Magnetoneurographic evaluation of peripheral nerve regeneration.

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Magnetoneurographic evaluation of peripheral nerve regeneration

Magnetoneurografische evaluatie van perifere zenuw regeneratie

Proefschrift

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Dedicated to:

my parents, Hans and Toetie
for leading me into intellectual pursuits

my wife, Caroline
for her endless devotion to our family

our children, Sam and Noa
for making it all worthwhile

Contents

List of abbreviations:

ALD	activity of daily living
AP	action potential
CNS	central nervous system
CV	conduction velocity
$CV^{1st\ peak}$	conduction velocity of the first peak
DDH	diameter distribution histogram
DF	dorsoflexion
DRG	dorsal root ganglion
HRP	horseradish peroxidase
MNG	magnetoneurography
NaCl	sodiumchloride
NCAC	nerve compound action current
NCAP	nerve compound action potential
NCAS	nerve compound action signal
NGF	nerve growth factor
NO	laughing gas
O_2	Oxygen
PNS	peripheral nervous system
PPD	paraphenylenediamine
SD	standard deviation
TS	toe-spread
ϕ	diameter

1. Introduction

When a peripheral nerve is reconstructed after it has been damaged, it is important to assess, in an early stage, whether the nerve is regenerating across the lesion. However, at present for this purpose an adequate method is not available. In this study short term changes in the proximal and distal segment of a transected and reconstructed peripheral nerve are evaluated using a new quantitative magnetic recording technique. For a general understanding, the anatomy and neurophysiology of peripheral nerves will be discussed in this introduction, followed by an overview of clinical aspects of peripheral nerve reconstruction and regeneration, and of the techniques used for evaluation of nerve regeneration.

1.1 Peripheral nerve lesions

Injuries to peripheral nerves are common. Approximately 1000 peripheral nerve injuries of the hand or wrist are treated in Holland every year (J.B. Jaquet, unpublished observations). The prolonged paralysis and anaesthesia that result are particularly disabling, especially if they impair the motor and/or sensory function of (part of) the hand. The capability of the peripheral nervous system to restore the connections between the nerve cell bodies and the target organs, after such a nerve lesion, is considerable. But a regenerating axon must take many hurdles before it can contribute to recovery of function. First, the transected axon will have to survive the trauma and seal off the open end of the fibre [Egeraat van et al., 1993; Egeraat van, Wikswo, 1990; Egeraat van, Wikswo, 1993; Yawo, Kuno, 1985]. Then it will have to form a growth cone [Ramon y Cajal, 1905] which will start to regenerate into the distal direction. The regenerating sprouts have to grow across the lesion and find the proper target organ to connect to [Fawcett, Keynes, 1990]. The number of possible connections to target organs is enormous and the specificity of the regenerating sprouts in finding the target organ to which the respective axon was connected prior to the transection is restricted [Brushart, 1988; Brushart, Seiler, 1987; Brushart et al., 1983]. By the time the regenerated neuron arrives at a target organ, it should still be able to perform its highly specialised function of conducting electrical signals at a high speed from the periphery to the spinal cord or vice versa. Finally, many regenerating sprouts will not find the target organ they were connected to prior to the transection. The capability of the brain to reorganise the information transported to and from the periphery through the new connections after nerve regeneration is less than complete. Furthermore, this plasticity of the brain decreases with increasing age of the patient [Dellon, 1990; Moberg, 1985]. It seems obvious that recovery of function after peripheral nerve lesions is not always as good as desired.

1.2 Anatomy

1.2.1 Gross anatomy

The nervous system can be divided into a central nervous system (CNS) and a peripheral nervous system (PNS). The CNS (brain and spinal cord) receives information

through the PNS (connections between spinal cord and target organs) from the sensory organs. This way the brain will have a continuous overview of posture, pressure, temperature, vibration and pain which is encountered at different parts of the body and the relation the body has to its direct and indirect surroundings. The CNS thus senses and analyses the environment in order to generate appropriate behaviour. The brain can influence the environment by changing the tone of different muscles resulting in posture and movement of the body. Also the commands from the CNS to the periphery are conducted along the PNS to the muscles.

1.2.2 Neurons

Neurons are the cells responsible for the transportation of information. A neuron consists of a nerve cell body and an axon. The nerve cell body is the metabolic factory of the neuron. Products of the cell body which are needed in other parts of the neuron are transported along the axon by use of a fast transportation mechanism based on the smooth endoplasmic reticulum [Droz, Leblond, 1962; Droz et al., 1975]or a slow

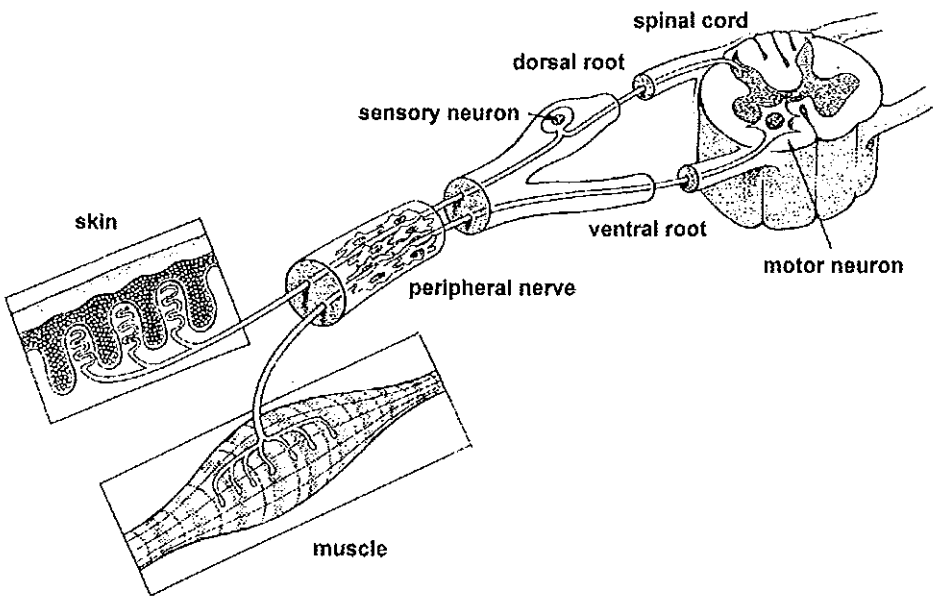

Fig 1: Components of the peripheral nervous system.
(from: Nerve injury and repair by G. Lundborg)

mechanism which uses the cytoskeleton [Frizell, Sjostrand, 1974; Sjostrand, Frizell, 1975]. Substances absorbed by the distal end of the axon in turn are transported to the nerve cell body [Droz et al., 1975]. The cell bodies of the motor neurons innervating the skeletal muscles lie in the anterior horn of the spinal cord, while the cell bodies of the sensory fibres lie in the dorsal root ganglia (DRG). These are located outside the spinal cord, in the dorsal root.

Nerve cell axons connect the nerve cell bodies in the anterior horn or the DRG to the peripheral target organs. These axons conduct action potentials at different speeds (varying from 120 to less than 1 m/s), over a length of up to 1.5 meter in man. The axons of motor neurons run through the ventral root to the peripheral nerves. The axons of the sensory neurons divide into central and peripheral branches. The peripheral branches will run through the dorsal root and join the motor fibres in the peripheral spinal nerves, while the central branches will enter the spinal cord.

The peripheral axons of the sensory neurons connect to several receptor types in the skin, joints, muscles and tendons. The motor neurons connect to muscle fibres (fig. 1).

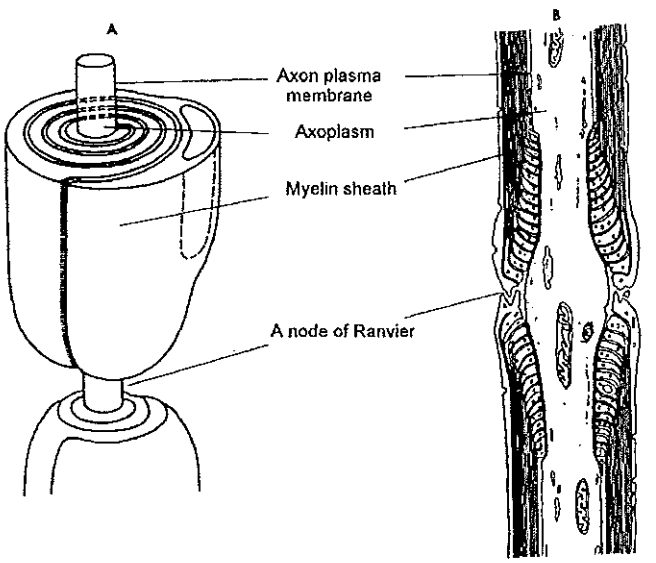

*Fig. 2:*A drawing of the myelin sheath and internode of a myelinated axon. A: 3 D view B: longitudinal section
(from Physiology by Guyton)

The axons of peripheral nerves are insulated by Schwann cells. In myelinated axons this coverage is present as a myeline sheath, consisting of the membrane of a series of Schwann cells winding concentrically around the axons. Each single Schwann cell electrically insulates a portion of the axon (internodium), but between two successive Schwann cells a small stretch of axon (the node of Ranvier) remains uninsulated (Fig.2). The unmyelinated axons are embedded in poaches of the Schwann cell membrane.

1.2.3 Peripheral nerve

The peripheral nerve bundle represents a composite tissue which maintains the continuity, nutrition and protection of the nerve fibres within the nerve trunk. These nerve fibres need a continuous energy supply which is provided by an extensive microvascular system in the nerve trunk [Lundborg, 1988; Sunderland, 1978; Sunderland, 1991]. The nerve fibres are closely arranged in the endoneurial tissue of the fascicles. The fibres will cross from fascicle to fascicle throughout the length of the nerve [Sunderland, 1991; Sunderland, Ray, 1948]. This endoneurial tissue consists of closely packed collagen fibres forming the supporting walls of the endoneurial tubes through which the nerve fibres run. A perineurial sheath which has considerable tensile strength covers the fascicles. This sheath is formed by up to 15 layers of flattened perineurial cells which are connected by tight junctions [Shantaveerappa, Bourne, 1963]. The fascicles are embedded in the epineurial connective tissue. This loose

connective tissue cushions the fascicles during movement of the extremity and protects them from external pressure [Sunderland, 1978; Sunderland, Ray, 1948]. Superficially the epineurium condenses to a sheath which covers the nerve trunk and is surrounded by paraneurium [Lundborg, 1988]. The peripheral nerve has well-developed interconnecting microvascularised systems in the endoneurium, perineurium and epineurium [Lundborg, 1979; Lundborg, 1982](fig.3).

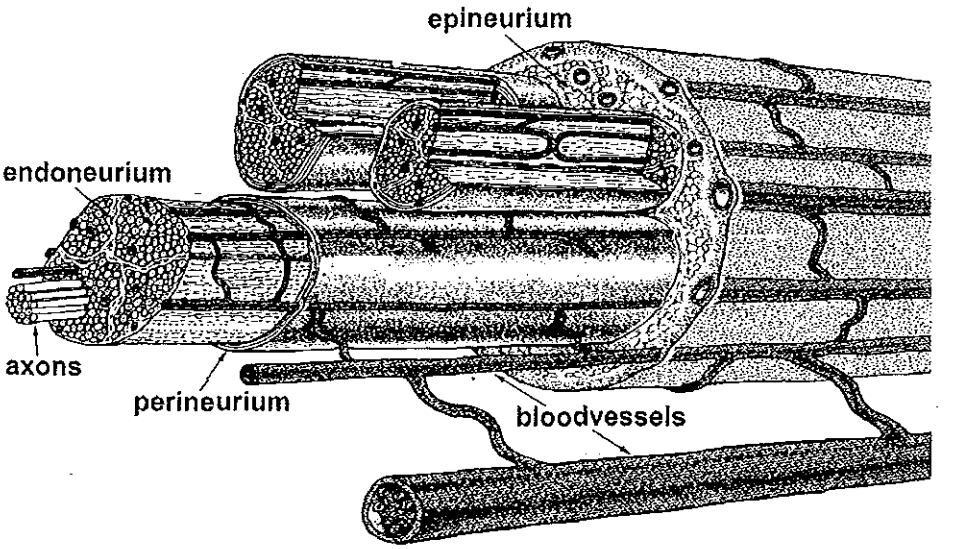

Fig. 3: Schematic drawing of the constituents of a peripheral nerve.
(From Nerve injury and repair by G. Lundborg)

1.3 Neurophysiology

1.3.1 Resting membrane potential

A cell membrane consists of a bilayer of lipoproteins. As ions are electrically charged, the axonal membrane is impermeable for ions except for some minor leakage. An axon, which is not conducting a signal, maintains a potential over its membrane. This resting membrane potential is established by active transport of positively charged sodium ions through the cell membrane to the exterior of the nerve fibre. Sodium is transported out of the nerve fibre by a special transporter mechanism, the so-called sodium/potassium pump. This pump will exchange sodium ions from the axoplasma for potassium from the extracellular fluid. As the pump transports two potassium ions into the axon for every three sodium ions that come out, there are always more positively charged ions being pumped outward than there are pumped inwards. This leaves a surplus of negatively charged ions on the inside of the nerve fibre while a surplus of positively charged sodium ions accumulate on the outside of the fibre. This results in a

membrane potential of approximately -90 mV (fig.4).

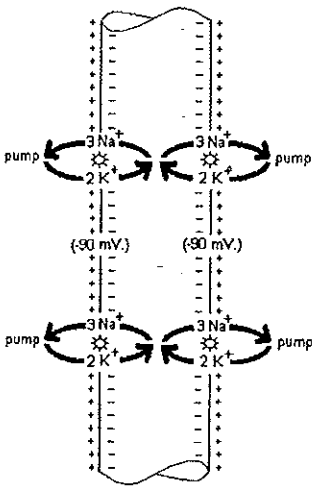

Fig. 4: The Na-K-pump in the membrane

potential back to near the resting potential of -90 mV. All of this occurs in less than 0.5 millisecond and is called an action potential (AP). After the AP the sodium/potassium pump will restore the resting membrane potential by pumping the sodium out and potassium back into the nerve fibre (fig.5). Recovering the resting membrane potential after an AP is an energy consuming process, for which mitochondria are transported into the axon. Furthermore an extensive vascular system will transport oxygen to the nerve fibres [Lundborg, 1979].

1.3.2 Action potential

If an axon membrane is disturbed, the membrane permeability for Na^+ and K^+ will change, thus depolarising the membrane. Such a depolarisation occurs in sequences. Initially the permeability increases greatly for sodium causing a flow of sodium into the nerve fibre. The membrane potential will rise from -90 mV to a positive value of +45 mV. Subsequently the permeability for potassium increases rapidly and the potassium runs out of the fibre. [Hodgkin, Huxley, 1952]. This results in a change of membrane

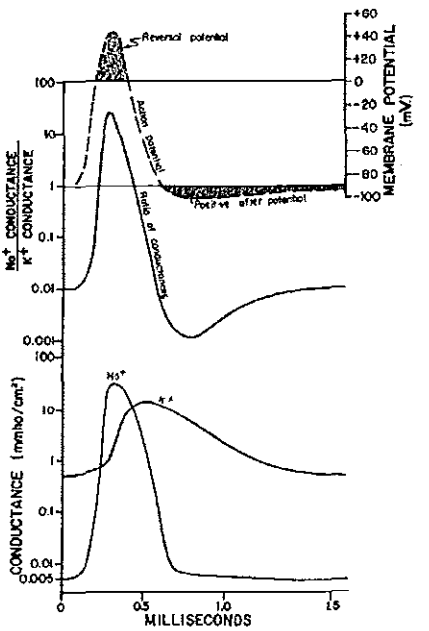

Fig. 5: The membrane processes contributing to an action potential

1.3.3 Propagation of action potential

1.3.3.1 Conduction of a signal

An action potential elicited at any point of an excitable membrane excites the adjacent portion of the membrane, resulting in propagation of the action potential over the length of the axon. If an axon is excited in its midsection, this part of the fibre will develop an increase in permeability to sodium. A positive current will flow inward through the depolarised membrane and the sodium pump will pump the positive ions out of the fibres through the adjacent resting membrane thus completing an electrical circuit. The decrease in potential over the membrane next to the depolarised section, due to the

intra-axonal current, will now cause a depolarisation at this part of the membrane. This depolarisation, in turn, lowers the potential over the following segment of membrane which will than depolarise. The depolarisation will thus be propagated through the nerve fibre in both directions away from the primary depolarisation (stimulation) site (fig.6) [Kutchai, 1983].

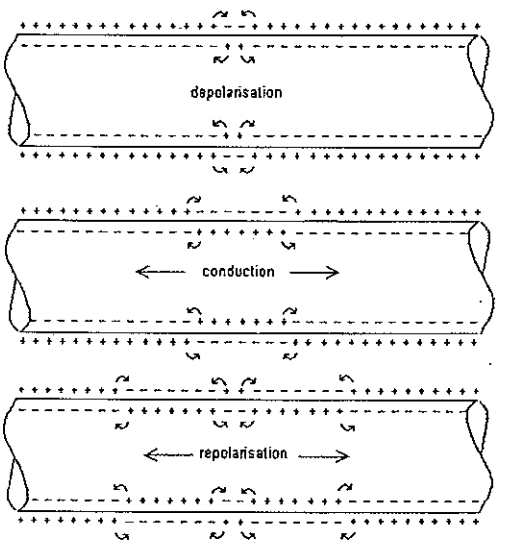

Fig. 6: Stages in the propagation of an action potential along an unmyelinated axon.

1.3.3.2 Saltatory conduction

In myelinated nerve fibres the internodia, produced by individual Schwann cells, consist of electrically highly insulated segments, which vary from 200 to 1600 µm. This length relates strongly to the axon diameter [Hursh, 1939]. Between each Schwann cell the myelin sheath is interrupted. Such an interruption is called a node of Ranvier. The depolarisation in such myelinated fibres only occurs at the nodes of Ranvier (fig.2, see:1.2.2). Therefore the electrical circuits described above (chapter 1.3.3.1) will run from node to node, thus depolarising the successive nodes. The impulse will jump from node to node down the fibre, greatly increasing the conduction velocity of the action potential through myelinated nerve fibres (Fig.7). The conduction velocity (CV) of the myelinated axons is related to the speed with which electrical charge can run through the axon. This is influenced by the intra-axonal resistance, which in turn is related to the diameter of the axon [Gillespie, Stein, 1983]. Furthermore, the CV will also increase with an increasing internodal distance [Hursh, 1939]. This distance is strongly related to the axon diameter.

1.4 Peripheral nerve regeneration

When a peripheral nerve is transected, several different phenomena will occur in the nerve cell body and in the proximal segment, at the lesion and the distal segment of the nerve trunk.

1.4.1 Nerve cell body

After a transection of a peripheral nerve approximately 5 to 7% of the nerve cells will not survive due to the trauma of the lesion [Horch, Lisney, 1981; Mackinnon et al., 1991; Shawe, 1954; Ygge, 1989]. The nerve cell body is the only part of the neuron that can synthesise the substances necessary for nerve regeneration. The surviving nerve cell bodies will change drastically after a transection [Goldstein et al., 1987; Hoffman, Lasek, 1980]. They increase in size due to an alteration in metabolic rate. The nucleus is displaced to the periphery of the cell body and an increase in the ribonucleic acid can

be demonstrated [Goldstein et al., 1987]. The metabolism of the nerve shifts from neurotransmitters and neurofilament production to the production of tubulin and actin which are necessary for growth in a regenerating sprout [Ducker, 1981; Sinicropi, McIlwain, 1983]. These changes equally occur in motor and sensory nerves. When the regenerating sprouts reach a proper target organ, the nerve cell body will start to perform its old task of producing neurotransmitters and neurofilament proteins [Hoffman, Lasek, 1980] and thus resume its original size.

1.4.2 Proximal segment

A transected axon will retrogradely degenerate over a distance of one or more nodes of Ranvier due to traumatic degeneration (traumatic degeneration of the central stump [Ramon y Cajal, 1928]. Except for some slight differences, the retrograde degeneration in the proximal segment is similar to the degeneration Waller described for the distal segment [Waller, 1850]. After an axon has degenerated over one or a few nodes of Ranvier it will seal off the open distal end to preserve its "milieu interieur" [Egeraat van et al., 1993; Egeraat van, Wikswo, 1990; Egeraat van, Wikswo, 1993; Yawo, Kuno, 1985]. Shortly after this sealing-off a growth cone is formed [Ramon y Cajal, 1905; Ramon y Cajal, 1928]. This has been demonstrated in "in-vitro" experiments to occur within minutes [Bunge, 1981; Fawcett, Keynes, 1990]. A growth cone consists of the broadened distal part of the proximal segment. At the tip of this "platform" numerous elongated processes arise. These filopodia will grow out and search for a surface to which they can attach. Such a surface would most likely be the basal lamina or a Schwann cell membrane. The filopodia will then draw the growth cone towards its site of

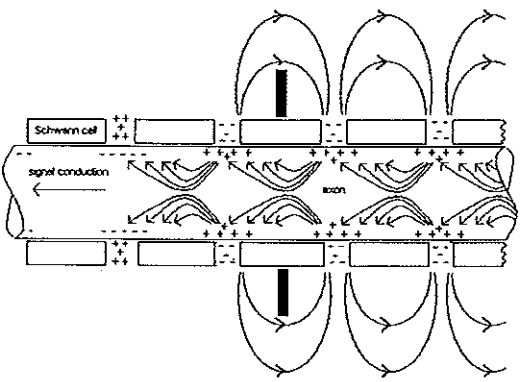

Fig. 7: A schematic view of the saltatory propagation of an action potential along a myelinated axon and its relation to the toroidal sensor

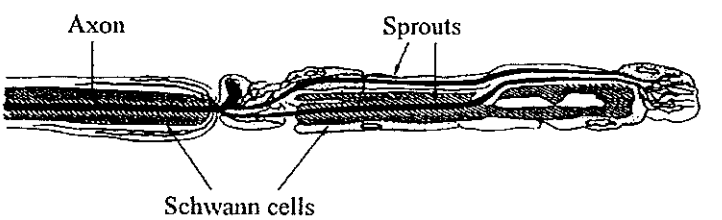

Fig. 8: Longitudinal section through a regenerating nerve.

attachment. As a result the sprout will elongate in the direction of the filopodium and will start to form a new axonal membrane [Bunge, 1981; Letourneau, 1975; Letourneau, 1978; Letourneau, 1979]). Each growth cone forms multiple filopodia. If a filopodium does not find a surface to which it can bind, it will degenerate; if more than one finds an appropriate environment they may all form separate sprouts. These sprouts will search for a pathway to the distal segment of the transected nerve through which they will try to reach a target organ (fig. 8). Such a nerve cell body with its proximal axon and all its sprouts form a regenerating neuronal unit [Mackinnon, Dellon, 1988]. If such a unit can also conduct a signal we consider it a functional neuronal unit [Kuypers et al., 1993].

1.4.3 The lesion

The regenerating sprouts grow along the remaining endoneurial tubes in the proximal segment towards the lesion. At the lesion these tubes are interrupted and a gap is formed between the proximal and distal segment. The regenerating sprouts will have to grow across this gap and are influenced by a complex of factors, like contact guidance offered by surfaces and structures in the lesion, and neurotropic factors (substances exerting an attraction, at a distance, on growing sprouts) produced by the distal segment [Lundborg, 1991]. The regenerating sprouts may demonstrate a motor-sensory specificity when regenerating across the lesion into the distal segment [Brushart, 1987; Brushart, 1988; Brushart, 1990; Brushart, 1993] due to these tropic factors [Lundborg, 1991]. Furthermore, by forming multiple sprouts which regenerate across the lesion into the distal segment, the chance of a proximal neuronal unit to reach a proper distal endoneurial tube with one of these sprouts is enhanced. The sprouts that reached an inappropriate distal endoneurial tube will later be "pruned" away [Lundborg, 1991; Mackinnon et al., 1991]. However, the regenerating sprouts will not always succeed in reaching the distal segment. A great number of sprouts will also grow into the extra-perineurial space at the lesion site. These sprouts will, therefore, not reach a proper target organ and thus will also not add to functional recovery. They will, on the contrary, form a suture line neuroma [Mackinnon et al., 1991].

