surviving motoneurones at different ages from the soleus muscle (rhombuses) and the anterior tibial muscle (squares). Error bars indicate the range.](image)
observed in two of the rats an increase far above the values found at the control side. These results were included in the data-set, as we did not have strong indications for leakage of the tracer to neighbouring muscles.

The average number of motoneurones in the right half of the spinal cord, labeled from the TA was 86.9 (range 15.7-122.0). Counting the numbers of the motoneurones at the left side and expressed as percentages of the numbers at the right side resulted at P4 into a figure of 25% (range 4.9-52.8%), at P7 of 18% (range 0.5-34.2%). At P10 this percentage had increased to 49% (range 23.8-89.6%) and at P15 to 44% (range 17.7-71.9%). From P20 we observed again an increase to 75% (range 36.3 – 132.0%) and at P40 the numbers of surviving motoneurones had increased to average values similar to those at the control side of the spinal cord.

The data on the cross sectional areas of the motoneurones (needed for calculating Abercrombie’s correction factor) allowed us to compare these dimensions

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**Figure 2/3:** Summated data, from all age groups, from cross sectional area of all motoneurones labeled from the soleus muscle (fig 2) or anterior tibial muscle (fig 3). Statistical analysis of these data in the respective groups of 250μm indicated that these differences were statistically significant, and related to the transection as such, and not to the age at transection.
at the affected and at the control side. They were classified into 7 categories of 250 μm², ranging from 0 - 1500 μm² and higher. The numbers in a certain category were expressed as percentages of the total number of motoneurones (figs 2 and 3). The sizes of the motoneurones on both sides appeared to be normally distributed at all ages. The distributions of these sizes both in the SOL and in the TA muscles showed a slight shift towards the left side irrespective of the age at lesioning. Obviously, both in the distributions of the SOL and TA motoneurones at the operated side, a preponderance of the smaller sizes occurred. In figures 2 and 3, the summated data from all the age groups are presented. Statistical analysis of these data in the respective groups indicated that these differences were statistically significant, and related to the transection as such, and not to the age at transection.

In sum, the numbers of motoneurones which escaped cell death after nerve transection is low at P4 and P7. At P10 these numbers have reached values of 59% in the SOL and 49% and the TA. At P15 these values had decreased in both muscles but from P20 values had increased again.

**DISCUSSION**

Numbers of motoneurones innervating the SOL and TA muscles were studied by retrogradely labeling with unconjugated CTB. CTB specifically binds to the GM1 ganglioside on the cell surface, and this enhances the specific labelling at axon ramifications in the injected muscle and minimizes the possibility of diffusion. Such diffusion occurs e.g. after Horse Radish Peroxidase (HRP) at the injection site and this tracer therefore might also label motoneurones from adjacent muscles (for extensive discussion and further references, see,42).

Important problems are whether all the motoneurones innervating a specific muscle are labelled after filling the muscle with CTB, and, secondly whether a transected and newly outgrown axon, transports this tracer as readily as normal nerve fibres. Earlier, Gramsbergen et al.9 discussed this problem in a study into the effects of a sciatic nerve transection in rats at adult age (i.e. about 120 days old). Motoneuronal numbers in the pools of the SOL, the TA and the gastrocnemius muscles at the control side were similar to those in previously denervated muscles9 and also to such counts in earlier research with CTB labeling10,44. It has been concluded in that study, therefore, that CTB, despite transection and reinnervation, readily labels the specific motoneurones. In the present study we investigated rats between the age of 60 and 70 days. The neuronal properties as to the transportation of CTB on those ages must be considered to be similar to those of rats at 120 days, and therefore, we propose that also in the present study CTB indeed is an appropriate method for studying numbers of surviving motoneurones.
The numbers of motoneurones labeled from the SOL and TA muscles after transection at P4 and P7 are low but at P10 these numbers have reached values of 59% in the SOL and 49% in the TA. Remarkably, the percentages in both muscles had decreased in the groups lesioned at P15 but from P20 they had increased again (Fig 1). The numbers of motoneurones labelled from the SOL muscle in two animals transected at P40 are high indeed. These exceptionally high values must have been due to leakage of the tracer to the surrounding muscles, although during the actual procedures this was not noticed.