1.4.4 Distal segment

After the trauma, Schwann cells and fagocytes will clear away the myelin and cell debris in the distal segment of the axon. Once the debris has been removed only the basal lamina and the Schwann cells will remain in place as endoneurial tubes which are called the "Bands of Büngner" [Mackinnon, Dellon, 1988; Waller, 1850]. These tubes, which were the pathway of the now degenerated axons, form a guide for the regenerating sprouts from the lesion to the target organs. It is essential that the regenerating sprouts find an endoneurial tube which is related to a target organ they can communicate with. If a regenerating sprout enters an endoneurial tube which used to be occupied by an axon with another function (for instance sensory instead of motor) this sprout will reach an inappropriate target organ and the neuronal unit will therefore not add to the functional recovery. Such a sprout will eventually degenerate (be pruned out) [Mackinnon et al., 1991] The endoneurial tubes remain susceptible to regenerating sprouts for relatively long periods of time. But after 6 months to a year the diameter of the tube will have decreased due to degeneration [Sunderland, Bradley, 1950a; Sunderland, Bradley, 1950b] and the basal membrane and Schwann cells will lose their capability to bind the filopodia of the regenerating sprouts [Li et al., 1997]. The capacity

for regeneration and functional recovery, therefore, will be decreased in case of a late nerve reconstruction.

1.4.5 Nerve repair

When confronted with a damaged nerve, the aim of the surgeon is to repair the nerve in such a way that a maximum number of axons regenerate through the site of injury, and that a maximal proportion of these neuronal units can grow on to the appropriate target. Causes of unsuccessful repair are twofold: failure of axons to regenerate across the lesion, and the innervation of incorrect targets. If all axons fail to cross a lesion this will result in persisting paralysis and anaesthesia. If the axons do regenerate across the lesion but reach an incorrect target organ this will lead, in the case of a motor nerve to poor muscular control, while for the sensory nerve this will result in qualitatively abnormal and poorly localised sensibility [Sunderland, 1978].

1.4.5.1 Simple approximation

If a nerve is cut cleanly, the two ends can be connected with epineurial sutures or, if necessary, perineurial or fascicular sutures while using optimised surgical techniques [de Medinaceli et al., 1983; Fawcett, Keynes, 1990]. The fixation of the two nerve ends can be performed either by use of sutures or by use of fibrin glues. In previous studies, using relatively gross tests to measure functional recovery, no significant difference between these methods has been demonstrated [Povlsel, 1994]. Presently, an experiment is undertaken to determine if fibrin glue is preferable over suturing of peripheral nerve lesions. The preliminary results seem to demonstrate a better regeneration across the lesion when glue is used instead of sutures.

1.4.5.2 Motor and sensory fibre matching

When a nerve is reconstructed, functional recovery will be influenced by the number of proximal neuronal units which regenerate into an endoneurial tube leading to a target organ with which this neuronal unit can communicate (motor nerves to motor target organs and sensory nerves to sensory organs) (chapter 1.4.4.). The functional recovery will therefore improve if the nerve ends can be sutured in such a way that the motor and the sensory fascicles are matched. For that purpose, motor and sensory fibres should be identified. This is presently performed based on three techniques. 1) *Anatomic mapping of the nerve.* An average map of motor and sensory fascicles in a certain nerve is made based on data from a population. When in a subject such a nerve is transected it is reconstructed in such a manner that, according to the average nerve map, the highest percentage of motor and sensory fibres will be coaptated [Sunderland, 1945; Watchmaker et al., 1991]. Due to the strong variation in the localisation of the motor and sensory fibres in the nerves [Sunderland, 1978] this technique does not add much to the functional recovery. 2) *Electroneurophysiological mapping of the nerve.* While the subject is in a very superficial state of anaesthesia, signals are recorded from the proximal segment with a needle electrode. In such a state the motor fibres will start to become active, probable due to pain. Their action potentials are measured and the fascicles with a high percentage of motor fibres can then be mapped. Subsequently the distal segment is stimulated with a needle electrode and muscle activity evaluated. This way the fascicles with many motor fibres are mapped and thus the coaptation of the proximal and distal segments can be optimised [Vandeput et al., 1969]. A disadvantage

of this technique is that the nerve conduction deteriorates after section of a nerve. Thus proximally, action potentials can no longer be found, while distally the threshold values for electrical stimulation increases. The time limit for this method seems to be at approximately 6 to 7 hours [Vandeput et al., 1969]. In clinical practice, patients are usually not operated within this period of time. A further disadvantage is that the localisation with this technique is still quite gross and therefore the question remains whether this technique will add much to the juxtaposition of the proximal and distal segments. 3) *Histochemical mapping of the nerve.* For this technique the motor and sensory fascicles in the distal and proximal segment of the transected nerve are determined based on the acetylcholinesterase activity, present only in the motor fibres [Deutinger et al., 1993; Yunshao, Shizhen, 1988]. Thus, the predominantly motor or sensory fascicles can be identified and coaptated. The eventual functional recovery was demonstrated to improve when peripheral nerves are reconstructed using this technique for motor/sensory differentiation [Deutinger et al., 1993]. A disadvantage of this technique is that the histological procedure takes up to one hour [Kanaya et al., 1991]. Furthermore, motor and sensory fibres are usually intermingled over most of their trajectory through the nerve trunk [Sunderland, 1945]. Therefore no predominantly motor or sensory fascicles will exist. Except just proximal to where a predominantly motor or sensory nerve branch leaves the main trunk. Thus, this method is only sufficiently reliable just proximal to such a division of the nerve.

1.4.5.3 Conduits

Often the nerve is damaged over a considerable length and if this segment is not excised it will be replaced by scar tissue which will impair nerve regeneration. After excision the gap generally is too large to suture the two ends together and a conduit is necessary to bridge the gap. Usually an autograft is used as conduit [Berger, Millesi, 1978; Millesi, 1977]. A disadvantage of the use of such autogenous nerve grafts is the donor site morbidity. Hence other techniques to cross a nerve gap are being explored. A nerve gap can for instance be bridged by freeze thawed muscle grafts. This procedure leaves an acellular tubular structure of muscle basal lamina through which axons can regenerate [Fawcett, Keynes, 1986; Glasby et al., 1988]. With this technique gaps of up to 4 cm can be crossed with a reasonable regeneration across the graft into the distal segment [Lawson, Glasby, 1995].

Several types of tubing techniques have been developed [Lundborg, 1991]. These tubes can be divided into biological and non-biological tubes. Biological tubes contain for instance mesothelial tubes [Lundborg et al., 1982], venous grafts [Chiu, Strauch, 1990] and collagen tubes [Mackinnon, Dellon, 1990]. The non-biological tubes can be divided into resorbable and non-resorbable tubes. The resorbable tubes, like for instance the polyglycolic acid tubes [Mackinnon, Dellon, 1990], have as advantage over the non-resorbable ones that they will not produce nerve entrapment syndromes. On the other hand the disintegration of the tubes can cause fibrosis or allergic reactions. Examples of the non-resorbable tubes are silicone tubes [Lundborg et al., 1994] and gore-tex tubes [Stanec, Stanec, 1998]. In general these tubes have been demonstrated to be able to help the nerves regenerate across gaps of approximately 3 cm. An increase in the gap within the tube will negatively influence the nerve regeneration [Lundborg, 1991]. It seems, therefore, that the conduits should not be viewed as an alternative for autologous nerve grafts. They rather serve to keep the proximal and distal

segment in line and retain any released neurotropic substance within the gap between the two segments. By leaving a small gap between the two nerve segments the regenerating axons may be able to find a proper distal endoneurial tube which connects to a motor or sensory target organ based on neurotropism [Brushart, 1987; Brushart, 1990; Brushart, 1993; Brushart, Seiler, 1987].

1.5 Recovery of function

Functional recovery after a peripheral nerve lesion is influenced by many factors. These factors can be divided into internal and external. A cascade of internal factors for instance starts with the number of functional neuronal units that survive in the proximal segment after a peripheral nerve transection. This number will be influenced by the distance between the nerve cell body and the axonal lesion. Of these surviving neuronal units only a portion will be able to regenerate with one or more sprouts across the lesion into the distal segment. The number will also be influenced by the quality of the reconstruction. Only a percentage of the functional neuronal units regenerating into the distal segment will reach proper target organs [Sunderland, 1978]. This percentage will theoretically be influenced by the precision of the approximation of the two nerve segments, and by the specificity of the regenerating sprouts [Brushart, 1987; Brushart, 1988; Brushart, 1993]. Functional recovery can also be influenced by external factors. Examples are the condition of the surrounding tissues, the patient's general medical condition and the capability of the brain to cope with the rigorous changes in the somatotopic organisation of the information transported to and from the brain. These changes in somatotopy are caused by the great number of *new* peripheral connections which are made between nerve cells and target organs. [Lundborg, 1993].

1.5.1 Evaluation of the nerve reconstruction

Of all factors contributing to recovery of function, the surgeon can influence only, at the present time, the quality of the nerve reconstruction. It is, therefore, important to be able to quantify the number of neuronal units regenerating across the lesion as an evaluation of the quality of the nerve reconstruction. Different methods are available for this purpose. Here the following will be described: function tests, histological techniques and neurophysiological techniques.

1.5.1.1 Function tests

The clinical function tests can be divided into three groups: sensory, motor and psychomotor tests. The sensory tests can be subdivided into two groups: threshold tests, which measure the minimal pressure or vibration that can be perceived by the patient, and spatial discrimination tests, which assess the minimal distance at which two pressure points can be sensed separately. The motor function tests evaluate the regeneration of motor neurons by measuring the power of specific muscle groups. The psychomotor tests quantify the integral hand function during everyday life.

1.5.1.1.1 Sensory function tests

Threshold tests: These tests measure the minimal stimulus to the skin, which triggers either the slow or the fast adapting pressure sensory organs. The slow adapting pressure organs can be stimulated by using the Von Frey hairs [von Frey, 1922] or Semmes-Weinstein monofilament [Semmes et al., 1960]. These tests are

based on a standardised increase in pressure applied to the skin by monofilaments with increasing diameters. The results of these tests do seem to relate to the number of reinnervated pressure organs in the skin [Dellon, 1990]

The tests for the fast adapting pressure organs measure whether a specific vibration can be sensed at a specific location on the skin. This is usually performed with the use of a tuning fork. Computerised devices have been developed to perform this test on the hand in an automated way [Lundborg et al., 1992; Lundborg et al., 1986]. The test results should theoretically relate to the number of fast adapting sensory organs which are reinnervated.

Complicating factors in the implementation of these tests are tremor of the patient or the testing person, pulsations of vessels underlying the tested skin, and the long lever arm of the testing device. These factors influence the pressure exerted on the skin, and thus blurr the measured threshold value. Moreover, they may transform an intended single stimulus into a stimulus train, mimicking a low frequency vibration [Macey, Burke, 1995]. Nevertheless, at the moment the monofilament tests are the best tests available. They do seem to be related to the number of reinnervated pressure sensory organs and are therefore used as a clinical indicator for recovery of sensory function [Macey, Burke, 1995].

Spatial discrimination tests: These tests evaluate the density of the pressure sensors by determining the minimal distance between two pressure points of which the stimuli can still be discriminated by the patient as two separate points. The static two point discrimination test has originally been described by Weber [Weber, 1835] and was later modified to a moving two point discrimination test [Dellon, 1978]. These tests are very difficult to perform properly. Asynchronous application of the pressure points and tremor may influence the outcome [Marsh, 1990]. Furthermore these tests do reflect the innervation density in the healthy digit but do not necessarily do so for the injured hand [Macey, Burke, 1995] and thus poorly predict function [Macey, Burke, 1995; Marsh, 1990].

An example of a related test in animal experiments is the pinch test. This test studies the sensory perception of the animals [Lubinska, Olekiewicz, 1959]. Such sensory tests are relatively insensitive and the results are difficult to interpret.

1.5.1.1.2 Motor function tests

These tests are designed to measure the number of reinnervated motor units in the muscles of the damaged hand. Many tests have been described; they all measure the muscle power and fatigue. The most widely used techniques measure the grip strength (Jamar dynamometer) and the pinch strength [Bechtol, 1954] and the abduction force of different fingers [Rosen, 1996]. Digital instruments to measure these muscle strengths have been developed. Examples are the "intrinsic dynamometer" and the "digital pinch and grip analyser". These instruments measure the strength, the fatigue rate and strength over time of specific muscle groups [Macey, Burke, 1995]. For the results to be a reliable indicator of function, the measuring instrument should fit the patients hand. Moreover, the patient should be well motivated, understand what to do and make a consistent and honest effort.

In animal experiments motor function is tested by use of reflex tests. An example is the toe-spread test [Lundborg, 1970]. With these tests a reflex is initiated and the resulting motor action is evaluated. As it is based on a reflex, the influence

of the brain is reduced and, as a consequence, such tests give a better estimate of peripheral motor function. A disadvantage of these tests is that they are relatively gross. However, in absence of better motor function tests they were used in our study.

1.5.1.1.3 Psychomotor tests

These tests are used to obtain a measure of the integrated hand function. Moberg developed the pick-up test [Moberg, 1985]. With this test the subject has to pick-up 10 small objects and place them in a container. The time it takes to perform this test with and without vision is measured. Thus the motor control as well as the sensory information from the hand and the degree in which the subject can combine the two are evaluated. Other tests are object and texture recognition tests [Marsh, 1990; Rosen, 1996; Rosen et al., 1994]. These tests measure the time it takes to identify small objects and textures placed in the blindfolded subjects hand. The Sollerman grip test puts the emphasis on the motor aspect of the integrated hand function [Sollerman, Ejeskar, 1995]. Other examples are the rapid pinch and release test [Jenq et al., 1997] and the Activities of Daily Living (ADL) test [Jerosch-Herold, 1993]. The psychomotor tests effectively evaluate the integrated hand function. But this function is influenced by many factors. Examples are the reinnervation density of both the motor and the sensory nerves, the amount of disruption of the somatotopy of the information transported to the brain due to new peripheral connections, and the cognitive ability of the patient [Rosen et al., 1994].

In animal experiments the integrated function is tested by use of the walking track analysis. The combination of sensory and motor function is evaluated by studying the positioning of the toes and foot while walking [Bain et al., 1989] These tests give a good indication of the integrated function but they can only be performed on rats and mice.

Neither of these function tests is sufficiently specific to quantify the number of functional neuronal units regenerating across a nerve lesion. The only test that seems to be reasonably specific for nerve regeneration is the Semmes-Weinstein monofilament test [Dellon, 1990; Macey, Burke, 1995; Rosen, 1996].

The main shortcoming of all function tests is that they only give reliable results after long regeneration times. By the time the results of these tests indicate that the regeneration across the nerve lesion is poor and a revision of the nerve reconstruction is indicated, the prognosis of such an intervention will be restricted due to atrophy of the "Bands of Büngner" [Sunderland, Bradley, 1950a; Sunderland, Bradley, 1950b] and loss of the ability off the Schwann cells to bind the filopodia of the growth cones [Li et al., 1997]. Therefore, tests evaluating early regeneration are important in terms of prognosis and indication for early reintervention.

1.5.1.2 Histological techniques

For histological techniques a section of the nerve or spinal cord is required. Therefore these techniques can not be used in a clinical setting. There is one exception, namely that at the time of a nerve reconstruction, a section from the lesion site can be taken for histological studies (see chapter 1.4.5.2).

In animal experiments the nerve regeneration across a lesion can be studied by means of histological methods. These can be divided into two types of techniques: direct staining and the labelling of retrogradely transported substances. With the direct staining techniques the number of myelinated axons proximal and distal to the lesion can be counted. By administering retrogradely transported substances to the distal segment of a reconstructed nerve and counting the number of stained cell bodies in the spinal cord or the dorsal root ganglia, the number of neuronal units which have regenerated across the lesion can be studied.

Direct staining techniques. With these techniques different organelles of the nerve cell can be stained, either in the peripheral nerve or in the central nervous system. Important techniques in this field are the staining of the myelin sheath of myelinated axons by use of osmium tetroxide in combination with toluidine blue [Lewis, Knight, 1977] or paraphenylenediamine (PPD) [Estable-Puig et al., 1965], or by means of antibodies against neurofilaments or acetylcholinesterase [Deutinger et al., 1993]. These direct staining techniques, when sufficiently robust, may give quantitative results when studying the number of axons or sprouts in the proximal and distal segment of the nerve. These methods on the other hand, have two disadvantages. The first is that they do not give information on the functional state of the axons (a segment of the nerve has to be removed from the animal before it can be studied under the microscope). The second disadvantage is that the transected axons may produce more than one regenerating sprout [Ramon y Cajal, 1890], resulting in higher axon counts than the actual number of neuronal units regenerating across the lesion. As a consequence, the number of sprouts counted histologically in the distal segment of a reconstructed nerve does not correlate to the number of functional neuronal units regenerating into that segment. Moreover, if multiple sprouts of an axon reach distal target organs this will lead to different motor units being stimulated at the same time or, when a sensory nerve cell is concerned, the cell can be stimulated at different sites of the skin [Sunderland, 1978]. This might well impair functional recovery, and in such a case a higher axon count is disadvantageous.

Retrograde labelling techniques. The nerve cell body may be labelled by substances transported retrogradely through the axon after being administered to the peripheral nerve. A frequently used tracer is horseradish peroxidase (HRP) [Brushart, 1988; Brushart, 1990; Peyronnard et al., 1988; Zachs, Saito, 1969]. The amount of HRP transported to the cell body seems to be influenced by the level of nerve activity. Therefore this method has been considered a semifunctional test. Also fluorescent substances have been developed as neuronal tracers [Bentivoglio et al., 1980; Kuypers et al., 1977]. The advantage of these tracers is that they can be combined, producing double labelled cells [Bentivoglio et al., 1980]. In this way specific reinnervation after a nerve transection and reconstruction can be studied [Brushart, 1990]. Recently neurotropic viruses have been introduced for neuronal tracing [Kuypers, Ugolini, 1990; Ugolini, 1995]. These viruses migrate retrogradely through the axon, multiply in the cell body and subsequently pass across a synapse to enter the adjacent neuron. They can therefore be used to study more complex neuronal networks.

1.5.1.3 Neurophysiological techniques

1.5.1.3.1 Electro-neurophysiological recording technique

Another way to evaluate nerve reconstructions is by use of electroneurophysiological tests. An example is the Electroneurography (ENG) which measures the extracellular nerve compound action potentials (NCAPs) from peripheral nerves. For these tests, the nerves can be stimulated and the signals recorded either transcutaneously (non-invasive) or directly from the nerve (invasive). These tests can be performed after relatively short regeneration times.

The non-invasive technique is the standard technique to study nerve regeneration but the results of these tests are strongly influenced by variations in tissue properties and geometry [Stegeman et al., 1988]. Furthermore, the relatively large distance between the nerve and the electrodes placed on the skin, results in an insufficient spatial resolution to detect short regeneration distances [Kuypers et al., 1993]. Therefore this test does not seem appropriate for short term evaluation and quantification of peripheral nerve regeneration.

For the invasive technique the electrodes are placed around the nerve; as a result the spatial resolution will increase such that nerve regeneration can be studied. Moreover the amount of tissue between the axon and the sensor will drastically decrease. This will also improve the reproducibility of the recorded signal amplitude which is very sensitive to changes in the impedance of the tissues between the axons and the sensors [Kuypers et al., 1993; Wijesinghe, Wikswo, 1991b].

To be able to evaluate the short-term regeneration across a nerve lesion, the nerve should be stimulated directly thus increasing the spatial resolution. Furthermore, since the signal amplitude of the electrically recorded nerve compound action signal is so strongly influenced by the amount of tissue between the axon and the sensor, the signal amplitudes will be poorly reproducible and can not be used for quantitative analysis. This is further demonstrated in chapter 2 [Kuypers et al., 1993].

1.5.1.3.2 Magnetoneurography

In 1980 Wikswo developed a new magnetic recording technique [Wikswo et al., 1980]. When an action potential is conducted through the axon of a nerve cell, this will result in an intracellular action current being conducted through the axon. Such an action current will produce a magnetic field outside the axon and nerve trunk. Changes in such magnetic fields will cause induction currents in a toroidal coil placed around the axon [Wikswo, van Egeraat, 1991]. From these induction currents the intracellular currents that are conducted through a stimulated axon can be derived [Roth, Wikswo, 1985].

Photo 1: A typical example of an openable and conventional toroidal sensor.

The toroidal coil sensors used for magnetoneurography (MNG) consist of a ferrite core which is enwound with insulated copper wire. [Wikswo, 1982; Wikswo, van Egeraat, 1991]. The great advantage of MNG is that magnetic fields, in contrast to electrical potentials, are not significantly influenced by biological tissues [Kuypers et al., 1993;

Wijesinghe, Wikswo, 1991b]. The magnetic signals are therefore theoratically far more reproducible than the electrical signals [Kuypers et al., 1993].

By stimulating a motor nerve with an increasing stimulus strength and measuring the twitch force of the muscle in combination with magnetically recording the compound action signal from the nerve and the muscle, it has been demonstrated that the signal amplitude is related to the number of functional neuronal units being stimulated [Gielen et al., 1991] Furthermore, since the nerves are stimulated directly, the spatial resolution of the recordings is sufficiently high to study nerve regeneration in the distal segment close to the nerve anastomosis, shortly after the reconstruction. Thus, MNG seems to be well suited to study early nerve regeneration following nerve reconstruction [Kuypers et al., 1993]. A major disadvantage of this technique is that the signal has to be recorded directly from the nerve which as a consequence makes it an invasive technique.

1.6 Goals

The first goal of this project was to produce a laboratory set-up in which early nerve regeneration can be studied after peripheral nerve lesion repair. The above mentioned MNG technique seemed to be a suitable technique for this purpose.

When studying nerve regeneration in a peripheral nerve based on MNG, the signals should be recorded from the proximal segment, because distal to a nerve lesion the fiber diameters of the regenerating sprouts, the myelin sheath thickness and inter-nodal distances vary strongly. This will result in a poorly defined Single Fiber Action Current, which can not be used for quantitative analysis. Furthermore, a regenerating neuronal unit may spawn multiple sprouts at the lesion site [Ramon y Cajal, 1890; Ramon y Cajal, 1905] and this would yield a result higher than the actual number of neuronal units. When recording the signals from the proximal segment, the amplitude will not be influenced by the number of sprouts, but only by the number of functional neuronal units which are stimulated.

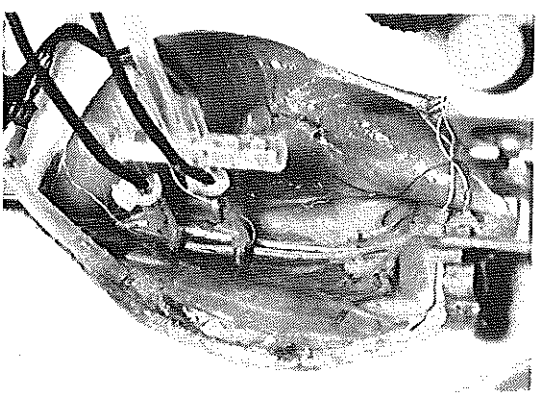

Photo 2: The setup used in the rabbit experiments.

The rabbit was chosen as animal model because of its size. The arguments for this are the following. When using the MNG, at least 35 mm of conduction distance is necessary to measure a signal that is not influenced by the stimulus artifact [Kuypers et al., 1993]. We also wanted to measure the signals at two different conduction distances, in order to be able to calculate the conduction velocity more precisely. Finally, if MNG is used the nerves have to be transected so they can be threaded through the sensors. To be sure that the "end of the stump" changes in the signals [Egeraat van et al., 1993; Egeraat van, Wikswo, 1993] do not influence the

signals, a 1 cm stretch of nerve was ensured proximal to the sensor. Thus, if a MNG signal is to be measured, without being influenced by a stimulus artifact, from a nerve when stimulated at 1 cm proximal and 1 cm distal to the lesion, at least an 8 cm length of nerve is required.
The rabbit is the smallest animal that has a nerve of such length easily available. Moreover, many histological data on rabbit peroneal nerve have been published.

1.7 Outline of the study

Chapter 2

In this rabbit model we compared the magnetoneurographic and the electroneurographic technique. Furthermore, the model was used to study the possibility of quantifying nerve regeneration shortly after peripheral nerve reconstruction. It was demonstrated that the magnetoneurographic technique was superior to the electrical technique and that it can be used to quantify nerve regeneration across a lesion shortly after the reconstruction (chapter 2, [Kuypers et al., 1993]).

Chapter 3

Based on the superiority of magnetoneurography over electroneurography, the magnetoneurographic technique was used to study the changes, which occur in the proximal segment of the transected and reconstructed peripheral nerve after 8 weeks of regeneration time. This analysis was based on a mathematical model to calculate the axon diameter histogram as described by Wijesinghe [Wijesinghe et al., 1991a; Wijesinghe, Wikswo, 1991b] (chapter 3,[Kuypers et al., 1995]).

Chapter 4

The changes, which occur in the proximal segment, are further compared to the histological data collected after 20 weeks of regeneration time from the stimulation sites, at 10 and 20 mm proximal to the lesion and at 50 mm proximal to the lesion. By using these data a hypothesis is developed to explain the loss of signal amplitude in the proximal segment of the reconstructed nerves (chapter 4, [Kuypers et al., 1995]).

Chapter 5

Finally, the decrease in signal amplitude, which occurs in the signal amplitudes measured from the proximal segment, was related to the changes in conduction velocity and to functional recovery (Kuypers et al. Submitted 1998).

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Chapter 2

A comparison of electric and magnetic compound action signals as quantitative assays of peripheral nerve regeneration.

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The evaluation of peripheral nerve regeneration is of great interest in clinical as well as in experimental situations. However, there are few techniques that give early and quantitative information on the status of the regeneration process. If quantitative assays would be available, different surgical techniques and medications could be evaluated more accurately in relation to axonal ingrowth and functional recovery. The purpose of this study was to investigate the merits of nerve compound action signals (NCASs) recorded electrically and signals recorded with a novel magnetic recording technique. We compared the two techniques in the rabbit peroneal nerve, 2, 4, 6, and 8 weeks after a nerve reconstruction. Our conclusions are that the signals recorded with the magnetic sensor are far more reproducible and less prone to stimulus artifact than the electrically recorded signals. Furthermore, the magnetic recording shows that the number of axons that have regenerated increases with time. Previously, this could only be determined with histological studies. Other ingrowth parameters that can be quantified are the average ingrowth distance, and the variation between axons in ingrowth velocity.
Key words: nerve regeneration • nerve compound action signals • quantitative analysis • electrophysiology • magnetic recording
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A COMPARISON OF ELECTRIC AND MAGNETIC COMPOUND ACTION SIGNALS AS QUANTITATIVE ASSAYS OF PERIPHERAL NERVE REGENERATION

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One of the challenges in the field of peripheral nerve regeneration is the early and quantitative evaluation of the regeneration process in terms of number of active axons in the bundle. In clinical situations, the success rate of a second nerve reconstruction may increase significantly when failing regeneration can be detected early after the initial nerve repair.[11,14] The decision to attempt a second reconstruction can be justified only if it is based on accurate, i.e., quantitative, information on the present state of the nerve. Under laboratory conditions, it is equally important to have a quantitative assay of nerve regeneration, for example, when medications or microsurgical techniques are compared. The evaluation of nerve regeneration is just an example of a situation where quantitative measurements would be desirable. Such a diagnostic tool would also be helpful, for example, to evaluate the extent of peripheral nerve trauma and/or compression or to assess the severity of peripheral neuropathies.

The existing techniques to evaluate nerve regeneration do not meet the criteria of sensitivity and/or accuracy necessary for early and quantitative evaluation. For example, functional recovery may not occur until many months after the reconstruction. Although functional recovery should remain the ultimate proof of the quality of regener-

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ation, it cannot detect regeneration in an early stage. Standard, noninvasive transcutaneous electrophysiological techniques may be useful to demonstrate nerve activity qualitatively, but a quantitative interpretation of the signals is hampered by variations in tissue properties and geometry.[16] In addition, the relatively large distance between the nerve and the electrodes is the reason that this technique has insufficient spatial resolution to detect short regeneration distances on the order of millimeters.

Therefore, we investigated two other invasive techniques that may overcome these problems. Both methods require surgical exposure of the nerve bundle. The first method is the traditional measurement of the extracellular nerve compound action *potential* with a ring electrode.[8] This method is compared with a relatively new technique of measuring the intracellular nerve compound action *current* with a magnetic sensor.[17,20] In principle, both techniques offer greatly improved sensitivity and accuracy compared to transcutaneous measurements. They can be used to measure axonal ingrowth well before functional recovery can be observed.[9,10]

MATERIALS AND METHODS

The common peroneal nerve in 26 New Zealand White rabbits, 12–14 weeks old and 2.8–3.0 kg of weight, was unilaterally transected and reconstructed in accordance with the Erasmus University guidelines for animal research. The animals were divided into four groups of 4, 7, 5, and 10 rabbits that survived 2, 4, 6, and 8 weeks, respectively.

For the recording of the signals, the following sensors and stimulation electrodes were used:

1. The magnetic toroidal sensor. The sensor consisted of a ferrite core which was wound 150 times with 50-μm diameter, insulated copper wire. This toroidal coil was insulated with silicone paint. The sensor had an inner diameter of 2.0 mm, an outer diameter of 4.8 mm, and was 1.5 mm wide.[4] The toroidal sensor measures intracellular action currents, and it has been shown that the signals are relatively independent of surrounding tissues.[15] The recordings must be done with the sensor submerged in saline.
2. The electric sensor. On the proximal side of the magnetic sensor coil, we mounted a ring of 0.5-mm diameter silver wire. This was used as a monopolar electrical sensor. The ground electrode was placed in the saline bath between the electric sensor and the stimulation electrode.
3. The stimulation electrode was composed of two 0.5-mm diameter silver wires, which were embedded in a 2.5-mm diameter epoxy semicylinder, forming a stimulator hook on which the nerve could be mounted. The two wires were placed 2.5 mm apart.

The experiment involved two operative procedures that were performed under general anesthesia for which we used a mixture of nitrous-oxide, oxygen, and enflurane gas. The first operation was performed to create a standardized nerve lesion and reconstruction, and in the second operation we measured the NCASs (nerve compound action signals) in the regenerating nerve.

In the primary operation a lateral incision was made just proximal to the knee and the common peroneal nerve was dissected and separated from the vascular bed over a distance of 20 mm. The nerve was transected with a pair of "Millesi micro scissors" (Inox FD20) at a position 15–20 mm proximal to where it inserts into the peroneus longus muscle. The nerve ends were approximated with a temporary reinforcement suture that was placed between two points at 5 mm proximal and distal to the respective cut ends of the nerve. The nerve was reconstructed with a standardized procedure using three or four epineural microsurgical 10-0 ethilon sutures as described by Millesi[13] and used by many others.[12] After the reconstruction, the skin was closed and the anesthesia was ended. The entire procedure took an average of 40 minutes. No postoperative protection was used and automutilation, wound infection, or wound dehiscention were not observed. The rabbits survived for variable times after the reconstruction operation as described above.

For the terminal (recording) surgery, we exposed the reconstructed common peroneal nerve from the sciatic notch to the knee. This was done through a lateral incision from the sacrum to a point lateral and just distal to the knee. By dividing the insertion of the biceps femoris muscle we could displace the muscle and expose the nerve over its full length. The common peroneal nerve was mobilized and then cut at a distance of approximately 7 cm proximal to the primary lesion. The cut end of the nerve was threaded through the recording sensors and stretched to its original length. The

sensors could be moved freely over the nerve from the proximal cut end to the lesion. The stimulator hook was placed near the lesion at the distal end of the nerve. With this hook the nerve could be stimulated at a position varying from 10 mm proximal to 20 mm distal to the lesion. Because the sensors and the stimulator electrodes were all mounted on XYZ micromanipulators, their positions could be determined with a 0.5-mm accuracy. We recorded with four stimulator/sensor configurations as indicated in Figure 1: B_0/A_1, B_0/A_2, B_1/A_2, and B_2/A_2.

During the recording procedure, the tissues were kept moist by creating a bath with skin flaps. The bath was continuously perfused with 0.9% NaCl solution (20 mL/min) which was maintained at a temperature of 37 ± 1°C. In this way, we had good temperature control. The saline is also required for the magnetic recording of the NCASs.[15] When measured in saline, the electrically recorded signals will decrease by 3 orders of magnitude in comparison to signals recorded with the nerve suspended in air. However, the signals recorded in saline will be more reproducible since the amplitude and shape of signals recorded in air varies greatly depending on the amount of moisture surrounding the nerve and the electrode.

The nerves were stimulated with a 50 μs, monopolar, rectangular pulse, sufficient in amplitude to stimulate all viable fibers in the bundle. The electric and magnetic signals were recorded simultaneously and in each recording session four batches of 256 consecutive NCASs were averaged. In this way we could evaluate the signal variability and eliminate artifacts in the recordings.

RESULTS

The results of this study can be divided into two topics. The first deals with a comparison between electrically and magnetically recorded NCASs in healthy nerves. We will focus on the merits of these signals for quantitative analysis, i.e., an analysis in terms of the number of active axons. The second topic deals with the actual evaluation of peripheral nerve regeneration.

Comparison between Electrically and Magnetically Recorded Signals. Figure 2 shows typical examples of magnetic (a) and electric (b) signals recorded in the configurations B_0/A_1 and B_0/A_2. The stimulator/sensor distances are indicated in the figure. Three aspects of these signals are of interest. The first aspect is the latency time, which is the main parameter traditionally extracted from electrically recorded signals. For example, the time of the signal onset is the time in which the fastest axons conduct a signal from the stimulation site to the recording site. Because the distance between the two is known, we can calculate a conduction velocity of these axons. This can be done with the magnetically as well as with the electrically recorded signals.

The second aspect of the signal that is of interest is the amplitude. Table 1 shows the average amplitude for both types of signals recorded in the

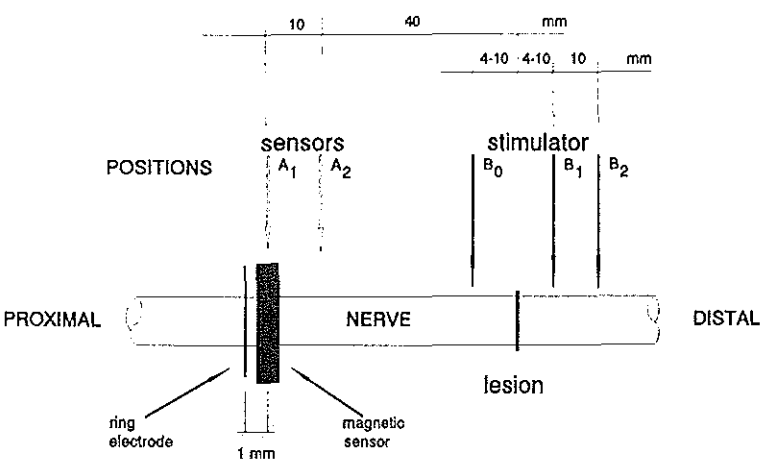

FIGURE 1. Schematic overview of the recording setup with typical locations of the recording **(A)** and stimulation **(B)** sites. The electric and magnetic sensors were mounted together at a fixed distance of 1.0 mm, with the electric sensor on the proximal side.

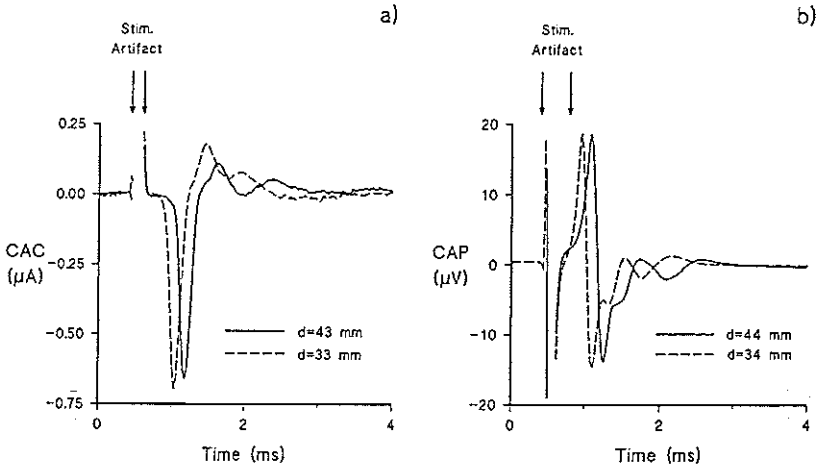

FIGURE 2. Representative examples of magnetically recorded compound action currents (CAC) **(a)**, and electrically recorded compound action potentials (CAP) **(b)** recorded from the same nerve in the B_0/A_1 and B_0/A_2 configurations. The distance, *d*, between the stimulator and recording sensors is indicated. As can be seen in Figure 1, the electric sensor was always 1 mm more proximal than the magnetic sensor.

B_0/A_1 and B_0/A_2 configurations. The relative variation in the magnetically recorded amplitude is less than in the electrical counterpart. Some variation may be expected due to differences between animals, but we expect a good correlation between the magnetic and electric signal amplitude when measured simultaneously in one animal. However, calculation of the correlation coefficient yields a low value of 0.5, demonstrating that the magnetic and electric signal amplitudes recorded in one animal are only weakly correlated. The following analysis shows that the magnetic recordings are more reproducible than the electric signals.

The magnetically recorded signals normally show a decrease in amplitude with an increasing distance between stimulator and sensor. This expected change in amplitude is the result of the differences in the conduction velocities of the axons which leads to more pronounced differences in latencies when the NCASs are conducted over a longer distance.[17] This dispersion leads to signals that are lower in amplitude and more stretched in time. The same argument should hold for the electrically recorded signals. Table 1 shows the average relative change in signal amplitude due to dispersion in percent per millimeter conduction distance for both signals. For this analysis, we used all B_0 recordings in the healthy proximal nerve segment with an average distance between the stimulus electrode and recording site of 40 mm. The average change in the magnetic signal amplitude is −1.4% per millimeter and, as expected, this value was consistently negative in all but one anomalous experiment. On the other hand, the equivalent value for the electric signals is positive (1.0% per millimeter), contrary to what is expected, and has a large variation between experiments. This is a well-known shortcoming of the electrically recorded signals, which are very sensitive to changes in the impedance of the surrounding tissues.[16] This parameter is hard to control under experimental conditions. The amplitude of the magnetic signal is determined mostly by the intracellular action current, and therefore, it reflects the number of active axons in the bundle.[18]

Table 1. Comparison of parameters derived from magnetically and electrically recorded signals in healthy nerves.

Parameter	Magnetic recording	Electric recording
Average signal amplitude	0.80 ± 0.32 μA (n = 26)	31.18 ± 18 μV (n = 19)
Average amplitude reduction due to dispersion (%/mm)	−1.4 ± 1.8 (n = 16)	1.0 ± 6.4 (n = 13)

A third aspect of interest that can be seen in Figure 2 is the duration of the stimulus artifact. In the magnetic recordings, the artifact is far shorter in time than in the electric recordings.

In conclusion, the timing parameters that can be extracted from the electric signal can also be

found from the magnetic signal. In addition, the magnetic signal has a reproducible amplitude that is virtually independent of the uncertain extracellular impedance under the given experimental circumstances.[4,15] Also, the stimulus artifact is smaller in the magnetic than in the electric signal.

The Evaluation of Nerve Regeneration. Figure 3 shows typical examples of magnetic and electric signals recorded in the B_0/A_1 and B_1/A_2 configurations (see Fig. 1). The conduction distance for both measurements was approximately equal. The solid line represents the B_0/A_1 measurement and looks similar to the signals in Figure 2a. However, the broken line was obtained with stimulation of the regenerating, distal nerve segment. There is a clear reduction of the amplitude of the magnetic signal of more than 50% and the latency of the first peak increased by 15%. Both effects were observed in all measurements. The amplitude reduction ranged from 30% to 100%, and the increase in first peak latency time varied from 0% to 30% when conduction distances were kept constant.

A 100% reduction, i.e., no signal, indicates that no excitable fibers have reached the stimulation point. Less severe reductions are due to two effects: (1) dispersion in the distal, regenerating part of the nerve is greater than in a healthy nerve; and (2) only a fraction of all proximal axons has reached the stimulation point. Dispersion does not only lead to amplitude reduction, but also to an increased width of the action signal. Comparing the two signals in Figure 3a, it is clear that the signal width has not increased greatly, demonstrating that the additional dispersion in the distal segment must be modest. Additional evidence that the dispersion effect is not the main factor causing the smaller signals comes from Table 1 which shows the average reduction in amplitude due to dispersion is 1.4% per millimeter conduction distance in a healthy nerve. If the entire amplitude reduction of 50% would be due to dispersion, this would correspond to an increase of conduction distance that is equivalent to 35 mm of healthy nerve. Especially in view of the fact that the first peak latency increased by only 15%, this seems a rather unrealistic situation. The large stimulus artifact and previously described irreproducibility of the electric signal do not allow such conclusions based on Figure 3b.

Figure 4 shows the results as recorded in configuration B_1/A_2 and B_2/A_2. Each dot represents the first peak amplitude of the magnetic signal of a recording in one animal normalized with the amplitude recorded from the same nerve when in the B_0/A_1 configuration (compare with Fig. 3). Each panel (a, b, c, and d) shows all signals recorded in one survival time group. The horizontal axis represents the distance, z, from the lesion to the distal stimulator. Due to the neuroma formed at the site of the surgically repaired lesion, the stimulator could not be moved closer than within 4 mm of the

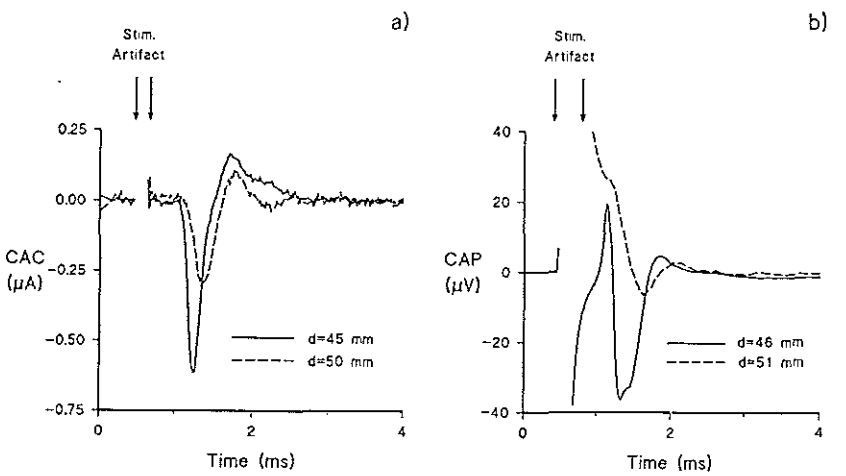

FIGURE 3. Representative examples of magnetically recorded compound action currents (CAC) **(a)**, and electrically recorded compound action potentials (CAP) **(b)** recorded from the same regenerated nerve in the B_0/A_1 (solid line) and B_1/A_2 (broken line) configurations. The distance, *d*, between the stimulator and recording sensors is indicated. The B_0 and B_1 positions were 5 mm proximal and 10 mm distal to the lesion, respectively.

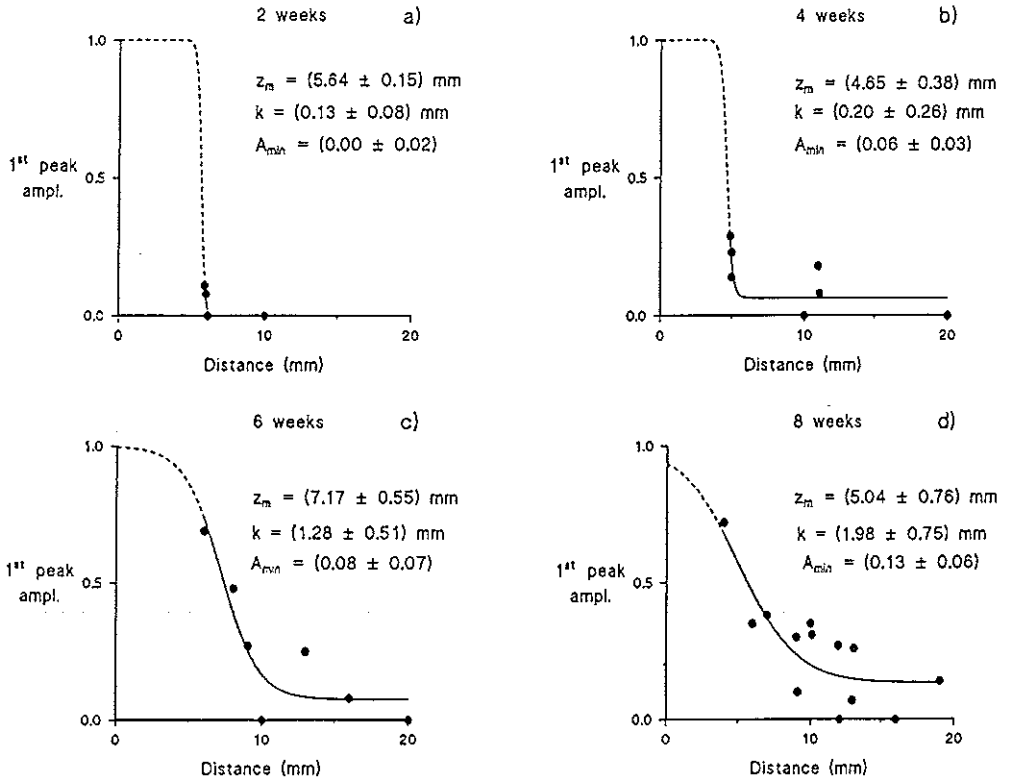

FIGURE 4. The normalized amplitude of the first peak of the magnetically measured compound action current in the regenerating common peroneal nerves of 26 rabbits as a function of the stimulus position. The lesion is at 0.0 mm. Due to neuroma, the stimulator could not be placed closer to the lesion than approximately 4 mm. Each panel gives all data recorded in one survival time group. Not all animals allowed two recordings with the stimulus distal to the lesion as indicated in Figure 1. The mean ingrowth distance, z_m, the variation of the ingrowth distance, k, and the fraction, A_{min}, of axons that have regenerated over the full 20-mm distance considered were used for the sigmoidal curves that were obtained as best fits to the data points.

lesion. The normalized amplitude of the first peak of the magnetic signal is plotted on the vertical axis. By choosing the B_0/A_1 configuration for normalization, the dispersion effect was kept minimal because the conduction distances are approximately equal for the B_1/A_2 and the B_0/A_1 configurations (see Fig. 1). A small error in the normalized amplitude, estimated at 15%, had to be accepted for the amplitudes recorded in the B_2/A_2 configuration (compare with Fig. 2).

To compare the results from the different survival time groups a curve was fitted to these data. This curve can be viewed as the fraction of all proximal axons that has grown a specific distance into the distal segment of the reconstructed nerve. In order to allow a comparison of the data, the equation describing the curve should contain a minimum number of parameters that are expected to vary with the survival time. We chose the following parameters: (a) the average distance that the axons have regenerated into the distal segment of the nerve; (b) the variation in ingrowth distance between axons, reflecting the fact that not all nerves grow with the same velocity; and (c) the number of axons that have regenerated over the full length of the nerve considered (20 mm).

Furthermore, the curve should have the property that the number of ingrown axons decreases with the distance distal from the lesion, independent of the choice of parameters. Since the number of axons distal from the lesion can be no more than a fraction of the number of axons on the proximal side, the curve should approach unity at the lesion. A curve that reflects all these properties

is the standard sigmoidal curve. The parameters in the equation for this curve are A, which represents the fraction of all axons proximal to the lesion that has grown into the distal segment of the reconstructed nerve. The parameter z_m represents the average regeneration distance of the axons, k represents the variation in the regeneration distance between axons, and $A_{\min}$ is a measure of the number of axons that has regenerated the full 20-mm length of the nerve.

$$A = A_{\min} + \frac{1 - A_{\min}}{1 + e^{\frac{z - z_m}{k}}}.$$

When the regeneration time increases from 2 weeks (Fig. 4a) to 8 weeks (Fig. 4d), the regeneration distance, z_m, initially increases, although at 8 weeks there seems to be a setback. As expected, the spreading in regeneration distance increases with time, which makes the curves less steep. Also, the number of axons that have completely regenerated increases with time. In the electrically recorded signals, the variations in amplitude, due to the changes in impedance of the interposing tissues, are so large that diagrams like the ones produced for magnetically recorded signals did not show any significant relationship between survival time, amplitude, and distance between the lesion and the stimulation site.