Our finding of high levels of motoneuronal cell death after transection of the sciatic nerve at the 4th or 7th day after birth is in agreement with data in the literature. Schmalbruch observed after sciatic nerve transactions at the day of birth the death of almost all motoneurones while after transection at P7 about half of the number of motoneurones died and transections, 4 weeks after birth did not lead to noticeable cell death34. Watanabe et al., by transecting the tibial nerve at the 6th day and the 22nd day found similar figures. Obviously, motoneurones in the early postnatal period are highly susceptible to axotomy and this vulnerability decreases with age. One factor is, that at early ages, the motoneurones are critically dependent upon their peripheral target for trophic support20,21. The administration of neurotrophic factors, as nerve growth factor (NGF), neurotrophin 4/5 (NT 4/5) and ciliary neurotrophic factor (CNF) are able to rescue considerable numbers of motoneurones after transection40. Also, neurotrophic factors which are expressed in the developing skeletal muscle as brain derived neurotrophic factor (BDNF), NT 3 and insuline-like growth factor-1, have been demonstrated to rescue most of the motoneurones after nerve transection22. Interestingly, these factors are only able to prevent motoneuronal death for a limited period6,38.

Greensmith and Vrbova13 proposed that next to the dependency on neurotrophic factors, also the interaction between the motoneuronal axons with their targets is crucial during development. Greensmith and Vrbova observed, after application of α-bungarotoxin, a toxin blocking the nerve-muscle interaction, that many motoneurones had died12, whereas application of 4-aminopyridine, enhancing the transmitter release at the neuromuscular junction, had rescued the motoneurones after transection of the nerve at the neonatal period11 (see also,40). Another factor undoubtedly is, that the immaturity of the cellular machinery at early ages makes young motoneurones more vulnerable to injury, than at later stages12. Also, a decreased trophic support from afferent input8,31 and not yet fully established contacts with glial cells in the spinal cord19 may add to the explanation of increased motoneuronal death after nerve transactions at early stages.

Recently, Moran and Graeber29 have proposed a “developmental switch” to explain the differential effects after transection at earlier and later ages. In the period before this “switch”, the neurons would be prone to cell death after injuries as transections, axon crushes etc, whereas thereafter they are able to
stand such interventions. Recently, it has been suggested that the timing of this switch is related to the induction of a small heat shock protein 27, Hsp27\(^3\). In adult motoneurones, sciatic nerve transection induces the production and the phosphorylation of this Hsp27 and this protects the neurons from cyochrome c release from the mitochondria as well it inhibits caspase-3, the molecule active in cell death. Transections at neonatal, in contrast, do not lead to the expression of Hsp27 in the majority of the motoneurones and as a consequence of that, it is suggested, these die. The few motoneurones that do survive lesioning at neonatal age, however, did express Hsp27 (for further discussion on this point see,\(^29\)).

A surprising finding in our study is the steady increase in the number of surviving motoneurones after transections until P10, but a decrease thereafter, at P15, and from then again an increase till control values at P40.

A factor which might explain a temporary increased susceptibility to axotomy around P15 is the important reorganizations taking place in the dendritic arborization of lumbar motoneurones between P14 and P16. Westerga and Gramsbergen\(^42\) observed that the dendrites of the SOL motoneurones before P14 are oriented in a seemingly random order whereas from P16 their dendrites run in so-called dendrite bundles directed in longitudinal and transversal directions. Probably, a similar reorientation takes place in parts of the motoneuronal pool of the gastrocnemius muscle (see,\(^10\)). Details on the dynamics involved in this rearrangement (pruning and regrowth, or, translocation of the dendrites) are unknown yet. Such reorganizations specifically do not take place in the TA motoneuronal pool, but, it seems feasible that the reorganizations in the SOL (and the gastrocnemius) motoneuronal pool also imply important synaptic rearrangements in the pool of the TA muscle. Westerga and Gramsbergen\(^41\) demonstrated that this rearrangement is related to a change in walking patterns around this same age, and in later research, Gramsbergen and IJkema-Paassen have demonstrated that this reorganization of dendrites is dependent upon descending fiber projections (results published in abstract form). A spinal cord transection before this reorganization, interferes with the development of bundles. In sum, this reorganization, normally taking place between P10 and P20, and probably requiring considerable metabolical capacity, might well add to the explanation of an increased susceptibility of the motoneurones to the effects of a sciatic nerve transection.

The aim of the present study was to identify the youngest age at which a sciatic nerve transection still leaves considerable numbers of motoneurones alive. After transections at P10, between 50 and 60% of the motoneurones in the pools of the SOL and the TA survive. At earlier ages, increased percentages die and this holds as well for transections at P15. On the basis of these results we therefore conclude that for studying the effects of sciatic nerve transections in young rats, the 10\(^{th}\) postnatal day offers the best compromise between young age and the survival rate of motoneurones.
REFERENCES