DISCUSSION

The goal of this study was to compare techniques that allow quantitative studies on peripheral nerves without taking histological sections, specifically after a nerve reconstruction. For example, the healing process after a nerve lesion can be subdivided into three steps: the reconstruction, the axonal ingrowth, and the reconnection with the proper target organs. Quantitative measurements of the ingrowth could be used as an indication of the probability of functional recovery. More detailed information on the ingrowth process may prompt specific interventions that eventually lead to improved results.

Quantitative assays would also be of interest in experimental research in which one would like to determine the effect of, for example, surgical techniques, nerve-growth-enhancing compounds, or other substances on ingrowing axons in a damaged nerve. The quantitative information needed consists of a fiber distribution diagram that would give absolute numbers of fibers for a number of fiber size classes. Electrically recorded NCASs have been used for this purpose,[2,8–10] but they can only provide the number of fibers in one fiber class relative to the number of fibers in other classes.[18]

Although this study does not present fiber distributions, we have demonstrated in this article that the magnetic technique is sufficiently reproducible to allow a quantitative analysis along the same lines as presented in the literature.[19] In this article, we have tacitly assumed that the amplitude of the magnetically recorded signal is a measure of the number of fibers in the bundle. This approach is valid as a first indication of the capabilities of this method. However, more accurate results may be expected from a more detailed analysis of the signals, for example, with a 2CAS method.[1,2,17–19] Our present efforts are directed toward this goal.

The electrically recorded signals do not only have irreproducible amplitude, but may also suffer from severe stimulus artifact interference. The stimulus artifact in our electrically recorded signals is between 0.4–0.6 ms in width, while the artifact in the magnetically recorded signals is only 0.2–0.3 ms wide. In order to avoid a coincidence of the nerve signal and the stimulus artifact, the stimulator must be placed at some distance from the recording site. When the stimulus artifact becomes very wide, such as in the electric signals, this distance may become impractically large. In human nerves this effect will be even more pronounced due to the higher conduction velocities.

With respect to the evaluation of the regeneration, several observations can be made from the magnetically recorded signals in the four survival time groups. The first observation is that the number of axons that can be stimulated decreases with an increasing distance between the lesion and the stimulation position. Second, the number of axons that have regenerated completely through the distal segment considered increases with time. These aspects of the nerve regeneration are well known and have been described based on histological studies.[5–7,11]

Furthermore, our data indicates that not all axons grow with the same velocity so that the variation in ingrowth distance increases with time. This is reflected by the parameter k in the fitted curves. The decrease of the average ingrowth distance, z_m, in the 8-week survival time group, compared with the 6-week group, seems paradoxical. However, this may be evidence of a degeneration of those axons that failed to reach the proper target organs.[3] Further research is required to elucidate this point.

In this study, we used antidromic stimulation and proximal recording for several reasons. First,

we found that the immature sprouts distal to the lesion produce considerably smaller signals than the healthy segments of the axons on the proximal side. Further, the signals of the ingrowing axons are probably not very well defined, which would hamper a 2CAS analysis mentioned earlier. Also, it is well-known that one proximal axon may produce more than one sprout in the distal segment[5–7] so that the functional number of axons could be overestimated when recording distally.

So far, the literature has not provided any method to evaluate nerve regeneration quantitatively in an electrophysiological way. This experiment shows that with the magnetic recording method it is possible to evaluate the regeneration of peripheral nerves without taking sections for histological studies.

The goal of this research project is to further develop this novel recording technique. Just recently, an openable magnetic sensor has been developed which can be clipped onto the nerve. This device has made it possible to record NCASs from nerves without having to transect the nerve to place them in the sensor. The characteristics of this device and its performance in comparison with the closed sensor are presently investigated. With this technique, the possibility has been created to do longitudinal in vivo studies in a single animal. In the near future, it should be possible to obtain diameter distribution histograms with absolute numbers of fibers in each fiber size class.

While this study indicates that magnetic measurements can be used to study the effects that different medication or surgical techniques have on regeneration, similar quantitative information would be useful under a variety of other conditions. For example, it would be possible to evaluate the state of nerves that suffer from neuropathies, physical trauma, or compression. When the magnetic recording techniques presented here progress to clinical application, both the physician and patient will benefit from this new, quantitative information.

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Chapter 3

Loss of viable neuronal units in the proximal stump as possible cause for poor function recovery following nerve reconstruction's.

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Loss of Viable Neuronal Units in the Proximal Stump as Possible Cause for Poor Function Recovery Following Nerve Reconstructions

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Function recovery after nerve reconstructions is often poor. Could this be caused by a loss of viable neuronal units proximal to the nerve reconstruction? The number of neuronal units (i.e., a motor or sensory neuron, including its axon and axonal branches) in the proximal segments of reconstructed peripheral nerves were studied using a novel magnetic recording technique. In five rabbits a common peroneal nerve was transected and microsurgically reconstructed. After 8 weeks regeneration time the nerve compound action signals were recorded magnetically from the reconstructed as well as from the healthy contralateral peroneal nerve and from peroneal nerves of five unoperated control animals. The amplitudes of the recorded signals were compared and the diameter distribution histograms were calculated. These calculations were based on the conduction distance between the stimulator and the sensor and the conduction velocities of 30 different axon diameter classes ranging from 3 to 18 μm. Our results indicate that there is a reduction of approximately 50% in the number of viable neuronal units at 10 mm proximal to a simple nerve reconstruction after 8 weeks regeneration time. The number of neuronal units innervating a hand is strongly correlated with clinical function in a healthy hand. The reduction in viable neuronal units after a reconstruction, demonstrated in our experiments, corresponds with a frequently clinically observed decrease in function after nerve reconstructions. Therefore, we suggest that the number of viable neuronal units may be a good indicator of final functional recovery. © 1995 Academic Press, Inc.

INTRODUCTION

From clinical practice it is well known that function recovery after nerve transection and reconstruction is often far from perfect.

Sensory hand function is usually evaluated with the two-point discrimination test (4, 13). This test shows a strong correlation between the number of functional tactile units and sensory hand function (7, 12). A functional tactile unit can be defined as a sensory ganglion cell, its axon, the related sensory organs, and the specific surface of skin which, indifferent of where it is stimulated, will send a signal through this axon to the cell body.

The quality of hand motor function depends on the accuracy with which certain motor tasks can be performed. The fine control of muscular contractions is related to the number of functional motor units per muscle (3). A functional motor unit can be defined as a motor nerve cell, its axon and axonal branches, and all muscle fibers innervated by this cell.

It thus can be concluded that a larger number of functional (motor or tactile) units innervating the hand will result in a two-point discrimination with a shorter interpoint distance and a better motor control. This will result in improved hand function.

A neuronal unit can be defined as a motor or sensory neuron, including its axon and all axonal branches (like for instance in a regenerating functional unit). When a neuronal unit is regenerating it may spawn multiple sprouts (2). If more than one sprout from one neuronal unit connects with different target organs, the neuronal cell/target organ ratio will decrease. This will result in an increase in the size of the functional motor or tactile unit. As a result the motor control will decrease and the two-point discrimination will deteriorate, neither of which is of benefit to hand function recovery. It can be concluded that the ultimate recovery of hand function is determined by the number of nerve cells functionally connected to the target organs rather than by the sheer number of ingrowing sprouts. The number of regenerating neuronal units in the proximal as well as the distal segment of reconstructed peripheral nerves will therefore be of paramount importance for the quality of the function recovery.

The goal of our experiment was to show that the number of viable neuronal units in the proximal segment of a peripheral nerve decreases significantly after a simple transection and reconstruction. This is of interest as they theoretically seem to be well related to function recovery. For this purpose a new quantitative magnetic recording technique was used to measure the

nerve compound action signals (NCASs), as described previously (8, 17).

MATERIALS AND METHODS

The common peroneal nerve of five New Zealand White rabbits, 12–14 weeks old and 3–4 kg in weight, was transected and microsurgically reconstructed. After 8 weeks of regeneration time, the reconstructed nerve was again mobilized and a stimulator was placed at 10 mm proximal to the lesion. The NCASs were magnetically recorded at a distance of approximately 40 mm proximal to the stimulator. The amplitudes of these signals were compared with those of signals recorded from the contralateral nerves and from control nerves. The NCASs from the reconstructed and the contralateral nerves were also analyzed to determine the number of active fibers and their diameter distribution.

Surgery. All the operations were performed under general inhalation anesthesia for which a mixture of nitrous-oxide, oxygen, and ethrane gas was used. Analgesia was produced with intravenous Fentanyl. All procedures where performed in accordance with the Erasmus University guidelines for animal research. After the recording procedure the animals were euthenized with a high dose of intravenous potassium.

The transections and reconstructions were performed following a standardized protocol. The nerve was transected at approximately 15 mm proximal to where it enters the long peroneal muscle and microsurgically reconstructed using four or five perineurial (10-0 ethilon) sutures. After the reconstruction the skin was closed and an intramuscular injection of antibiotics was given.

Recording. For the recording procedure the nerve was again approached through a lateral incision now running from the sciatic notch to the tibial tuberosity. The insertion of the biceps femoris muscle was divided and the muscle displaced, thereby exposing the nerve. Two markers were placed 70 mm apart on the nerve before the nerve was dissected. The nerve was transected and a ligature placed as far proximal as possible to accommodate the threading through a toroidal sensor with which the NCASs were recorded magnetically (5, 18). The nerve was stretched in such a way that the distance between the two markers was the same as before the dissection. A bipolar stimulator was placed at a distance of 10 mm proximal to the lesion, while the toroidal sensor was placed at approximately 40 mm proximal to the stimulator (see Fig. 1).

During the recording procedure the nerves were kept moist by creating a bath with the skin flaps. The bath was continuously perfused with 0.9% NaCl solution (20 ml/min) and maintained at 37 ± 1°C.

The signals recorded from the reconstructed nerves were compared with signals recorded from healthy common peroneal nerves. For this purpose we measured

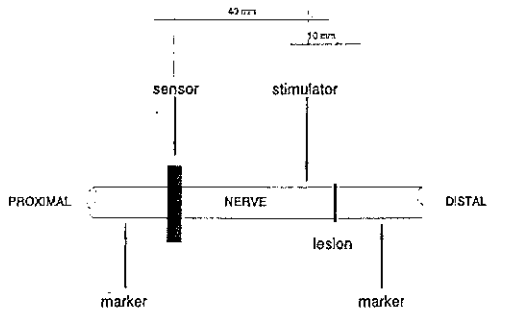

FIG. 1. Schematic overview of the recording set-up in reconstructed nerves. The nerves were stimulated at 10 mm proximal to the lesion and the magnetic signals recorded at approximately 30 mm proximal to the stimulation site.

the signals from the contralateral peroneal nerve in the operated animals and in addition in five healthy control animals. In these animals the nerves were approached in the same way as described for the recording procedure of the reconstructed nerves. The nerves were stimulated distally and the signals recorded approximately 40 mm proximal to the stimulation site. The location of the stimulation site on the healthy peroneal nerve does not influence the number of axons stimulated and therefore the amplitude of the recorded signal, since the peroneal nerve does not have any branches in the thigh. The nerves were stimulated with a monopolar rectangular current pulse, sufficient in amplitude to stimulate all viable fibers in the bundle. This was found to be three times the threshold value. The signals were averaged by recording them in four batches of 256 consecutive NCASs. In this way the signals could be evaluated and artifacts could be eliminated from the recordings.

To investigate the changes in signal amplitude we calculated the diameter distribution histograms (DDH) from the recorded signals by using a method described by Wijesinghe *et al.* (14–16). This method of calculation is based on the conduction distance, the conduction velocity of 30 fiber diameter classes, and the dispersion of the recorded signals.

RESULTS

The amplitudes of the magnetically recorded NCASs relate to the number of active fibers. Therefore, the amplitudes of the magnetically recorded NCASs can be used to analyze the number of viable neuronal units in a nerve bundle (8, 16).

In the five animals with a reconstructed nerve the signals were also recorded from the "healthy" contralateral nerve. The amplitudes of the two recorded signals were compared per animal. In this group the mean decrease in NCAS amplitude on the reconstructed side

was 60% compared to the amplitudes recorded from the contralateral side. These results are statistically significant using the paired Student t test (Table 1; $P < 0.005$).

Furthermore, we compared the mean amplitude of the signals recorded from the reconstructed nerves with the mean amplitude of the signals recorded from nerves in the control group. The reconstructed nerves averaged a 55% lower NCAS amplitude than the control nerves, and this was also statistically significant according to the Wilcoxon test (Table 1; $P < 0.001$).

We analyzed all signals recorded from the reconstructed and the healthy contralateral nerves using the method described by Wijesinghe *et al.* (14–16). The averaged DDH is shown in Fig. 2. There is a significant decrease in the number of axons throughout the diameter classes in the reconstructed nerves compared to the healthy nerves and the total number of axons has decreased by 50%.

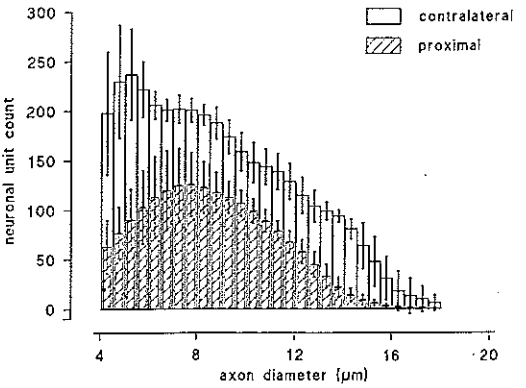

FIG. 2. Comparison of the average axonal diameter distribution in the reconstructed "dark bars" and contralateral "white bars" peroneal nerves of five rabbits, calculated according to action signal analysis (Wijesinghe *et al.* (14–16)). The error bars indicate the standard deviation between animals.

DISCUSSION

The goal of this experiment was to study changes in the number of viable neuronal units in the proximal segment of peripheral nerves after a simple transection and reconstruction. The results show a significant decrease in the NCAS amplitude and therefore in the number of stimulated axons proximal to the lesion. This decrease was found to be 60% after 8 weeks of regeneration time.

It could be argued that, if retrograde degeneration had occurred over a considerable distance proximal to the stimulation site, immature sprouts would have grown from the proximal axon into the nerve. These sprouts have a smaller diameter and may be partially unmyelinated (2) and therefore have a lower conduction velocity. This would lead to a higher degree of dispersion, which could explain the decrease in signal amplitude found in our recordings. In order to investigate this point, we compared the latency times of the onset of the NCASs in the healthy and reconstructed nerves. This comparison can be used to estimate whether degeneration has occurred over a considerable distance proximal to the stimulator, i.e., whether there is a large conduction distance that has a lower-than-normal conduction velocity. It appeared that no substantial increase in latency time occurred in the regenerating bundles compared to the healthy ones, and therefore, the conduction distance through immature axons is zero or small compared to the total conduction distance. This is in agreement with the notion that the axons in the proximal stump of a reconstructed nerve either retrogradely degenerate completely or degenerate over just a short distance proximal to the trauma (2, 9).

Analysis of the NCASs according to the method described by Wijesinghe *et al.* (14–16) produce a diameter distribution histogram. The calculations are based on the conduction distance, the conduction velocity of the different axon diameter classes, and the dispersion of the recorded signals. The calculations done on our recordings show that axons from all diameter classes are reduced in number with similar percentages. Therefore, all axon diameter classes seem equally sensitive to functional axonal loss after transection (see Fig. 2).

The number of nerve cells in the dorsal root ganglion have been shown to decrease after peripheral nerve transection with 7 to 30% (1, 19). In those experiments, however, a part of the distal segment was excised and a ligature had been placed on the proximal segment to prevent regeneration. This also prevents the axon protecting nerve growth factors (NGFs), which are produced in the distal segment, to reach the axons in the proximal segment. In our experiments, on the other

TABLE 1

Statistical Comparison between Magnetically Recorded NCASs

Recordings from	n	Mean amplitude (μA)	Standard deviation	Uncertainty P
Control group	5	0.66	±0.13	<0.001
Reconstructed nerve	5	0.30	±0.03	
Contralateral nerve	5	0.76	±0.10	<0.005

Note. NCASs were recorded magnetically from the proximal segment of five reconstructed common peroneal nerves, five contralateral, healthy, common peroneal nerves, and five common peroneal nerves of unoperated control rabbits. The uncertainty P is calculated with the paired student t test when comparing the signals recorded from the proximal segment with the signals from the contralateral nerve. The Wilcoxon test was used for comparing the signals recorded from the proximal segment of a reconstructed nerve with the signals recorded from unoperated animals.

hand, the nerves were immediately reconstructed after transection, thus, allowing NGFs from the distal segment to reach the proximal axons. Moreover, Ygge (19) also showed that an increase in distance between the nerve cell body and the lesion from 25 to 47 mm will reduce the cell death from 26 to 7%. In our experiment the distance from the nerve cell body to the lesion was at average 120 mm. For these reasons one would not expect much cell and axonal death in our experimental set-up.

If cell death occurs, a decrease in number of axons counted proximal to the lesion could be expected. Studies based on counts of histologically stained myelinated axons proximal to the lesion (10, 11), often used for quantifying peripheral nerve regeneration, also show results that seem to be very different from ours. The number of axons counted at 10 mm proximal to the lesion in a reconstructed peripheral nerve of a rabbit (11) was found to have increased, during the first 7 weeks after the reconstruction, to a maximum of approximately 160% of the number of axons in a control nerve. After a 32-week regeneration period the number of axons was reduced to near normal again. Similar changes in axon counts were found in the rat (10), though they occurred in a 104 weeks time-span.

The explanation for this can be found in the fact that after an axon has degenerated to a certain point proximal to the location of the trauma it will seal the open end and form a growth cone with multiple filopodia. They will start to grow into the distal direction. When one of these filopodia finds a good receptive environment it will grow out to form a sprout which has its own growth cone. More than one sprout can develop from one growth cone and therefore from one axon (2). This will result in an increase in number of sprouts when counted distal to the site of retrograde degeneration of the proximal nerve stump. Therefore, counting of the sprouts just proximal to the lesion does not give much information about the actual change in number of neuronal units.

Since the retrograde degeneration of the axons in the proximal segment occurs over a short distance (2, 9), the sprouts will only be formed in the proximity of the lesion and the number of axons counted further proximal to the lesion should better represent the number of neuronal units. The number of axons counted at a greater distance proximal to the lesion indeed does not differ much from the number of axons counted in the contralateral nerve (6). From this we may conclude that a decrease in the number of neuronal units in a peripheral nerve after transection and immediate reconstruction at a considerable distance from the nerve cell body is small or absent.

Our recording method stimulates all sprouts. The number of sprouts, however, does not influence the amplitude of the recorded signal since the signal was recorded 50 mm proximal to the lesion and thus from the healthy part of the axon (6, 9), proximal to where the sprouts were formed. This number should be similar to the number of nerve cell bodies sending an axon into the peripheral nerve and therefore to the number of neuronal units. When analyzing our results in view of studies using histological nerve counts (6, 10, 11). The decrease in the number of viable neuronal units found with the magnetic recording technique combined with an unchanged number of histologically found axons, far proximal to the lesion, suggests that although the nerves are still alive they are not capable of conducting electrical signals and are therefore of no benefit to hand function recovery.

In summary, since the number of functional (motor and tactile) units innervating a hand seems so closely related to the hand function (7, 12). It is more important to study the number of viable neuronal units growing into a hand after a nerve reconstruction, than the number of ingrowing sprouts or the axons and nerve cell bodies surviving the transection. The new magnetic recording technique used in this study measures these viable neuronal units.

Furthermore, it seems likely that the deficit in function recovery is not only caused by loss of sprouts at the lesion or in the distal segment during the regeneration process. But much of this deficit will be due to nerve function loss in the proximal segment, which also reduces the number of neuronal units capable of functionally connecting to the distal target organs.

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Chapter 4

A magnetic evaluation of peripheral nerve regeneration I: The discrepancy between magnetic and histologic data from the proximal segment.

Muscle & Nerve 1998; 21: 739-749

ABSTRACT: Histologic techniques can quantify the number of axons in a nerve, but give no information about electrical conductibility. The number of functional myelinated neuronal units in a nerve can be quantified based on a *magnetic* recording technique. When studying reconstructed peripheral nerves a significant difference between the results found with these two techniques can be observed. A comparison was made between the long-term changes in the number of histologically and magnetoneurophysiologically measured neuronal units proximal to a nerve reconstruction. This study was performed on 6 New Zealand White rabbits, 20 weeks after the peroneal nerve had been reconstructed. The contralateral nerves were used as a control. Histologic examination demonstrates a statistically significant decrease of approximately 5% in the number of myelinated fibers. The magnetoneurophysiological results demonstrate a decrease which is estimated to be caused by the loss of approximately 50% of the functional myelinated neuronal units in the nerve. Therefore we conclude that of the initially available myelinated neuronal units, 5% degenerate completely, 45% are vital but lose their signal conducting capability, and the remaining 50% are vital and continue to conduct signals. Apparently, only this latter group of 50% of the initially available functional neuronal units appears to remain available for functional recovery. © 1998 John Wiley & Sons, Inc. *Muscle Nerve* **21:** 739–749, 1998
Key words: nerve regeneration; magnetic recording; histologic axon count; proximal segment; loss of functionality

A MAGNETIC EVALUATION OF PERIPHERAL NERVE REGENERATION: I. THE DISCREPANCY BETWEEN MAGNETIC AND HISTOLOGIC DATA FROM THE PROXIMAL SEGMENT

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Two criteria have to be met by a peripheral axon that has been transected, to restore its functional neuronal connection between the central nervous system and a peripheral target organ. The first is that the axon has to reestablish a physical connection with the target organ. To accomplish this the axons will have to regenerate to the target organ. Second, the axon will have to be able to conduct electric signals.

Histologic techniques can quantify the number of vital axons in a nerve at the time the nerve biopsy was taken. Such studies have demonstrated that the number of myelinated axons proximal to a peripheral nerve transection and reconstruction is only slightly lower than in a control nerve.[13,21,31] Also, other histologic studies have demonstrated that the number of vital sensory nerve cells in the dorsal root ganglia (DRG) only decreases 7% after a peripheral

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nerve transection performed at a significant distance from the nerve cell bodies.[40] This decrease is due to degeneration.

The number of functional myelinated neuronal units in a nerve can be estimated using a novel magnetic recording technique.[16,17,36–38] A neuronal unit is defined as a motor or sensory nerve cell body, its axon, and all sprouts growing from this axon. Such a neuronal unit is functional when it can be stimulated, and as a reaction to the stimulus will conduct a depolarization wave to the nerve cell body and to the distal end of the unit. In a previous study we demonstrated that 8 weeks after a peripheral nerve reconstruction, the number of functional myelinated neuronal units at 10 mm proximal to the nerve lesion has decreased approximately 50%.[17]

The goal of this experiment was to measure the long-term decrease in number of functional myelinated neuronal units in the proximal segment of a reconstructed peripheral nerve, using the new magnetic recording technique, and to compare this to the decrease found in the number of histologically counted myelinated axons.

MATERIALS AND METHODS

Reconstruction Operation. Six New Zealand White rabbits 12–14 weeks of age (3–4 kg in weight) were used. The common peroneal nerve was unilaterally transected, and microsurgically reconstructed. To this end a lateral incision at the thigh was made, and the nerve was partially mobilized. The nerve was transected at 15 mm proximal to where it enters the long peroneal muscle and reconstructed, tension free, with 4 or 5 × 10-0 ethilon perineurial sutures. These reconstructions were performed with the aid of an operating microscope and according to a standardized protocol. After the nerve reconstruction the wound was closed.

Recording Operation. Twenty weeks after the first operation, the reconstructed nerves were again mobilized through a similar incision, and transected as far proximal as possible. The nerve was threaded through two toroidal sensor coils, and a double metal hook served as a bipolar stimulation electrode. During the recording procedure, the tissues were kept moist by creating a bath with the skin flaps. The bath was continuously perfused with 0.9% NaCl solution (20 mL/min), which was maintained at 37 ± 0.5°C.[16]

Recording Technique. The magnetic recording technique measures the changes in magnetic fields caused by the intraaxonal electric currents which occur when a signal is propagated through a stimulated peripheral nerve.[37,38] The changes in magnetic fields caused by the stimulated nerves (nerve compound action currents or NCACs) are recorded through induction currents in the toroidal coils placed around the nerves.[10,37,38] Each coil consisted of a ferrite core, which was wound with insulated copper wire (diameter 50 μm). The coil was 4.8 mm in diameter and 1.5 mm thick.[10,37,38] Figure 1 shows typical examples of such magnetically recorded

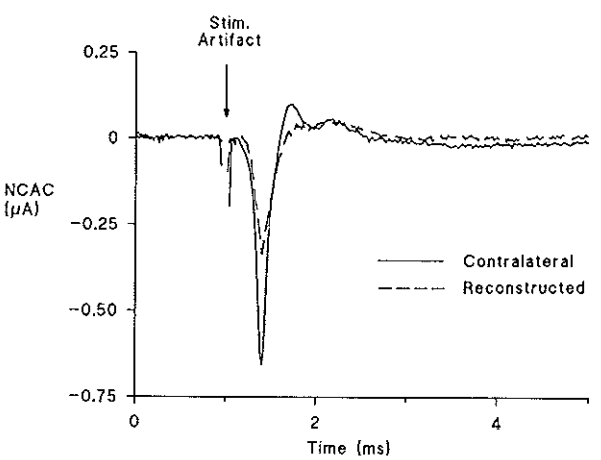

FIGURE 1. Typical examples of magnetic signals recorded from a reconstructed nerve (dashed line), and from a contralateral control nerve (solid line). No increase in first peak latency time was observed. Stim., stimulus.

NCACs measured from the healthy and reconstructed peroneal nerves of a rabbit after 20 weeks of regeneration time.

The nerves were stimulated at 10 and 20 mm proximal to the lesion (Fig. 2); 50-μs, monopolar, rectangular pulses were delivered. The pulse amplitude was supramaximal (three times the specific threshold amplitude for each nerve) in order to stimulate all viable axons in the bundle. For each measurement 1024 NCACs were averaged to increase the signal/noise ratio. The signals were simultaneously recorded at 40 and 56 ± 0.5 mm proximal to the stimulation site[16] (Fig. 2). After measuring the signals, two 5-mm segments of the nerve were taken for histologic examination at 10 and 50 mm proximal to the lesion (Fig. 2). For control purposes NCACs were recorded in the intact contralateral nerve simultaneously at 40 and 56 ± 0.5 mm proximal to the stimulation site and histologic sections were taken from the contralateral nerve, following the same procedures as on the reconstructed side.

All operations were executed under general inhalation anesthesia (O_2, NO, and enflurane). After the recording operation the animals were euthanized. All procedures were performed in accordance with the Erasmus University guidelines for animal experiments.

Histology. The segments of the nerves assigned for histologic study were immediately fixed in glutaraldehyde. After 48 h the segments were washed in phosphate buffer, postfixed with osmium tetroxide, and imbedded in Durcupan[k] ACM, Fluka. Sections 1.5 μm thick were cut and stained for myelin with paraphenylenediamine. Light microscopic photographic prints with a magnification factor of 1000 were made (Fig. 3) and assembled to display the complete section. Using these compositions all myelinated axons were counted by hand.

RESULTS

The results from this experiment can be divided into histologic and neurophysiological data. To remove the interanimal variability, the axon counts as well as the magnetic recordings from each reconstructed nerve were normalized with the data from the contralateral control nerve of the same animal.

Histologic Analysis. The number of myelinated fibers counted in the contralateral control nerves was on average 5151 (Table 1). The axon counts at 10 and at 50 mm proximal to the reconstruction site were on average 95% of the number of axons found in the contralateral nerve (Table 1). For both the

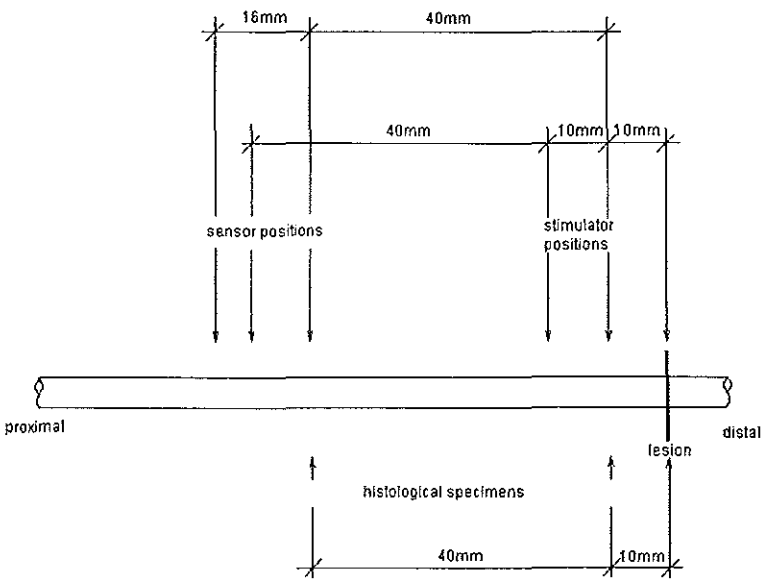

FIGURE 2. A schematic overview of the recording setup for measuring the magnetic signals from the reconstructed nerves and the locations from where the histologic sections were taken. The nerves were stimulated at 10 and 20 mm proximal to the lesion, and the magnetic signals were recorded 40 and 56 mm proximal to these stimulation sites. After recording of the signals, two sections of the nerve were taken for histologic studies. This was done at 10 and 50 mm proximal to the lesion.

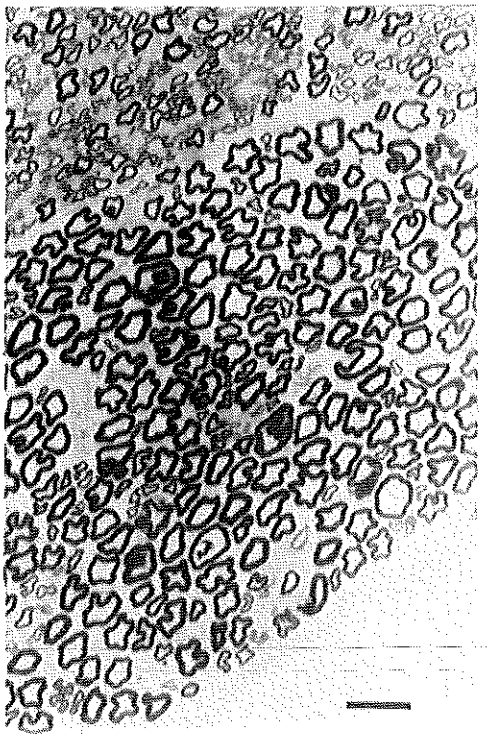

FIGURE 3. A typical example of a photographic print showing a part of a histologic section. Several such prints were made per nerve section, and assembled to display the complete histologic section. From such complete sections all myelinated axons were counted. Bar = 25 μm.

axon counts at 10 and 50 mm proximal to the lesion, the decreases were statistically significant ($P < 0.05$ using the Wilcoxon test). However, the difference between the number of axons counted at 10 and 50 mm proximal to the lesion was not statistically significant.

Therefore approximately 5% of the axons in the proximal segment of the reconstructed nerve degenerated completely. These results are compatible to the results found in the literature.[13,31,40]

Table 2. Comparison of the dispersion rate measured from the proximal segment of the reconstructed nerves and from healthy control nerves in a previous experiment.

	Reconstructed nerves	Control nerves[7]
Average amplitude reduction due to dispersion (%/mm)	−1.7 ± 0.5 (n = 6)	−1.4 ± 1.8 (n = 16)

Neurophysiological Analysis. The amplitude of the magnetic signals recorded from the control nerves at 40 mm proximal to the stimulation site was on average 0.938 μA (Table 1). The signal amplitudes recorded 40 mm proximal to the stimulation site when stimulating the reconstructed nerves at 10 and 20 mm proximal to the lesion were approximately 40% (Table 1) of the amplitudes recorded from the contralateral control nerves (Fig. 1). This decrease is statistically significant ($P < 0.005$ using the paired Student's t-test). The difference in signal amplitudes recorded when stimulating the nerve at 10 and 20 mm proximal to the lesion was not statistically significant.

For the purpose of analyzing the cause of decrease in amplitude, the dispersion rates (the decrease in amplitude per extra millimeter conducting nerve) were calculated for the operated nerves. This ratio was within the normal range as calculated from healthy control nerves (Table 2).[17] These data are discussed in the Appendix.

A third aspect of the recorded signals that is of interest, is the latency time. The first peak latency times of the signals measured from the reconstructed nerves and from the control nerves were studied. The latency times measured on the reconstructed side were on average 0.456 ± 0.028 ms and on the control side 0.429 ± 0.044 ms when recorded at 40 mm proximal to the stimulation sites. No significant increase in latency time was observed ($P < 0.05$ using the Student's t-test). Thus, it can be concluded that the average conduction velocity of the thicker axons in the reconstructed nerves has not changed significantly.

Table 1. Results of histology and magnetic recordings in the proximal segments of the six transected and reconstructed nerves, in comparison to the contralateral nerves.

	Axon counts			Amplitude		
	Control side (n)	10 mm proximal (% of control)	50 mm proximal (% of control)	Control side (μA)	10 mm proximal (% of control)	20 mm proximal (% of control)
Average	5151	94%	95%	0.938	41%	43%
±SD	±416	±5	±5	±0.175	±12	±12

DISCUSSION

Until recently nerve conduction studies were based on the recording of extracellular potentials. These signals are highly sensitive to changes in geometrical factors. The present study, however, was based on a new, quantitative recording technique which measures nerve compound action currents based on the changes in magnetic fields produced by the variations in the intraaxonal currents which occur when a nerve conducts a signal.[37,38]

Previous studies have demonstrated that this magnetic recording technique, in contrast to the electrical technique, is insensitive to sensor radius and extracellular conductivity.[10,35] In the Appendix we discuss the variables which may influence the NCAC amplitudes. Only four of them remain valid in these experiments. These are the temperature, conducting distance, number of functional neuronal units, and their diameter distribution.[17,34–36] The temperature and conducting distance varied only within a small range (±0.5°C and ±0.5 mm) in these experiments. Thus it is assumed that the signal amplitudes are only influenced by the number of fibers and their diameter distribution.

In a previous experiment, a 60% decrease in signal amplitude was observed when recording signals from the proximal segment of a reconstructed nerve were analyzed.[17] The analysis was performed by calculating the diameter, distribution histograms from the recorded signals by using the method described Wijesinghe et al.[35,36] This method of calculation is based on the conduction distance, the conduction velocity of 30 fiber diameter classes (ϕ: 4–19 μm), and the dispersion of the signals. The results demonstrate a decrease in number of functional myelinated neuronal units which was approximately equal for all axon diameter classes. Furthermore the total loss of functional neuronal units proximal to the lesion was approximately 50%.[17] In the present study as well we found a 60% decrease in signal amplitude. We therefore assume that this decrease in signal amplitude is caused by a loss of approximately 50% of the functional myelinated neuronal units proximal to the nerve lesion.

In the present study, unmyelinated axons were not taken into account. Histologic analysis was only performed on myelinated axons. The unmyelinated axons are also not considered when measuring the NCACs; due to the far smaller single fiber action current amplitude of the unmyelinated fibers, when compared to the myelinated fibers, their influence on the compound signals is too small to enable a quantitative measurement when using this technique. However, since this study was initiated by the phenomenon of poor functional recovery after peripheral nerve reconstructions, and the unmyelinated sensory fibers (class C) are slow-adapting thermo- and nociceptors,[32] variations in the number of neuronal units of this class will not significantly influence the recovery of motor and sensory function after nerve reconstruction. Similarly, variations in the number of sympathetic fibers will not influence these functions.

A 5% loss in the total number of axons was found histologically. Since it has been demonstrated by other authors[23] that the histologically acquired axon diameter distribution histograms of the proximal segment of a reconstructed nerve and that of the contralateral nerve are indistinguishable after longer regeneration times, it was concluded that the loss of axons due to degeneration is evenly distributed over the axon diameter classes.

Our results demonstrate that the number of myelinated fibers, counted histologically, proximal to the reconstruction site, decreases with a much lower percentage than the number of functional myelinated neuronal units measured magnetically. How can such a mismatch between the histologic and neurophysiological results be explained?

We are tempted to pose the hypothesis that only 5% of the neuronal units has degenerated, but that a much larger percentage has lost its electrical conductibility. To discuss this hypothesis, it is essential to review the degenerative and regenerative phenomena that can conceivably occur after transection and reconstruction of a peripheral nerve.

Phenomenon I. Some of the transected axons and their nerve cell bodies will degenerate completely (Fig. 4I). Such a degeneration will result in a decrease in the number of histologically counted axons as well as in the amplitude of the magnetically recorded signal. Furthermore, due to this degeneration the number of nerve cell bodies counted in the central nervous system will also decrease. Previous histologic experiments have demonstrated a decrease of approximately 7% in the number of nerve cell bodies in the dorsal root ganglia, after a peripheral nerve had been transected.[40] If a comparable cellular loss would occur in the motor neurons, the total number of axons, and therefore also the total number of neuronal units in the proximal segment of the reconstructed nerve, would decrease 7%. In our experiment similar decreases (5% and 6%) were found histologically, but a far greater decrease was found neurophysiologically.

Phenomenon II. After transection, the axons in the bundle that do not degenerate may seal off the open end, and start to form a growth cone (Fig. 4II).

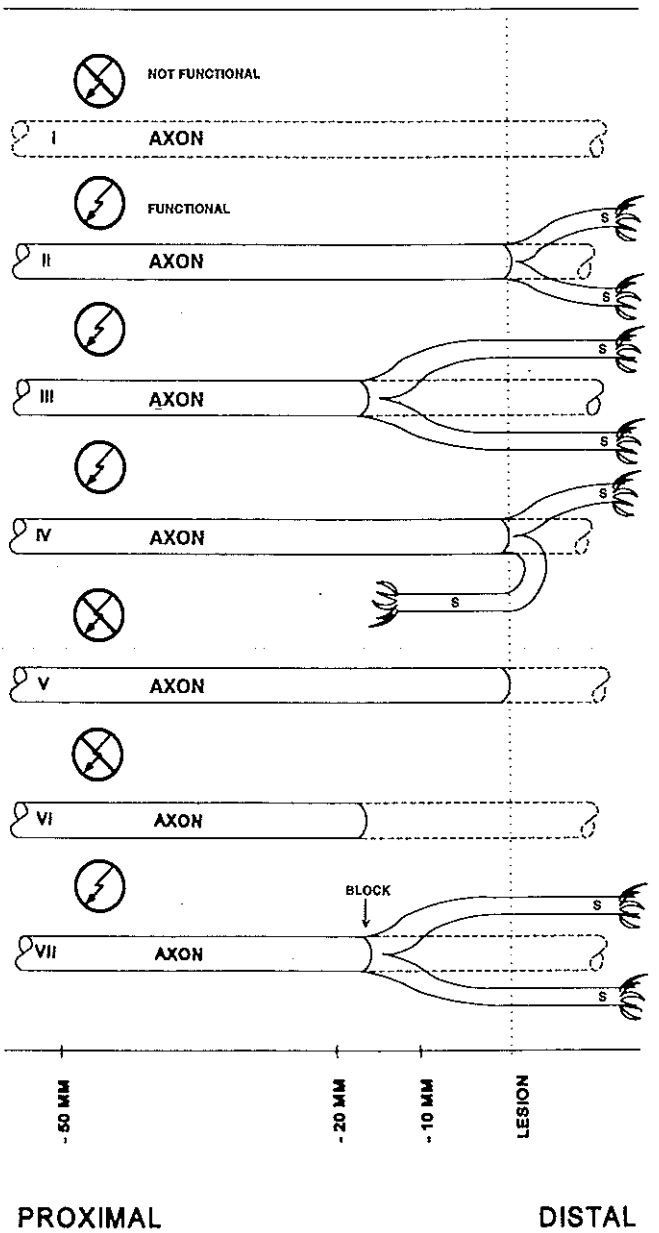

FIGURE 4. A schematic illustration of the degenerative and regenerative phenomena that could occur in a peripheral nerve after a transection and reconstruction. I, Total degeneration. II, Degeneration of the distal segment, forming of multiple anterograde sprouts (S). III, Degeneration of the proximal (partially) and distal segment, forming of multiple anterograde sprouts. IV, Degeneration of the distal segment, forming of anterograde and retrograde sprouts. V, Degeneration of the distal segment, loss of signal conducting capability; no regeneration. VI, Degeneration of the proximal (partially) and distal segment, loss of signal conducting capability; no regeneration. VII, Degeneration of the proximal (partially) and distal segment, forming of multiple anterograde sprouts, with a conduction block at the axon/sprout junction.

Each growth cone may spawn multiple sprouts.[3,4,14] This will influence the axonal counts distal to the lesion, but it will not change the histologic counts or the magnetic recordings proximal to the lesion as studied in our experiment.

Phenomenon III. Some of the axons may show retrograde degeneration before sealing off their distal end and forming a growth cone and sprouts[4,20] (Fig. 4III). This is generally accepted to occur over a distance including one or several nodes of Ranvier,[4,20] which does not exceed several millimeters. If the retrograde degeneration should occur over more than 10 mm and the sprouts grow distally again, this would result in an increase in the number of histologically counted axons at 10 mm. On the other hand it would not influence the axon counts at 50 mm proximal to the lesion. The magnetic signals would not be influenced by this phenomenon, since the total number of functional neuronal units in the nerve does not change.

Phenomenon IV. When the nerve is transected and the axons have sealed off the open end and formed a growth cone, some of the newly formed sprouts might grow retrogradely into the proximal segment of the reconstructed nerve[4,21] (Fig. 4IV). But since sprouts growing from a transected axon are attracted by neurotropic substances produced by the distal segment of the nerve and the target organs,[20,24,26,30] they will not grow very far into the proximal segment. The sprouts will soon be drawn back into the direction of the distal segment.[2] These retrogradely growing sprouts may therefore increase the histologic axon counts near the lesion, but they will not influence the magnetic signals.

Phenomenon V. Some axons may not degenerate but nevertheless lose their signal conducting capability after the transection (Fig. 4V). This phenomenon has not been previously described. It would, at any location in the nerve, decrease the number of magnetically recorded functional neuronal units but will not influence the number of histologically counted axons.

Phenomenon VI. Some axons may degenerate over a longer distance and not regenerate at all (Fig. 4VI). Such a phenomenon has also not been described previously. Our results from the magnetic recordings demonstrate that the number of *functional* neuronal units at 10 and 20 mm are equal. From this we conclude that there are no *functional* neuronal units that degenerate over a distance between 10 and 20 mm proximal to the lesion without again regenerating into the distal direction. Thus, it also seems highly unlikely that there are *functional* neuronal units that did partially degenerate over a distance larger than 20 mm, but less than 50 mm without again regenerating into the distal direction. Therefore, if this phenomenon does occur, the axons must not be able to conduct a signal. The amplitudes of the magnetic signals will be decreased throughout the proximal segment, while the histologic counts will only be influenced near the lesion. The histologic counts at 50 mm proximal to the lesion will not vary due to this form of degeneration.

Phenomenon VII. Some axons may degenerate over a longer distance proximal to the lesion (>10 mm) before forming a growth cone and sprouts as was described as phenomenon III. Of these neuronal units, some may demonstrate a conduction block at the axon/sprout junction. In such a case, a signal will be recorded when these nerves are stimulated proximal to the junction, but not when stimulated distal to the junction. The histologic axon counts will be influenced at 10 mm proximal to the lesion but not at 50 mm, as such a long retrograde degeneration does not seem likely to occur (see phenomenon III). Furthermore, no difference in amplitude was found between the signals recorded when stimulating at 10 and at 20 mm proximal to the lesion. Such a difference would have occurred if a significant number of neuronal units existed with a conduction block between 10 and 20 mm proximal to the lesion. It therefore also seems highly unlikely that a considerable number of neuronal units exist with a neuronal block further than 20 mm proximal to the lesion. If the retrograde degeneration is less than 10 mm, this phenomenon will only influence the recordings from the distal segment of the reconstructed nerves, as is discussed in the accompanying study.[18]

In our experiment the histologic study was performed at 10 and 50 mm proximal to the lesion. For the magnetic recordings a conduction distance of at least 40 mm is required between the stimulation electrode and the sensor. When stimulating the nerves at 10 and 20 mm proximal to the lesion, as we have done, the signals must be recorded at 50 and 60 mm proximal to the lesion. To stimulate the nerves at 50 mm proximal to the lesion, the signals would have to be recorded at 90 mm proximal to the lesion. This appeared to be impossible due to the anatomical configuration of the nerve.

However, as the results obtained by stimulation at 10 and 20 mm proximal to the lesion were equal, this must indicate that either no retrograde degeneration took place over more than 10 mm, or, if it occurred, the axons must have regenerated again until they reached the segment within 10 mm from the transection. In either case it is unlikely that stimulat-

ing at 50 mm would give a result different from those at 10 and 20 mm.

The number of axons counted at 10 and 50 mm proximal to the lesion are equal. Thus, an *increase* due to phenomena III, IV, and VII at 10 mm proximal to the lesion must be compensated by a *decrease* due to phenomenon VI. It seems highly unlikely that such a compensatory system could, so accurately, reproduce the 5% decrease in number of axons found at 50 mm proximal to the lesion, in each of the 6 rabbits. Furthermore, as was previously mentioned, it does not seem likely that phenomena III, IV, and VII will influence the axon counts far proximal to the lesion. From this it was concluded that phenomenon VI also does not occur in considerable numbers.

A significant decrease of 5% was observed in the number of histologic neuronal units at 10, as well as at 50 mm proximal to the lesion. From this we conclude that 5% of the initially available neuronal units had degenerated completely (phenomenon I).

Our results from the magnetic recordings demonstrate that after a nerve transection and reconstruction, 50% of the initially available myelinated neuronal units have lost their signal conducting capability, with or without total nerve degeneration (phenomenon I or V).

Combining our histologic data with the magnetic recording data we conclude that, of the approximately 50% loss of functional myelinated neuronal units found magnetically, 5% is due to total degeneration of the neuronal units (phenomenon I) and the remaining 45% is due to neuronal units that histologically survive the transection but lose their signal conducting capability (phenomenon V). This 45% loss of signal conducting capability compares well to the 40% loss of horseradish peroxidase transporting capability of histologically viable axons in the proximal segment of transected and reconstructed peripheral nerve as demonstrated by other authors.[19,23]

The remaining 50% of the initially available functional neuronal units will seal off their transected end, and might start to form distally growing sprouts at the lesion site, as was described previously as phenomenon II (Figs. 4 and 5). These appear to be the only neuronal units available for eventual recovery of function.

Clinically the recovery of function after a nerve reconstruction is often far from perfect. The existence of such a large number of the neuronal units in the proximal segment which are histologically vital but not functional initiates the hope that this loss of the signal conducting capability could be reversed, e.g., by administering specific neurotropic agents during the reconstruction. If thus the number of functional neuronal units in the proximal segment would be increased, this in turn might result in an increase in the number of functional neuronal units in the distal segment of the reconstructed nerve. Since theoretically the number of neuronal units that grow into the distal segment relates to functional recovery,[15,33] which is also supported by our accompanying study,[18] increase of the number of functional units in the proximal segment might eventually improve functional recovery.

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APPENDIX

In this Appendix the factors which may influence the amplitudes of the NCACs recorded magnetically from the proximal segment of a reconstructed peripheral nerve are discussed. This will demonstrate that the amplitudes in our experiments are mainly influenced by temperature, conduction distance, number of functional fibers, and their diameter distribution.

Morphological Changes in the Regenerating Nerve Fibers. *Intracellular Conductivity.* The single fiber action current (SFAC) amplitude approaches a linear relation to the intracellular conductivity of a single fiber.[39] The signal latency time is also related to the intracellular conductivity.

Until now no studies have been published reporting a change in intracellular conductivity in the proximal segment of a reconstructed nerve. Furthermore, since the first peak latency time in our experiment does not change significantly, we assumed that if there were variations in the intracellular conductivity, they were not of such a dimension that they significantly influenced our signal amplitudes.

Axon Diameter. The SFAC amplitude relates approximately to the square of the axon diameter.[39] Therefore, the SFACs from the thicker axons will influence the NCAC amplitude far stronger than those from the smaller axons. Thus, a variation (shift from thick to thin fibers) in the diameter distribution histogram with the same total number of fibers could result in a significant decrease in signal amplitude. On the other hand, the signal latency has an approximately linear relation to the axon diameters.[1,9,11] Therefore, loss of all the fast (thick) fibers will result in an increase in the signal latency.

Our histologic data counted after 20 weeks of regeneration time demonstrate only a 5% decrease

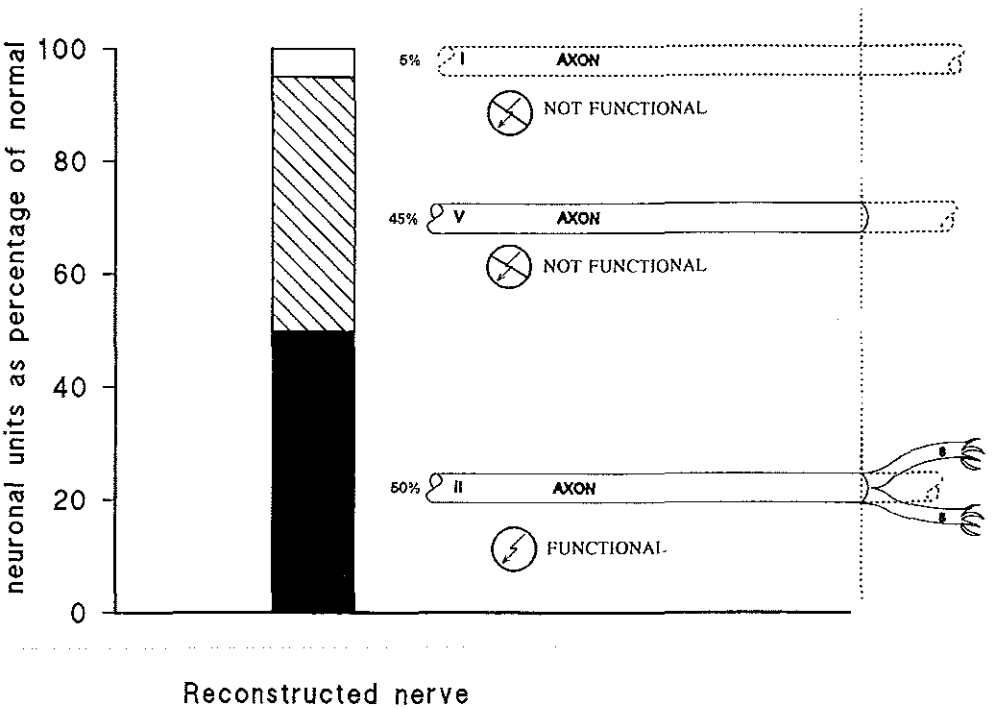

FIGURE 5. Distribution of the functional myelinated neuronal units (black), the histologically vital but not functional myelinated neuronal units (shaded), and the histologically degenerated myelinated neuronal units (white) in the proximal segments of the reconstructed nerves compared to the contralateral control nerves. S, sprout.

in the total number of myelinated axons in the proximal segment of a reconstructed nerve. Histologic data produced by other authors have demonstrated that the diameter distribution histograms from the proximal segment of reconstructed nerves are indistinguishable after longer regeneration times from those of the contralateral control nerves.[23] Furthermore, the conduction velocity in the proximal segment studied after long regeneration times, as in our experiment, was found not to have decreased.[7,11,12] Thus, it is concluded that the 5% loss of myelinated axons found histologically is equally distributed over all the axon diameter classes, and cannot explain the decrease in NCAC amplitude as found in our experiment.

Myelin Sheath Thickness. The myelin sheath thickness has a strong influence on the conduction velocity but only a minor influence on the signal amplitude.[9,11] An exception to this rule is the total conduction block caused by a total loss of myelin. This will decrease the signal amplitude to nil.

The decrease in signal amplitudes in our experiment cannot be explained by changes in the myelin sheath thickness, since it has been demonstrated that the myelin sheath thickness proximal to a nerve lesion does not change significantly.[11]

Sodium Channels. Computer simulations have demonstrated that a *decrease* in the number of sodium channels at the nodes of Ranvier will cause a significant decrease in the NCAC amplitudes.

It has been demonstrated that the ratio of sodium channels per weight of proximal nerve segment in a transected and reconstructed peripheral nerve will first *increase* and then return to normal in the first 3 weeks after the nerve reconstruction when compared to the contralateral control nerves.[8]

These changes in the number of sodium channels might initially cause an increase in signal amplitude rather than a decrease as was demonstrated in our experiment.

Nodes of Ranvier. Based on a theoretical model,[25] an increase in nodal gap will result in a decrease in axonal insulation at the nodes. This will result in a reduced signal conduction velocity. The

signal amplitude is, theoretically, far less sensitive to such a variation. Moreover, it has been demonstrated that the nodal gaps in the proximal segment do not change.[5]

Changes in Nerve Bundle Configuration. *Dispersion and Cancellation.* A decrease in signal amplitude due to *dispersion* will occur with an increasing conduction distance. This decrease is based on a more pronounced difference in latency of axons with different conduction velocities.[34] Thus the dispersion is related to the conduction distance, the conduction velocity, and therefore also to the temperature.[22,27]

A decrease in signal amplitude can also be caused by cancellation. This is the decrease in NCAC amplitude due to the positive and negative peaks from signals of different axon diameter classes cancelling each other at a specific conduction distance.

In our experiment the conduction distances in all nerves were kept equal. Furthermore, the dispersion rates (the decrease in amplitude per extra millimeter conducting nerve) were calculated for the operated nerves. This ratio was within the normal range as calculated from healthy control nerves (Table 2).[16]

Thus, neither dispersion nor cancellation is the cause of the 60% loss of signal amplitude as was measured in our experiment when comparing the operated nerve with the contralateral control nerves.

Perineurial Sheath Conductivity, Nerve Bundle Radius, and Anisotropy of the Bundle. Reliable values of these parameters are poorly known, because the influences of these parameters are difficult to measure. At present no indications for a variation of these parameters due to nerve regeneration have been reported.

Variations in Parameters of the Recording Setup. *Conductivity of the Bath Surrounding the Nerve.* The conductivity of the bath may influence the NCAC amplitudes if it approaches the conductivity of the nerve bundle.[35] In our experiments the conductivity of the bath is far smaller than that of the nerve bundle.

The Difference in Diameter of the Sensor and That of the Nerve Bundle. As long as these variables are within the same magnitude, as was the case in our experiment, the difference will not considerably influence the recorded signal amplitudes.[28,39] Furthermore, there are no indications that the bundle diameter of the proximal segment of the reconstructed nerve is much different from that of the contralateral control nerve.

Recording Temperature. The relation between the temperature and conduction velocity is approximately linear.[22,27] A decrease in conduction velocity will increase the rate of dispersion. This in turn will decrease the signal amplitude.[36] Therefore, the temperature in our experiments was kept within a small range (37 ± 0.5°C) and did not considerably influence our recordings.

Conduction Distance. An increase in conduction distance will result in an increase in the rate of dispersion.[36] This will result in a decrease in signal amplitude. Therefore in our experiment the conducting distance in all nerves was kept within a small variation range (40 ± 0.5 mm).

Based on these data we conclude that only the temperature, conduction distance, number of functional axons, and their diameter distribution will determine the NCAC amplitudes in our experiments. Since the temperature and conduction distances can be considered equal in all recordings, the number of functional axons and their diameter distribution are the only variables influencing the NCAC amplitudes. These parameters can be estimated based on the conduction distance, the conduction velocity, and the amount of dispersion.[6,29,36] In a previous experiment[17] we estimated the diameter distribution histogram based on the deconvolution technique. Preliminary results indicate a 50% decrease in the number of functional neuronal units recorded proximal to a peripheral nerve lesion.[17]

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Chapter 5

A magnetic evaluation of peripheral nerve regeneration II: The signal amplitude in the distal segment in relation to functional recovery.

Muscle & Nerve 1998;21:750-755

ABSTRACT: Motor and sensory function in a healthy nerve is strongly related to the number of neuronal units connecting to the distal target organs. In the regenerating nerve the amplitudes of magnetically recorded nerve compound action currents (NCACs) seem to relate to the number of functional neuronal units with larger diameters regenerating across the lesion. The goal of this experiment was to compare the signal amplitudes recorded from the distal segment of a reconstructed nerve to functional recovery. To this end, the peroneal nerves of 30 rabbits were unilaterally transected and reconstructed. After 6, 8, 12, 20, and 36 weeks of regeneration time the functional recovery was studied based on the toe-spread test, and the nerve regeneration based on the magnetically recorded NCACs. The results demonstrate that the signal amplitudes recorded magnetically from the reconstructed nerves increase in the first 12 weeks from 0% to 21% of the amplitudes recorded from the control nerves and from 21% to 25% in the following 23 weeks. The functional recovery increases from absent to good between the 8th and the 20th week after the reconstruction. A statistically significant relation was demonstrated between the signal amplitude and the functional recovery ($P < 0.001$). It is concluded that the magnetic recording technique can be used to evaluate the quality of a peripheral nerve reconstruction and seems to be able to predict, shortly after the reconstruction, the eventual functional recovery. © 1998 John Wiley & Sons, Inc. *Muscle Nerve* 21: 750–755, 1998
Key words: nerve regeneration; magnetic recording; distal segment; functional recovery; evaluation of nerve reconstruction

A MAGNETIC EVALUATION OF PERIPHERAL NERVE REGENERATION: II. THE SIGNAL AMPLITUDE IN THE DISTAL SEGMENT IN RELATION TO FUNCTIONAL RECOVERY

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Clinically, the quality of a peripheral nerve reconstruction is evaluated based on the functional recovery. This can be studied with the two-point discrimination test,[5,17] which measures the innervated receptor density in the skin, and by muscle contraction, which measures the reinnervation of motor end-plates by the appropriate neuronal units.[6,9] In the healthy nerve, the number of neuronal units innervating the sensory end-organs strongly relates to sensory function.[8,18] A similar relation can be demonstrated for motor function.[9]

An important drawback of functional recovery tests is that they can only be performed after long regeneration times. If a Schwann cell in the distal segment of a transected nerve is not contacted by a regenerating sprout within 6 months, it will start to lose its axon binding and guiding capability.[3] There-

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fore, once the eventual functional recovery proves to be poor this leaves only a short period of time to correct the nerve reconstruction while leaving sufficient time for the sprouts to again regenerate into the distal segment.

In the complex process of restoring the function after a nerve lesion, the only factor that, at present, can be influenced by the surgeon is the quality of the nerve reconstruction. A method to evaluate the nerve regeneration across a lesion, and thus the quality of the reconstruction, shortly after the operation is therefore desirable.

Previous experiments have demonstrated that a new quantitative magnetic recording technique can be used to estimate the number of functional neuronal units regenerating into the distal segment of a reconstructed peripheral nerve.[10]

The goal of this experiment is to demonstrate that the signal amplitudes, recorded with this magnetic recording technique, relate to the functional recovery, and that this technique can be used to evaluate the quality of the nerve reconstruction shortly after the operation.

MATERIALS AND METHODS

Reconstruction Operation. In 30 New Zealand White rabbits, 12–14 weeks old and 3–4 kg in weight, the common peroneal nerve was unilateraly transected and microsurgically reconstructed, according to a standardized protocol.[12] After the reconstruction operation the rabbits were divided into five groups which had different regeneration times: 6, 8, 12, 20, and 36 weeks, respectively. Each group contained 6 animals.

Recording Operation. After the respective regeneration times, the reconstructed nerves were again mobilized through a lateral incision of the thigh, and transected as far proximal as possible. The proximal segment of the nerve was threaded through a toroidal sensor coil, and the nerve was stimulated with a double metal hook distal to the lesion.[10–12] During the recording procedure, the tissues were kept moist by continuously perfusing them with 0.9% NaCl solution (20 mL/min), which was maintained at a 37 ± 0.5°C.[10–12]

Recording Technique. The novel recording technique measures the changes in magnetic fields caused by the variation in intraaxonal electric currents which occur when a signal is propagated through a nerve.[10,20] The nerves were stimulated at 10 mm distal to the lesion, and the pulse amplitude was supramaximal in order to stimulate all viable axons in the bundle.[10–12] For each measurement 1024 nerve compound action currents (NCACs) were averaged to improve the signal/noise ratio. The signals were recorded 40 mm proximal to the stimulation site, from the proximal segment of the nerve (Fig. 1). The NCACs were also recorded from the intact contralateral control nerve, following the same procedures as on the reconstructed side. Figure 2 demonstrates a typical example of NCACs measured from the healthy and reconstructed peroneal nerves of a rabbit after 20 weeks of regeneration time.

All operations were performed under general inhalation anesthesia. For further specifications of the recording technique see the accompanying article.[12]

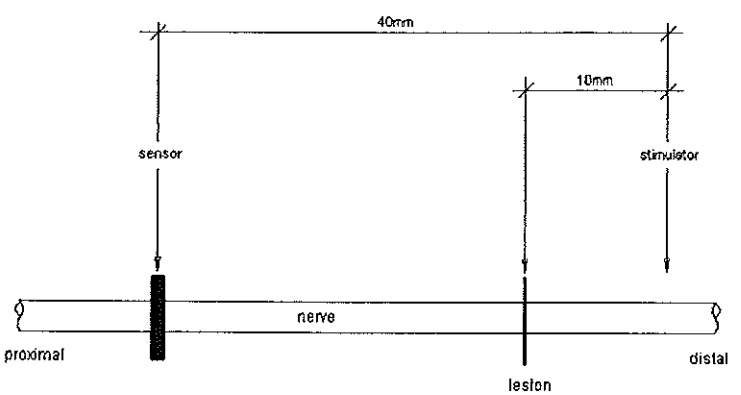

FIGURE 1. Schematic overview of the recording setup for measuring the magnetic signals from the reconstructed nerves. The nerves are stimulated at 10 mm distal to the lesion and recorded at 30 mm proximal to the lesion.

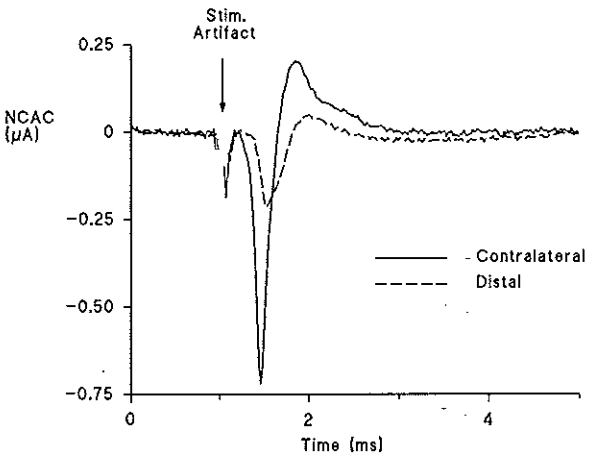

FIGURE 2. Two typical examples of magnetically recorded nerve compound action currents (NCACs) measured in a rabbit from the 20 weeks regeneration time group. Dashed line: signal recorded from the reconstructed peroneal nerve when stimulated 10 mm distal to the lesion. Solid line: signal recorded from the contralateral (control) nerve. Stim., stimulus.

Functional Recovery. The functional recovery was evaluated prior to the recording operation, by use of the toe-spread test.[17] This test evaluates the reflective dorsoflexion (DF) of the ankle and the spreading of the toes (TS) induced by "dropping" the rabbit over a distance of approximately 20 cm while holding the animal by the skin of the neck. These reflexes are predominantly controlled through the peroneal nerve.

The muscle contractions are classified per animal as: "absent" if no reaction is seen, "good" if the dorsoflexion of the ankle and the toe-spread are maximal (as in the unoperated leg), and "poor" for all results in between (Table 1).

Table 1. Neurophysiological (NCAC) and functional (toe-spread test) recovery after nerve reconstruction.

Time after reconstruction (weeks)	*n*	NCAC amplitudes (% of control ± SD)	Functional recovery (% of regeneration group)		
			Absent	Poor	Good
6	6	8 ± 4	100	0	0
8	5	15 ± 3	40	40	20
12	6	21 ± 5*	0	16.6	83.3
20	4	21 ± 5	0	0	100
36	6	25 ± 5	0	0	100

NCACs recorded from the reconstructed nerves are presented as percentage of the signals recorded from the contralateral nerves. The functional recovery was tested with the toe-spread test and is presented as percentage of the regeneration time group with absent, poor, or good function.
**P < 0.005 as compared to the 6-weeks group (Student's t-test).*

RESULTS

For each animal the top/top amplitude of the NCACs recorded from the reconstructed nerve was calculated as a percentage of the signal amplitude recorded from the contralateral control nerve. These values are averaged per regeneration time group (Table 1). Three animals were excluded from the experiment (1 from the 8-weeks and 2 from the 20-weeks regeneration time groups) due to technical disorders during the recording procedures.

The increase in signal amplitudes from 8% after 6 weeks to 21% after 12 weeks of regeneration time is statistically significant ($P < 0.005$ using the paired Student's *t*-test). In the following 24 weeks of regeneration time the signal amplitudes recorded from the reconstructed nerves did not increase significantly (Fig. 3, Table 1).

Functional recovery was studied based on the toe-spread test. First signs of recovery were seen after 6 weeks of regeneration time. A 100% "good" response to the test was first observed in the 20 weeks regeneration time group (Table 1, Fig. 4).

The signal amplitudes of the magnetically recorded NCACs in this experiment demonstrate a strong correlation ($P < 0.001$ based on a linear regression analysis) with the functional recovery measured per animal (Fig. 5).

DISCUSSION

One of the challenges in the field of peripheral nerve surgery is the early and quantitative evaluation

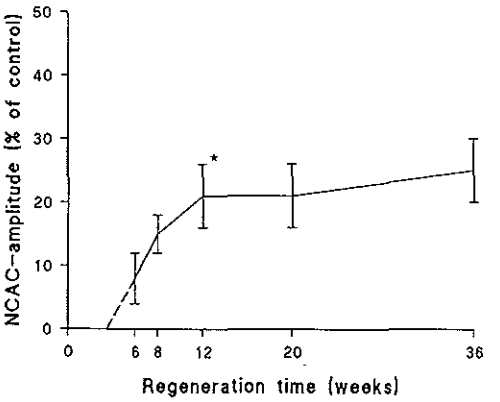

FIGURE 3. The average signal amplitude per regeneration time group, calculated as a percentage of the amplitude recorded from the contralateral (control) nerves, with standard deviation. *$P <$ 0.005 as compared to the 6-weeks group (Student's *t*-test).

of nerve regeneration across a lesion. Clinically it would be of significant interest to be able to quantify the quality of a peripheral nerve reconstruction shortly after the operation. This could be done based on the number of functional neuronal units which have regenerated across the lesion. Such quantitative measurements could be used as an indication for the probability of long-term functional recovery.[10,14]

In the laboratory setup the Sciatic Function Index (SFI) is considered the golden standard to evalu-

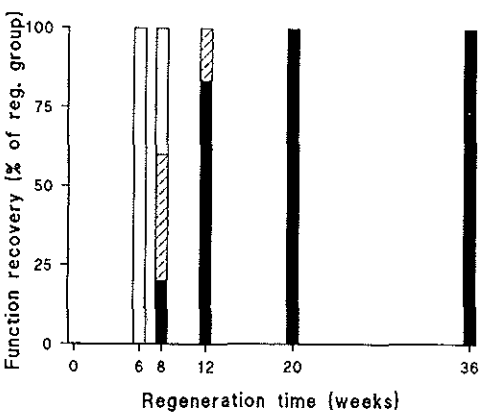

FIGURE 4. The functional recovery as tested with the toe-spread test after 6, 8, 12, 20, and 36 weeks of regeneration time and classified as absent (white), poor (hatched), or good (black) function. The functional recovery is calculated as the percentage of animals per regeneration time (reg.) group.

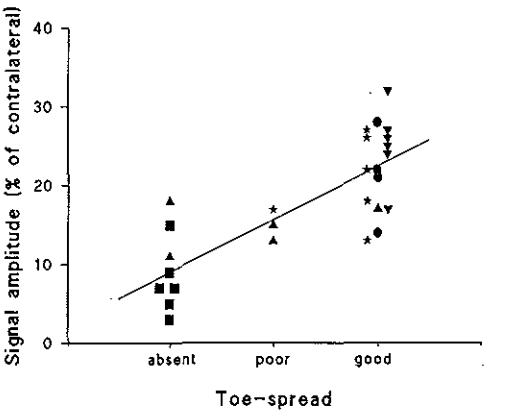

FIGURE 5. Linear regression analysis of the relation between signal amplitude and functional recovery. Each point represents the data of 1 animal. Squares: animals from the 6 weeks regeneration time group; triangles: 8-weeks group; stars: 12-weeks group; circles: 20-weeks group; inverted triangles: 36 weeks regeneration time group. $P < 0.001$.

ate the functional recovery in the rat.[9] The SFI is based on a comparison of the footprint length, the toe-spreading, and the intermediate toe-spreading of the operated and unoperated legs of the rat.[1] In the rabbit such a function test is not possible because of their mechanism of locomotion: hopping instead of walking. A functional system that however does involve the sciatic nerve in the rabbits is the toe-spread reflex. If the animal is dropped over approximately 20 cm while held by the skin of the neck, it will dorsoflex the ankle and spread the toes[17] to ensure a safe landing. This reflex is conducted predominantly through the common peroneal nerve. The spreading of the toes can be studied in a qualitative manner and classified as absent, good (i.e., as in the healthy contralateral leg), and poor for everything in between.

It is clear that the toe-spread test is less sensitive than the SFI in the rat. But since the toe-spread in the SFI represents the major weight of the index, we consider the toe-spread test a reasonable alternative to study functional recovery in the rabbit model.

Our results demonstrate that the toe-spread, absent immediately after the nerve reconstruction, will start to recover after 6 weeks of regeneration time. It will reach a maximum between 12 and 20 weeks of regeneration time (Table 1, Fig. 4). The increase in function over these 6–14 weeks can be explained by an increasing number of regenerating neuronal units reaching the target organs.[8,16]

Other authors have demonstrated that the mag-

netically recorded NCAC can provide a quantitative determination of the conduction velocity distribution (CVD) in a nerve bundle.[18–21] In a previous experiment[10] we have demonstrated that, if the amplitudes of the magnetic signals recorded from the proximal segment of a reconstructed nerve, while stimulating the nerve distal to the lesion, are assumed to be a measure of the number of neuronal units regenerating into the distal segment, this technique can detect an increasing number of neuronal units regenerating into the distal segment with an increasing regeneration time.[10]

We are currently validating a 2-NCAC method[4,18–21] to analyze the NCACs in more detail. In this method, NCACs are simultaneously recorded at two different locations in the nerve, and thus have two different conduction distances. With this method an estimation of the conduction distribution histogram of the nerve can be made based on the changes in dispersion rate found in the simultaneously recorded NCACs. At present we therefore can only assume that the signal amplitudes are a measure for the number of stimulated neuronal units regenerating into the distal segment.

The top/top amplitudes of the magnetically recorded NCACs are measured from the proximal segment of the reconstructed nerves while the distal segment is stimulated. With this technique only the functional neuronal units which have sprouts growing across the lesion into the distal segment will be stimulated and thus conduct a signal into the proximal segment of the nerve. The proximal segment of the reconstructed nerves are considered to be healthy with regard to the magnetic recordings (see Appendix in Ref. 12).

Due to the great variation in the axon and fiber diameters and the nodes of Ranvier in the regenerating sprouts distal to a lesion,[2,13] many neurophysiological parameters will also vary.[12] But only the conduction velocity (CV) or a conduction block may influence the amplitude of the proximally recorded signals. If a conduction block exists distal to the sensor, the neuronal unit, which then is not functional, will also not contribute to the NCAC. A change in the CV may result in a variation in the dispersion rate and therefore influence the signal amplitude. It has been demonstrated that the CV in the regenerating sprouts distal to a nerve transection, during the first 12 weeks after the reconstruction, is decreased but approximately equal for all fibers.[7] We therefore assume that no or nearly no dispersion occurs in the distal segment in the first 12 weeks after the reconstruction, and that the conducted signals of all regenerating sprouts will pass the lesion site delayed, but at approximately the same time. The signal dispersion will rather be caused by the differences in conduction velocities in the proximal segment. The signals recorded from the reconstructed nerves therefore have an increased first peak latency time, which is not associated with an increased dispersion rate (Fig. 2). Therefore we normalized the signal amplitudes measured from the reconstructed nerves with the amplitudes recorded from the control nerves.

The present results demonstrate that the amplitudes of the recorded signals start to increase before the sixth week after the reconstruction (Table 1, Fig. 3). In a previous experiment we have already demonstrated that the signal amplitude will start to increase between 2 and 4 weeks of regeneration time.[10] The signal amplitudes reach a maximum between 8 and 12 weeks after the reconstruction. This maximum amplitude is approximately 25% of that recorded from the contralateral nerve. After the first 12 weeks of regeneration time the signal amplitudes will not change significantly.

The gradual increase in signal amplitude between weeks 2 and 12 (Fig. 3) can be explained by an increasing number of neuronal units reaching the stimulation site at 10 mm distal to the lesion. Of interest is the similarity between the time span during which both the signal amplitudes and the functional recovery increase from nil to maximal; for the magnetic signal amplitudes it takes 6–10 weeks to reach a maximum and for the functional recovery 6–14 weeks.

When comparing the results of the function test with the data from the magnetic recordings, one may notice a delay in the onset of the recovery. This delay can be explained by the difference in regeneration distance between the stimulation site (10 mm distal to the lesion) and the intrinsic muscles of the foot (approximately 70–90 mm distal to the stimulation site). In our experiment this distance is bridged in 4 weeks (28 days). Therefore, the sprouts must regenerate with a rate of 2.50–3.21 mm/day. This rate compares well to the regeneration rate found histologically by Cajal, which was approximately 2.64 mm/day for a sprout regenerating down the distal segment of a reconstructed nerve of the rabbit.[2] This further indicates that the signal amplitude and the functional recovery are related to each other by way of the number of functional neuronal units regenerating into the distal segment.

The amplitudes of the signals recorded from the reconstructed nerves reach a maximum at 25% of those recorded from the healthy contralateral nerves. This suggests that only a small portion of the

initially available functional neuronal units will regenerate across the lesion. Clinically it is known that the functional recovery after a nerve transection and reconstruction is often far from perfect.[13] This might be explained by the restricted number of functional neuronal units regenerating across the lesion into the distal segment and thus contributing to the functional recovery.

The toe-spread test on the other hand suggests a functional recovery of 100%. This discrepancy between the signal amplitudes and the functional recovery can be explained by the fact that the toe-spread test only tests gross function. The first motor neurons regenerating into the muscles will increase the gross motor function until all motor end-plates are innervated. After that, a further increase in the number of functional neuronal units reaching the muscle will start to reduce the size of the "giant motor units" and therefore increase the fine motor function; gross motor function will not change any more. This gross function may be good while the fine motor function is still poor. In such a situation, "functional recovery" is dependent upon the type of function which is tested.

It is concluded that the signal amplitudes measured with this new magnetic recording method strongly relate to the "functional recovery" after a peripheral reconstruction, and that this technique also seems to be able to predict, shortly after the reconstruction, the eventual functional recovery. Therefore, it might be developed toward a useful technique for early evaluation of the quality of a peripheral nerve reconstruction in a clinical situation.

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Chapter 6

Changes in the compound action current amplitudes in relation to the conduction velocity and functional recovery in the reconstructed peripheral nerve.

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Changes in the Compound Action Current amplitudes in relation to the Conduction Velocity and Functional Recovery in the reconstructed peripheral nerve.

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Running title: NCAC amplitude v.s. C.V$^{1st\ peak}$ and func.recovery.

key words: *Nerve regeneration, magnetoneurography, proximal segment, functional recovery, Conduction Velocity*

Abstract:

The average axon diameter in the proximal segment of a transected and reconstructed peripheral nerve will decrease shortly after the transection and increase again when the regenerating axons make contact with their targets. The magnetically recorded nerve compound action current (NCAC) amplitude and the conduction velocity (CV) are directly related to the axon diameters. In this experiment the peroneal nerve was unilaterally transected and reconstructed in 42 rabbits. After 3, 4.5, 6, 8, 12, 24 and 36 weeks of regeneration time, hindleg motor function recovery, NCAC amplitude and $CV^{1st\ peak}$ were studied. Our results demonstrate a significant decrease in signal amplitude and CV in the first 8 weeks after the reconstruction ($P<0.05$). After 8 weeks of regeneration time, motor function and the CV of the recorded signals start to recover, but the signal amplitudes do not. Based on the correlation of the CV and the signal amplitude with the axon diameter they would both be expected to increase with recovering function. As an explanation for this lack of increase of signal amplitude we suggest that, at the same time as some axons reach their target organs and start to mature, a number of the axons which have not reached a proper target organ will loose their signal conducting capability. This will cause a decrease in compound signal amplitude, which cancels out the expected increase in NCAC amplitude due to axonal maturation.

Introduction:

Magnetoneurography (MNG) has been developed to measure Nerve Compound Action Currents (NCACs) from peripheral nerves [14-17]. The signals are highly reproducible and can be used to quantify the number of functional neuronal units i.e. an axon and all sprouts growing from it, which will conduct a signal after being stimulated [17] in a nerve [7;16;25;26;28].

Former studies concerning the evaluation of transected and reconstructed peripheral nerves in the rabbit with the MNG, have demonstrated the following:

1) Eight weeks post reconstruction the amplitudes of the signals recorded from the proximal segment had decreased with approximately 60% compared to the signals recorded from the healthy contralateral control nerves [17].
2) There was no function during the first 6 weeks after the nerve reconstructions. After 6 weeks of regeneration time the function started to recover and reached a maximal at 20 weeks of regeneration time [14].
3) The decrease in signal amplitude measured from the proximal segment of the reconstructed nerves at 8 weeks post reconstruction persisted after 20 weeks of regeneration time [15].

Thus, the signal amplitude recorded from the proximal segment did not alter during function recovery.

This is in contrast to what would be expected, since the Single Fibre Action Current (SFAC) amplitude is related to the axon diameter [29]. Histological studies have demonstrated that the average axon diameter in the proximal segment of a transected and reconstructed nerve decreases shortly after the reconstruction [3;5;10]; after long term regeneration times the axon diameters regain their normal values [5;20]. In theory such a recovery of axon diameters should also result in a recovery of signal amplitudes [29].

The goal of this experiment was to study short and long term changes in the NCAC amplitudes recorded from the proximal segment of transected and reconstructed peripheral nerves in the rabbit using MGN, and to relate these values to the Conduction Velocity (CV^{1st}

^{peak}) and the recovery of motor function in the hindleg.

Materials and methods:

Reconstruction operation:

42 New Zealand White rabbits of 12-14 weeks of age (3-4 kg body weight) were used. The common peroneal nerve was unilaterally transected, and micro-surgically reconstructed. To this end a lateral incision at the thigh was made, and the nerve was partially mobilized. The nerve was transected at 15 mm proximal to where it enters the long peroneal muscle and reconstructed, tension free, with 4 or 5 x 10-0 ethilon perineurial sutures. These reconstructions were performed with the aid of an operating microscope and according to a standardised protocol [15]. After the reconstruction the wound was closed. Following this operation the animals were divided into 7 groups of 6 animals. The survival times were 3, 4.5, 6, 8, 12, 24 and 36 weeks respectively, after which the signals were recorded from the reconstructed and the healthy contralateral nerve. In the 4.5 weeks regeneration time group the signals of one animal could not be measured due to technical disorders. The animals of the groups of 6 – 36 weeks were also used to collect data from the distal segment [14].

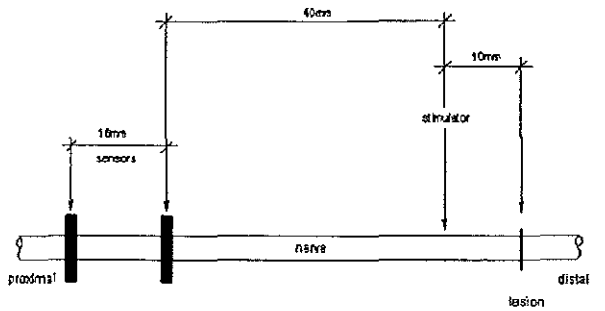

Fig. 1: A schematic overview of the recording setup. The nerves were stimulated at 10mm proximal to the lesion and recorded simultaneously at 40 and 56mm proximal to the stimulation site.

Recording operation:

After the respective regeneration times, the reconstructed nerves were again mobilised through a similar incision, and transected as far proximal as possible. The nerve was threaded through two toroidal sensor coils placed 16 mm apart. A double metal hook served as a bipolar stimulation electrode (see fig 1). During the recording procedure, the tissues were kept moist by creating a bath with the skin flaps. The bath was continuously perfused with 0.9% NaCl solution (20ml/min) which was maintained at 37 ± 0.5 °C [16].

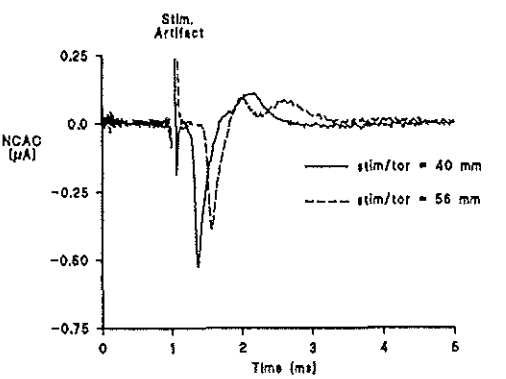

*Fig. 2:*Typical examples of NCACs measured at 40 (black line) and 56mm (dashed line) proximal to the stimulation site in the proximal segment of a reconstructed nerve after 36 weeks of reg. time.

Recording technique:

The MNG technique measures the changes in magnetic fields caused by the intra-axonal electric currents which occur

when a signal is propagated through a stimulated peripheral nerve [27;28]. The changes in magnetic fields caused by the stimulated nerves are recorded through induction currents (NCACs) in the toroidal coils placed around the nerves [8;27;28]. Each coil consisted of a ferrite core which was wound with insulated copper wire (diameter 50 μm). The coil was 4.8 mm in diameter and 1.5 mm thick [16].

The nerves were stimulated at 10 mm proximal to the lesion (fig.1); 50 μs, monophasic, rectangular pulses were delivered. The pulse amplitude was supra-maximal (three times the specific threshold amplitude for each nerve) in order to stimulate all viable axons in the bundle. A further increase in stimulus amplitude did not influence the signal amplitude. For each measurement 1024 NCACs were averaged to increase the signal/noise ratio. The signals were simultaneously recorded at 40 and 56 ± 0.5 mm proximal to the stimulation site [16] (Fig.2). For control purposes NCACs were also recorded from the intact contralateral nerve, following the same procedures as on the reconstructed side. Fig.3 shows typical examples of mag-netically recorded NCACs measured from the healthy and reconstructed peroneal nerves of a rabbit after 20 weeks of regeneration time.

All operations were executed under general inhalation anaesthesia (O_2, NO and enflurane). After the recor-ding operation the animals were euthanised. All procedures where performed in accordance with the Erasmus University guidelines for animal experiments.

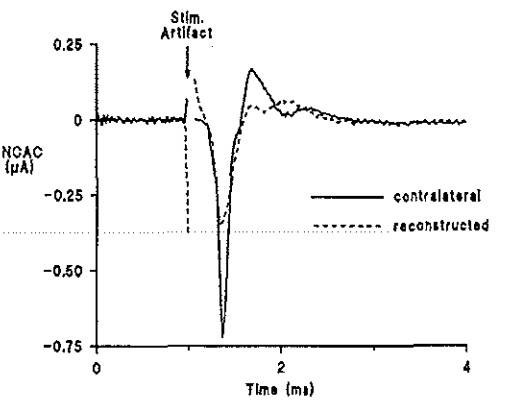

Fig. 3: Typical examples of magnetic signals recorded from a reconstructed nerve (dashed line) and the control nerve (black line) after 20 weeks of regeneration time.

Functional recovery:

Prior to the recording operation the functional recovery was evaluated by using the toe-spread test. This test evaluates the reflective dorso-flexion (DF) of the ankle and the spreading of the toes (TS) induced by dropping the rabbit over a distance of approximately 20cm while holding the animal by the skin of the neck. These reflexes are predominantly controlled through the peroneal nerve [14;24].

The functional recovery is presented as the percentage of animals in a regeneration time group in which the reflex was either absent (no reflex), good (maximal dorso-flexion of the ankle and spreading of the toes) or poor (all remaining results)[14].

Results:

For each animal the signal amplitude was measured at 40 mm proximal to the stimulation site and normalised with the amplitude measured with the same conduction distance from the contra-lateral control nerve. The conduction velocities in the reconstructed nerves were calculated from the signals measured with both sensors and normalised with the CV calculated from the contra-lateral nerves.

The signal amplitudes measured from the proximal segment of the transected nerves after 3 weeks of regeneration time were on average 74% of those measured from the control nerves (table 1, fig.4). This decrease is statistically significant ($P<0.02$,

one tailed t-test). In the 4.5 weeks group the amplitudes further decreased to an average of 62%, and subsequently to 50% and 40% after 6 and 8 weeks of regeneration time, respectively. The amplitudes did not change significantly after more prolonged periods (table 1, fig.4).

The conduction velocity of the first peak ($CV^{1st\ peak}$) of the signals measured from the proximal segment of the operated nerves was calculated by dividing the distance between the two sensors by the time difference between in the first peak latency times measured by the two sensors. The $CV^{1st\ peak}$ in the control nerves was on average 95 m/s. The $CV^{1st\ peak}$ in the operated nerves 3 weeks after the reconstruction had decreased to 88% ± 14 of the value measured from the contralateral control nerves (table 1, fig.4). The $CV^{1st\ peak}$ after 4.5 weeks had decreased to 71% ± 2. This difference of 17% between the two groups is statistically significant ($p<0.05$; two-tailed t-test). After 8 weeks of regeneration time, the $CV^{1st\ peak}$ in the reconstructed nerves had further decreased to 66% ± 9 of the value measured from the contralateral nerve. At 12 weeks the $CV^{1st\ peak}$ was again returned to control level and remained so for the following 24 weeks, until the end of the experiment (table 1). The increase between 8 and 12 weeks of regeneration time is statistically significant ($P<0.002$; two tailed t-test).

A statistically significant correlation ($p=0.05$; Spearman correlation test) was found between the signal amplitude and the $CV^{1st\ peak}$ per animal over the first 8 weeks after the reconstruction.

Regeneration time *(weeks)*	N	NCAC amplitude *(% of control + SD)*	$CV^{1st\ peak}$ *(% of control + SD)*	Func rec. *(non-poor-good)*
3	6	74 ± 15	88 ± 14	100 - 0 - 0
4.5	5	62 ± 10	71 ± 2	100 - 0 - 0
6	6	51± 16	71 ± 13	100 - 0 - 0
8	6	42 ± 4	66 ± 9	40 -40- 20
12	6	38 ± 7	102 ± 12	0 - 20- 80
24	6	45 ± 11	98 ± 10	0 - 0 - 100
36	6	44 ± 2	96 ± 16	0 - 0 - 100

Table 1: The functional recovery, conduction velocity and NCAC amplitude as measured 3, 4.5, 6, 8,12, 24 and 36 weeks after a peripheral nerve transection and reconstruction. The NCAC amplitude measured from the reconstructed nerves is presented as a percentage of the signal amplitude measured from their contralateral control nerve. The $CV^{1st\ peak}$ is given as a percentage of the $CV^{1st\ peak}$ measured from the contralateral control nerve. The data are calculated per animal and given as an average per regeneration time group. The recovery of function as tested with the toe-spread test is presented as the percentage of the regeneration time group with absent, poor and good function.

Motor function recovery as measured in our experiment was found to be absent after the transection and reconstruction. The function started to recover after 6 weeks of regeneration time and was maximal after 20 weeks (table 1, fig.4) [14]. When correlating the changes in $CV^{1st\ peak}$ with the functional recovery per animal over the period from 8 to 36 weeks after the reconstruction also a statistically significant correlation ($p<0.01$; Mann/Whitney

correlation analysis) was found.

Discussion:

The Magnetoneurografic technique (MNG) was used to further analyse the nerve regeneration in peripheral nerves after a transection and reconstruction [16;27;28]. It has been demonstrated that a positive correlation exists between the MNG signal amplitude and the number of stimulated functional neuronal units exists [7;16;17;26]. In previous experiments we have measured the signal amplitude from the proximal segment of reconstructed nerves at

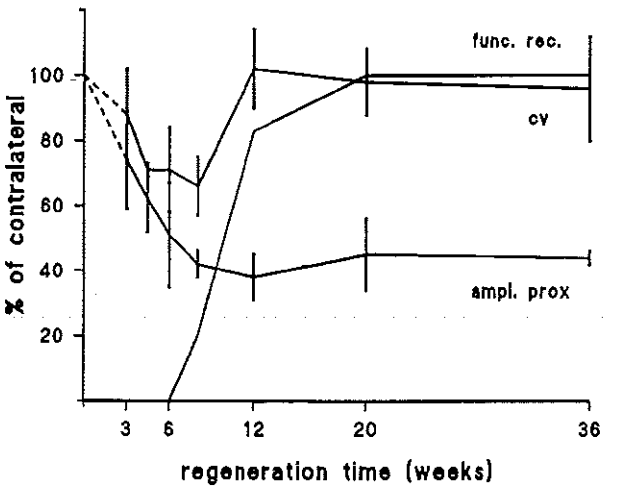

Fig.4: The functional recovery, conduction velocity and NCAC amplitude as measured at 3, 4.5, 6, 8, 12, 24 and 36 weeks after a peripheral nerve reconstruction. Functional recovery is presented as the percentage of animals in the corresponding regeneration time group that reached "good" function. Conduction velocity and signal amplitude in the reconstructed nerve are presented as percentage of the signal from the contralateral control nerve and are averaged per group.

8 and 20 weeks after the reconstruction. At 8 weeks the amplitudes had decreased to approximately half of those measured from the contralateral control nerves [17]. This decrease still persisted at 20 weeks after the reconstruction [15].

In the present experiment the signal amplitudes were studied after 3, 4.5, 6, 8, 12, 20 and 36 weeks of nerve regeneration. The amplitude measured from the proximal segment of the reconstructed nerves decreases significantly during the first 8 weeks after the reconstruction (table 1, fig.4). Also the $CV^{1st\ peak}$ decreased significantly during this period (table 1, fig.4). The reductions in $CV^{1st\ peak}$ and signal amplitude during these 8 weeks are found to be correlated (P=0.05; Spearman correlation analysis).

A reduction in conduction velocity proximal to a peripheral nerve lesion shortly after the reconstruction has also been demonstrated by other authors [3;11]. Conduction velocity is correlated to axon diameter [1]. The loss of conduction velocity shortly after the reconstruction has therefore been explained by a histologically confirmed reduction in the average axon diameter proximal to the lesion [3;4;9].

Also the amplitude of Single Fibre Action Current (SFAC) has been demonstrated to

be related to the axon diameter [29]. Therefore the decrease in axon diameter, as described for the proximal segment of the reconstructed nerves, would also be expected to result in a decrease of NCAC amplitude. Such a decrease indeed occurred in our experiment. The notion that CV and signal amplitude are related by means of their relation to axon diameter seems to be supported by the correlation between the $CV^{1st\ peak}$ and the NCAC amplitude in the first 8 weeks after the reconstruction as found in our experiment.

Regenerating axons in a transected and reconstructed peripheral nerve are known to first form thin sprouts which will regenerate into the distal segment and try to find a target organ. During this regeneration phase also the axons proximal to the lesion will reduce in diameter [3;10] Once a sprout has made contact with a proper target organ it will start to mature. An important aspect of this maturation is the increase in the diameter of the regenerated sprout [2] After long regeneration times the maturation phase will have been completed. This corresponds with the axon diameters in the proximal segment, which also are demonstrated to have also returned to normal values [3;5;20].

In our experiment the $CV^{1st\ peak}$ increases from 66% back to normal (100%) in the period from 8 to 12 weeks after the reconstruction. This change is correlated in time ($P<0.01$) to the recovery of motor function, which increases from absent to good between 6 and 20 weeks after the reconstruction (Table 1, fig. 4). It is tempting to explain this correlation in recovery of function and CV by the increase in axon diameter after the axon reaches its target and matures.

Besides recovery of function and increase in CV, at the same time an increase in the NCAC amplitude would be expected, since both amplitude and CV are related to the axon diameter [29]. However, this was not the case in our experiment. The NCAC amplitude remained at approximately 45% of the signal amplitude measured from the contralateral nerve (Table 1, Fig. 4) while the $CV^{1st\ peak}$ returns to normal.

Other authors [4] have tried to explain this lack of recovery of signal amplitude while the $CV^{1st\ peak}$ regains normal values, hypothesising that only a small number of thick axons reach their target organs and will therefore mature. This will result in an increased $CV^{1st\ peak}$ One would also expect the signal amplitude also to increase. But since the other axons, which are unsuccessful in reinnervating a distal target organ, continue to conduct the signals slowly, this will result in an increase in the dispersion of the compound signal. An increase in dispersion will cause a decrease in the compound signal amplitudes. This would compensate for the increase in signal amplitude due to the maturation. Thus, although the peak latency may recover, the signal amplitude will not [4].

This theory however does not seem a plausible explanation for the lack in recovery of the signal amplitudes as found in our experiment. The amplitude of signals measured from single nerve fibres vary approximately as the square of the CV [4;21]. A change in the axon diameters would therefore have a much higher impact on the NCAC amplitude than on the $CV^{1st\ peak}$. In our experiment one would thus expect an increase in signal amplitude even when a major increase in dispersion occurs. This was not the case. Furthermore, such an increase in dispersion would also result in an increase in duration of the first and second peak. In the signals measured in this experiment however no increase in signal duration was observed (See fig. 3).

In previous experiments, we have suggested that after 8 weeks of regeneration time, a considerable number of the neuronal units in the proximal segment of the transected and reconstructed nerves loose their signal conducting capability [14;16]. Similar conclusions have been drawn in histological studies [20]. In long-term experiments the number of peripheral

nerve cells labelled with horseradish peroxidase (HRP) after applying the dye to the proximal segment of a transected and reconstructed peripheral nerve, decreased to approximately 60% of the number found in the contralateral control nerves [20]. Based on the previous finding that the amount of HRP absorbed by peripheral nerves is influenced by the electrical activity of the nerve [18;19], this suggests a decrease in electrical activity of approximately 40% of the neuronal units in the proximal segment of a reconstructed peripheral nerve. Furthermore Cajal and others [2;6;22] already noticed a great number of so called 'steril axons' in the proximal segment of a transected and reconstructed peripheral nerve between 1 to 28 days of regeneration time. These 'steril axons' do not form functional growth cones and are therefore unable to regenerate. They become varicose and the axonoplasm contains many vacuoles. It might be that these axons also do not transport HRP and may have lost their signal conducting capability. These results support our conclusion that a great number of functional neuronal units lose their signal conducting capability while remaining histologically viable [14].

This could explain the lack of recovery of the signal amplitudes in the proximal segment after 8 weeks of regeneration time as found in our present experiment. At about this time an increasing number of neuronal units start to lose their signal conducting capability [17]. This would cause a decrease in signal amplitude. But, at the same time, other regenerating axons reach their target organs and will add to function recovery. Due to the subsequent maturation their axon diameter will increase, which in turn will result in an increase in their signal amplitude (and $CV^{1st\ peak}$). The decrease of signal amplitude due to the loss of functional neuronal units will thus be compensated for by the gain in signal amplitude due to axon maturation.

Conclusion:

The loss of signal amplitude measured from the proximal segment of a nerve shortly after transection and reconstruction, is related to the decrease in the $CV^{1st\ peak}$. These two phenomena are both caused by a decrease in axon diameter which is due to axon regeneration. The $CV^{1st\ peak}$ will regain normal values after the regenerating neuronal units have reached their target organs and start to mature. The persisting low NCAC amplitude after more than 12 weeks of regeneration time, while the $CV^{1st\ peak}$ has normalised, may be explained by a significant number of functional neuronal units losing their signal conducting capabilities. If such a loss of the conductibility could be reversed, the number of *functional* neuronal units regenerating across the lesion into the distal segment could also increase. Since theoretically the number of functional neuronal units that grow into the distal segment relates to functional recovery [12;13;23], an increase in the number of functional units in the proximal segment of a reconstructed peripheral nerve could be of clinical importance.

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7 General discussion

This thesis discusses the data collected from healthy and regenerating peripheral nerves by use of a new magnetoneurografic (MNG) technique. Two of the most challenging issues in the field of peripheral nerve surgery at the present time are how to quantitatively evaluate regeneration across a nerve lesion in an early stage, and how to improve this regeneration. Until now no satisfactory method was available to quantify peripheral nerve regeneration, either in the clinical or in a laboratory setting. At present, nerve regeneration is either studied by functional recovery tests, electroneurography (ENG) or histology. Functional recovery tests can only be used after long regeneration times and are influenced by many factors other than the regeneration across the lesion. ENG techniques are not quantitative and have a poor spatial resolution. As to histological techniques, both direct staining and retrogradely transported tracers have their disadvantages. Direct staining methods quantify the number of axons and axonal sprouts regenerating into the distal segment of a reconstructed nerve. However, as one neuronal unit might produce several sprouts, direct staining can not be applied as a quantitative method to asses the number of regenerating neuronal units. Application of retrogradely transported neuronal tracing substances in the distal segment labels the cell of origin, thus identifying not more than one stained cell body for each regenerating neuronal unit. But not all possible cell bodies will be stained, and therefore the use of retrogradely transported tracers is only a roughly quantitative method. On the other hand, the uptake and transport of these tracers is intensified by neuronal activity. Therefore this method could also be considered as a semi-functional method. The major disadvantage of histological techniques is that a section of the nerve has to be removed; therefore they can only be used for evaluation in animal experiments.

We have developed an experimental model to study nerve regeneration by use of MNG. This model uses the rabbit peroneal nerve, which is transected and reconstructed.

In this study the magnetic and electric signals measured from a healthy nerve were compared. Subsequently functional recovery was tested and MNG signals measured from the proximal, distal and contralateral control nerves after 2 to 36 weeks of regeneration time. Furthermore, the histological axon counts in the proximal segment were compared to the contralateral control nerve after 20 weeks of regeneration time. The different aspects of the collected data and their correlations will be discussed in the following pages.

Magnetic vs Electric signals

In the first experiment (chapter 2) we have demonstrated that the signals measured by use of MNG are far more reproducible then the ENG signals. The MNG signals are thus more suited then the ENG signals to be used to quantify the number of functional myelinated neuronal units in a nerve trunk (chapter 2, [Gielen et al., 1991; Kuypers et al., 1993]). The unmyelinated axons are not considered when measuring the NCACs; due to the far smaller single fiber action current amplitude when compared to the myelinated ones. Their influence on the compound signals is too small to enable a quantitative measurement when using this technique.

The MNG signals measured from the proximal, distal and contralateral nerves were used to study nerve regeneration in the following experiments.

Signal amplitude in the proximal segment

It was demonstrated that during the first 12 weeks after reconstruction the signal amplitudes measured from the proximal segment of the transected and reconstructed nerves decreased to approximately 45% of those measured from their contralateral control nerves (chapter 3 and 6, [Kuypers et al., 1998a; Kuypers et al., 1995] Kuypers et al. Submitted 1998). Furthermore, the signal amplitudes were found to remain decreased until 36 weeks after reconstruction which was the end of our experiment (chapter4 & 6, [Kuypers et al., 1998a](Kuypers et al. Submitted 1998). The decrease of approximately 55% in signal amplitude was estimated to be caused by a reduction of approximately 50% in the number of excitable neuronal units in the proximal segment of the transected and reconstructed peripheral nerve (chapter 3 & 6, [Kuypers et al., 1995](Kuypers et al. Submitted 1998)(fig.9).

Histological axon counts in the proximal segment

When comparing the histologically counted number of myelinated axons in the proximal segment of the transected and reconstructed nerve with the number counted in the contralateral control nerve after 20 weeks of regeneration time, a statistically significant decrease of only 5% was found. This was approximately the same percentage as reported in the literature (Chapter 4, [Kuypers et al., 1998a]).

Conduction Velocity in the proximal segment

Conduction velocity of the first peak ($CV^{1st\ peak}$) of the magnetically recorded signals in the proximal segment of the reconstructed nerve was compared with that in the contralateral nerve. The $CV^{1st\ peak}$ was demonstrated to decrease with approximately 35% during the first 8 weeks after the reconstruction. This decrease is statistically significant (chapter 6, Kuypers et al. Submitted 1998). Between 8 and 12 weeks after reconstruction the $CV^{1st\ peak}$ increases back to normal and remains normal till the end of the experiment fig.9).

Functional recovery

Immediately after transection and reconstruction the motor function, as studied with the toe-spread test, was absent. At 8 weeks after the reconstruction the motor function started to recover and had returned to maximal at 20 weeks of regeneration time (chapter 5[Kuypers et al., 1998b](fig.9).

Signal amplitude measured after stimulation of the distal segment

MNG signals were also measured in the proximal segment after stimulating the nerve 10 mm distal to the lesion. The signal was absent until 4 weeks after the reconstruction operation. Subsequently the signal amplitudes started to increase to reach, after 12 weeks of regeneration time, approximately 20% of those measured in the contralateral control nerves. In the following 24 weeks these amplitudes further increased only slightly to reach approximately 25% of the amplitudes measured from the control nerves (chapter 5[Kuypers et al., 1998b])(fig.9). (fig.9).

The correlations between the different data will be discussed below.

Signal amplitude vs CV $^{1st\ peak}$ in the proximal segment during the first 8 weeks after reconstruction.

In the first 8 weeks after reconstruction the signal amplitude and the $CV^{1st\ peak}$ measured after stimulation of the proximal segment decrease significantly. During this regeneration period, the decrease in signal amplitude and $CV^{1st\ peak}$ are correlated significantly (chapter 6, Kuypers et al. Submitted 1998). The most plausible explanation is that the signal amplitude and the CV are known to relate directly to the axon diameter. Furthermore, the axon diameter in the proximal segment is known to decrease during the regeneration phase. This decrease is caused by a reduction in the production of neurofilaments by the nerve cell body after nerve lesions [Hoffman, Lasek, 1980]. The decrease of signal amplitude and the CV are thus related through the axon diameter during this period of regeneration.

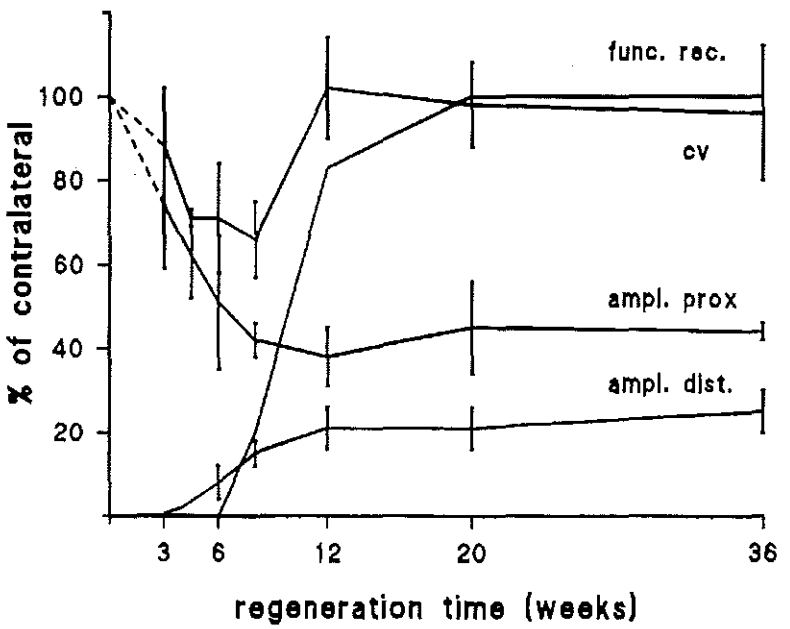

Fig.9: The functional recovery, conduction velocity and NCAC amplitude measured from the proximal segment and the NCAC amplitude measured when stimulating the distal segment of a transec-ted and reconstructed peripheral nerve. Measurements were performed after 3, 4.5, 6, 8, 12, 24 and 36 weeks after the reconstruction. Functional recovery is presented as the percentage of animals in the corresponding regeneration time group that reached "good" function. Conduction velocity and signal amplitude in the reconstructed nerve are presented as a percentage of the signal from the contralateral control nerve, averaged per group.

Functional recovery vs CV in the proximal segment after 8 weeks of regeneration

At 6 weeks after the reconstruction motor function, when measured with the toe-spread test, is absent. But from that moment on it will recover (Chapter 5 & 6, [Kuypers et al., 1998b],Kuypers et al. Submitted 1998). In this same period of regeneration time the $CV^{1st\ peak}$ will increase from 65 to 100% of the CV measured from the contralateral control nerve. Between 8 and 36 weeks after reconstruction the change in $CV^{1st\ peak}$ and functional recovery are correlated significantly (chapter 6, Kuypers et al. Submitted 1998). This correlation can be explained through the increase in axon diameter which is known to occur when the neuronal units reach their target organs and start to mature because, as mentioned in the previous section, the CV is directly related to the axon diameter (chapter 6, Kuypers et al. Submitted 1998). Thus functional recovery is a result of neuronal units reaching their target organs. This will cause axon maturation and a subsequent increase in axon diameter, resulting in a recovery of the CV.

Functional recovery vs signal amplitude in the distal segment

The signal amplitude measured in the proximal segment when stimulating the distal segment seems to relate to the number of functional neuronal units regenerating across the lesion (chapter 2, [Kuypers et al., 1993]). This amplitude increases from nil to its maximum between 4 and 12 weeks after reconstruction. Functional recovery occurs between 6 and 20 weeks. It was demonstrated that the increase in signal amplitude is correlated to functional recovery (chapter 5, [Kuypers et al., 1998b]). Therefore the signal amplitude measured when stimulating the distal segment may be used to predict the eventual functional recovery even after relatively short regeneration times.

The lack of increase in signal amplitude measured from the proximal segment

In the reconstructed nerves the signal amplitude measured after stimulating the proximal segment remained approximately 45% of the value measured from the contralateral nerves, even after long regeneration times (chapter 6, Kuypers et al. Submitted 1998). This suggests a major loss of functional neuronal units in the proximal segment. The histological axon counts in the proximal segment after 20 weeks of regeneration time on the other hand, had only decreased with approximately 5% compared to the contralateral nerves (chapter 4, [Kuypers et al., 1998a]). Furthermore, the $CV^{1st\ peak}$ increased between 8 and 12 weeks after reconstruction from 65% back to normal, due to maturation of neuronal units that have reached their target organs. The lack in recovery of signal amplitude after approximately 8 weeks of regeneration time, while the $CV^{1st\ peak}$ regained normal values (chapter 6, Kuypers et al. Submitted 1998) and only a slight decrease in the histological axon counts (chapter 4, [Kuypers et al., 1998a]), has been explained by the presence of a significant number of histologically viable neuronal units in the proximal segment losing their signal conducting capability (chapter 3, 4, 6). The decrease in amplitude due to this loss is compensated by other neuronal units regaining normal axon diameters due to maturation, causing an increase of the amplitude (chapter 6, Kuypers et al. Submitted 1998).

This loss of the signal conducting capability in the proximal segment of a transected and reconstructed peripheral nerve seems particularly interesting to us. Such a loss of functional neuronal units has also been suggested based on horseradish peroxidase (HRP) experiments. It has been demonstrated that the uptake of HRP is influenced by the electrical activity of a neuron. In transected and reconstructed peripheral nerves the number of nerve cell bodies stained with HRP applied to the proximal segment was decreased significantly [Peyronnard, Charron, 1983; Peyronnard et al., 1986; Peyronnard et al., 1988]. This too suggests a decrease in the number of electrically active neuronal units. Furthermore, Cajal and others [Ramon y Cajal, 1928; Stroebe, 1893] already noticed a number of so called "sterile axons" in the proximal segment of a transected and reconstructed peripheral nerve of the rabbit after 1 to 28 days of regeneration time. These "sterile axons" do not form functional growth cones and are therefore unable to regenerate. They become varicose and the axonoplasm contains many vacuoles. It could be that these axons do not transport HRP and have lost their signal conducting capability.

Finally, the number of neuronal units regenerating into the distal segment is related to functional recovery (chapter 5, [Kuypers et al., 1998b]). If it were possible to restore signal conduction and growth cone formation of these poorly functioning "sterile axons", the number of functional regenerating neuronal units in the proximal segment would increase. This should theoretically positively influence the chance of eventual functional recovery.

8 Conclusion

It has been demonstrated that the relatively new MNG technique can be used to quantify the number of functional neuronal units regenerating across a peripheral nerve lesion shortly after reconstruction. The rabbit model is suitable to study peripheral nerve regeneration.

Also, many clinical dilemmas can be studied in a quantitative manner with this technique. For example: Is the regeneration across a sutured peripheral nerve lesion equal to that of a lesion reconstructed by use of fibrin glue or any other method? Or: Does a delay between the transection and the reconstruction influence the number of functional neuronal units regenerating into the distal segment?

In a clinical setting the MNG recording technique could also be a useful instrument. At the present time, the quality of a peripheral nerve lesion is studied clinically by use of function tests and the ENG technique. The function tests can only be performed after long regeneration times, and neither the function tests nor the ENG give reliable quantitative results. A decision to re-reconstruct a nerve lesion based on these conventional evaluation techniques can therefore only be made after a long regeneration time. It has been demonstrated that the binding capability of the regenerating sprouts to the basal membranes of the Schwann cells decreases after 6 months [Li et al., 1997]. This will seriously reduce the chances for the late sprouts to reach a target organ. We have demonstrated that the eventual regeneration across the lesion can be quantified with the MNG at 3 months after the reconstruction and that these results can be used to predict the functional recovery. If an insufficient increase in the MNG signal amplitude would prompt a decision to redo a nerve reconstruction after 3 months of regeneration time, this will leave a relatively long period of useful time for the regenerating sprouts to reach their

targets, and thus improve eventual recovery.

Another interesting observation from these experiments is the controversy of the major loss of functional neuronal units in the proximal segment, while only a small percentage of the proximal axons have degenerated histologically. This loss of function might well be related to the number of transected axons in the proximal segment that have not been able to produce viable regenerating sprouts. It would be interesting to study this phenomenon and investigate whether neurotropic substances might increase the number of functional neuronal units.

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9. Summary:

In the study underlying this thesis peripheral nerve regeneration was examined in a rabbit model based on a Magnetoneurografic (MNG) recording technique. First it was demonstrated that the signals measured with the MNG technique are far more reproducible than those measured with the conventional Electroneurografic technique and that they can be used to quantify the number of functional neuronal units in a nerve.

Subsequently, the MNG technique was used to study nerve regeneration in the rabbit. For this purpose the common peroneal nerve was unilaterally transected and reconstructed and MNG signals were measured after various regeneration times with a maximum of 36 weeks. The MNG signals were measured in the reconstructed nerve at 40 mm proximal to the anastomosis while stimulating the nerve at 10 mm proximal and distal to the anastomosis; signals from the intact contralateral nerve served as control values. Stimulation in the proximal segment should thus yield the percentage of excitable neuronal units, whereas stimulation in the distal segment gives the fraction of excitable neuronal units that regenerated across the anastomosis. Prior to the recording operation, functional recovery was measured for each animal using the toe-spread test. Furthermore the number of myelinated axons in the proximal segment of the reconstructed nerve and in the contralateral nerve was counted histologically after 20 weeks of regeneration time.

The results demonstrate that the motor function starts to recover after 6 weeks of regeneration time. The signals measured when stimulating the distal segment increase from absent to 20% of those measured from the contralateral nerve, between 4 and 12 weeks after the reconstruction. The correlation between these changes is statistically significant. This led us to the conclusion that measuring MNG signal amplitude while stimulating the distal segment of a reconstructed nerve is a suitable method to predict, in an early stage, eventual functional recovery.

The signal amplitude measured after stimulation in the proximal segment decreased after the reconstruction, to reach approximately 45% of the amplitude measured from the control nerve after 8 weeks of regeneration time. From then on, the amplitude did not increase significantly till the end of the experiment at 36 weeks after the reconstruction. The CV decreased in the first 8 weeks after the reconstruction to approximately 65% of the value measured from the control nerves. The correlation between the decreases in the signal amplitude and in CV during the first 8 weeks after the reconstruction is statistically significant. This can be explained by the decrease in axon diameter known to occur during the nerve regeneration phase.

Between 8 and 12 weeks the CV increases to normal values again. During that period, changes in CV parallel those in functional recovery, such that the correlation between the CV and the functional recovery from the 8th week on is statistically significant. This relation seems to be due to the increasing axon diameter caused by the maturation of the neuronal units which reach their target organs.

At 20 weeks after the reconstruction the histological axon count in the proximal segment of the transected nerve had decreased with approximately 5% when compared to the axon counts in the contralateral control nerve. Thus 95% of the initially available neuronal units will remain viable after the transection and reconstruction. This is in contrast to the decrease in MNG amplitude measured from the proximal segment which suggests a major loss in number of functional neuronal units after the transection.

The discrepancy between the changes in CV and MNG amplitude from the 8th week

on is surprising, since the increase in axon diameter, which causes an increase in CV, should theoretically also result in an increase in signal amplitude. We put forward the hypothesis that the MNG signal amplitude is decreased due to a growing number of neuronal units in the proximal segment which loose their signal conducting capability while remaining histologically viable. This decrease cancels out the expected increase in signal amplitude due to the increase in axon diameter caused by maturating neuronal units. Therefore theoretically the eventual functional recovery after reconstruction of a transected nerve may be improved by reducing the number of neuronal units loosing their signal conducting capability.

10 Samenvatting:

Voor dit promotie onderzoek is de perifere zenuwregeneratie in het konijn bestudeerd. Hiervoor is gebruik gemaakt van een Magnetoneurografische (MNG) meetmethode. Als eerste is gedemonstreerd dat de signalen gemeten met behulp van deze MNG meetmethode veel reproduceerbaarder zijn dan de signalen gemeten met behulp van de electroneurografische (ENG) meetmethode. Deze MNG signalen kunnen gebruikt worden om een schatting te maken van het aantal functionele neuronale eenheden in een zenuw.

Om perifere zenuwregeneratie te bestuderen werd een konijn model gekozen. De regeneratie werd met behulp van de MNG meetmethode geëvalueerd. Hiervoor werd de nervus peroneus communis unilateraal doorgesneden en microchirurgisch gereconstrueerd. De MNG signalen werden gemeten na regeneratietijden oplopend tot 36 weken na de reconstructie.

De zenuw werd gestimuleerd op 10 mm proximaal, resp. distaal van de reconstructie, waarbij de signalen telkens werden gemeten op 40 mm proximaal van het stimulatie punt. Tevens werd het signaal gemeten van de contralaterale controle zenuw. Juist voordat de konijnen onder narcose gingen werd het functieherstel geëvalueerd met behulp van de "toe-spread test". Van de konijnen uit de groep met 20 weken regeneratietijd zijn, na de metingen, het aantal gemyeliniseerde axonen histologisch bepaald in het proximale segment van de gereconstrueerde zenuw en vergeleken met het aantal gemyeliniseerde axonen in de contralaterale controle zenuw.

De resultaten tonen aan dat na 6 weken regeneratietijd het functieherstel begint. In de periode tussen 4 en 12 weken na de reconstructie neemt de amplitude van de signalen gemeten na stimulatie van het distale segment toe van nul tot 20% van de signalen gemeten van de controle zenuw. De correlatie tussen de veranderingen in functie en signaal amplitude zijn statistisch significant. Hieruit hebben wij geconcludeerd dat de MNG meetmethode gebruikt kan worden om het uiteindelijke functieherstel al kort na een perifere zenuwreconstructie te voorspellen.

De amplitude van de signalen gemeten na stimulatie van het proximale segment verminderen, gedurende de eerste 8 weken na de reconstructie, in toenemende mate tot ongeveer 45% van de amplitude gemeten van de contralaterale zenuw. In de periode tussen 8 en 36 weken na de reconstructie blijft de amplitude ongeveer 45% van die van de controle zenuwen. De geleidingssnelheid van de axonen in het proximale segment daalt ongeveer 35% in de eerste 8 weken na de reconstructie. De afname in signaal amplitude en geleidingssnelheid in het proximale segment gedurende deze 8 weken zijn statistisch significant gecorreleerd. Dit kan verklaard worden door een afname van de diameters van de axonen in het proximale segment welke in het distale segment aan het ingroeien zijn.

Acht weken na de reconstructie begint de functie te herstellen. Dit gaat gepaard met een herstel in geleidingssnelheid in het proximale segment. De functie en de geleidingssnelheid bereiken beide normale waarden en zijn in de periode van 8 tot 36 weken na de reconstructie statistisch significant gecorreleerd. Dit kan verklaard worden door een toename, in het proximale segment, van de diameters van de axonen welke distaal een doelorgaan bereikt hebben en zijn gaan matureren.

Twintig weken na de reconstructie is, histologisch, het aantal gemyeliniseerde axonen in het proximale segment met 5% afgenomen ten opzichte van de contralaterale controle zenuw. Van de initieel aanwezige axonen was dus na de transsectie en

reconstructie nog 95% histologisch onveranderd aanwezig. Dit in tegenstelling tot de amplitude van de met behulp van het MNG gemeten signalen, welke met 55% waren afgenomen.

De discrepantie tussen de veranderingen in signaal amplitude en geleidingssnelheid is verrassend, met name omdat een verandering in axondiameter welke een verandering in geleidingssnelheid veroorzaakt, een veel grotere verandering in signaal amplitude zou moeten veroorzaken. Wij poneren de hypothese dat de vermindering in signaal amplitude gemeten van het proximale segment met behulp van de MNG, wordt veroorzaakt doordat in de tijd dat een deel van de axondiameters toeneemt door maturatie ten gevolgen van het berijken van een doelorgaan, er ook een groot aantal axonen in de proximale segment hun signaal geleidende kwaliteit verliezen. Hierdoor wordt de verwachte toename in signaalamplitude tgv de maturatie, teniet gedaan door de uitval van andere axonen. Theoretisch zou het uiteindelijke functieherstel na perifere zenuw reconstructies kunnen worden verbeterd door deze uitval in geleidingsfunctie te verminderen.

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Curriculum Vitae

Paul Kuypers was born in Baltimore, USA on the March 23, 1962. After finishing high school in 1981 at the "Montessori Lyceum" in Rotterdam, he studied medicine at the Rijksuniversiteit Leiden till 1989. During this time he did research electives at the Department of Plastic and Reconstructive Surgery of the Erasmus University in Rotterdam (Prof. Dr. J.C. van der Meulen) and at the Department of Thoracic Surgery of the Academic Hospital in Leiden (Prof. Dr. H. Huysmans). During his medical training he also did an elective period at the Department of General Surgery at Adenbrooks University Hospital in Cambridge, England (W. Smellie FRCS and D. Dunn FRCS). After graduating from medical school he became a demonstrator at the Department of Anatomy of the University of Cambridge, England (Prof. H. Ellis FRCS). During this time he also worked as a supervisor at Pembroke College, Cambridge. In 1989 he worked as a senior house officer at the department of orthopedic surgery of New Market General Hospital (D. Dandy FRCS and C. Constant FRCS). From 1991 till 1995 he worked as a research fellow at the Department of Plastic and Reconstructive Surgery of the Erasmus University in Rotterdam (Prof. Dr. S.E.R. Hovius) and from 1995 till 1998 he worked as a junior registrar at the Department of General Surgery of the Onze Lieve Vrouwe Gasthuis in Amsterdam (H. Hoitsma). In 1998 he started as a senior registrar at the Department of Plastic and Reconstructive Surgery of the Academic Hospital Dijkzigt in Rotterdam (Prof. Dr. S.E.R. Hovius).

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