End-to-side nerve repair - A study in the forelimb of the rat

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End-to-side nerve repair
A study in the forelimb of the rat

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Normally…

To my parents

But to them

I owe my whole life…

To my grandfathers

CONTENTS

7:7 Ask, and it shall be given you; seek, and ye shall find; knock, and it shall be opened unto you:
7:8 For every one that asketh receiveth; and he that seeketh findeth; and to him that knocketh it shall be opened.

Mathew

Abbreviations

NPY	Neuropeptide Y
CPON	C-terminal flanking peptide of neuropeptide Y
DRG	Dorsal root ganglion
ATF3	Activating transcription factor 3
CREB	cAMP responsive element binding protein
DY	Diamidino yellow
FB	Fast blue
TNFe	Total number of fibers (extrapolated)
MMA	Mean myelinated area
M-ratio	Myelination ratio
PBS	Phosphate buffered saline

List of Publications

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Bontioti EN, Kanje M, and Dahlin LB. Regeneration and functional recovery in the upper extremity of rats after various types of nerve injuries. Journal of the Peripheral Nervous System 8: 159-168 (2003)

II. Bontioti EN, Kanje, Lundborg G, and Dahlin LB. End-to-side nerve repair in the upper extremity of rat. Journal of the Peripheral Nervous System, 10(3): 58-68 (2005)

III. Bontioti EN, Kanje M, and Dahlin LB. End-to-side nerve repair: attachment of only a distal versus a proximal and a distal nerve segment. Submitted

IV. Bontioti EN, Dahlin LB, Kataoka K, and Kanje M. End-to-side nerve repair induces nuclear translocation of the activating transcription factor 3 (ATF3). Submitted

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THESIS AT A GLANCE

Paper I

Questions: Is the brachial plexus and its branches in the forelimb of the rat a suitable model to study regeneration and functional recovery after various types of nerve injuries and repair? What is the cell body response after a proximal and a distal nerve injury?

Method: A crush injury, a conditioning lesion, i.e. a test lesion preceding a nerve lesion, a transection and end-to-end nerve repair, or a graft interposition were performed to the median, ulnar and radial nerves in rats.

Evaluation times: 0 – 60 days.
Evaluation methods: Pawprints (for functional recovery) immunocytochemistry (staining for neurofilament and C-terminal flanking peptide of neuropeptide Y; CPON – marker for cell body reaction) and histology.

Conclusion: The brachial plexus with its branches offers an excellent experimental model to study nerve regeneration after injury and repair, where pawprints can be used as a simple and convenient way to evaluate functional recovery, which is fast in the forelimb. The pattern of axonal outgrowth and functional recovery is reflected by the severity of the lesion. An injury of both motor and sensory axons or only of preferentially sensory axons preceding a nerve lesion acted as a conditioning lesion. CPON expression in DRG and functional recovery are dependent on the level of the lesion from the cell bodies.

Paper II

Questions: Is end-to-side nerve approximation an appropriate method for nerve repair? What is the source, the type and the number of regenerating nerve fibers in the end-to-side attached nerve segment?

Method: The distal segments of the transected radial or both the median and ulnar nerves (recipient nerves) were attached in an end-to-side fashion on an epineurial window of the musculocutaneous nerve (donor nerve) in rats.

Evaluation time: 0 - 180 days.
Evaluation methods: Pawprints, tetanic muscle force, muscle weight, immunocytochemistry (neurofilament staining), retrograde labeling, histology and morphometry.

Conclusion: An end-to-side attached nerve attracts motor and sensory axons, which become myelinated. The repair method results in functional recovery up to around 60-70% of preoperative value with respect to pawprints and tetanic muscle force. Collateral sprouting is one of the mechanisms by which nerve fibers are recruited to the end-to-side attached nerve segment. One donor nerve can be used to reinnervate the territory of two end-to-side attached nerves.

Paper III

Questions: Has end-to-side attachment of also the proximal nerve segment of an injured nerve to a donor nerve any advantage over attachment of only the distal nerve segment, eg. are two potential sources of axons better than one?

Method: The distal or both the distal and the proximal nerve segments of the radial nerve were attached end-to-side to the musculocutaneous nerve.

Evaluation times: 0 - 240 days.
Evaluation methods: Pawprints, immunocytochemistry, retrograde labeling, routine histology and morphometry.

Conclusion: Both repair methods resulted in axonal outgrowth and some functional recovery. The attachment of also the proximal nerve segment as an extra source of axons did not improve recovery, but there was a difference in the distribution of retrograde labeled cell profiles indicating different sources of axons in the two models.

Paper IV

Questions: What type of injury or manipulation to a nerve trunk related to end-to-side nerve repair can result in activation of neurons and non-neuronal cells in the donor nerve?

Method: Various types of manipulations of the donor nerve were used: 1) a piece of nerve or muscle placed close to a 'donor' nerve; 2) an epineurial window and/or sutures were applied; 3) a nerve segment was attached end-to-side to the 'donor' nerve.

Evaluation times: 7 days.
Evaluation methods: Immunocytochemistry for the activating transcription factor 3 (ATF3), a marker of cell activation, was used for the DRGs, the donor nerve and for the motor neurons of the spinal cord.

Conclusion: A piece of nerve or muscle close to the donor nerve did not induce ATF3 locally in the donor nerve and rarely in its sensory and motor neurons. In contrast, an epineurial window/sutures with or without end-to-side attachment of a nerve induced a distinct ATF3 activation at several levels. An injury to a peripheral donor nerve may be a main prerequisite for activation of neurons and non-neuronal cells in end-to-side nerve repair inducing axonal sprouting.

«Ιητρός γάρ φιλόσοφος ισόθεος. Πολλή γάρ διαφορή επί τά έτερα' και γάρ ένι τά πρός σοφίην, εν ιητρική πάντα, αφιλαργυρίη, εντροπή, ερυθρίασις, καταστολή, δόξα, κρίσις, ησυχία, απάντησις, καθαριότης, γνωμολογίη, είδησις των πρός βίον χρηστών και αναγκαίων, ακαθαρσίης απεμπόλησις, αδεισιδαιμονίη, υπεροχή θεία.»

Ιπποκράτης (Περί Ευσχημοσύνης, παρ.5)

Όταν ο ιατρός είναι και φιλόσοφος γίνεται ισόθεος, γιατί μεταξύ των δύο αυτών ιδιοτήτων δεν υπάρχει μεγάλη διαφορά, εφόσον, ότι αποκτάται με την φιλοσοφία χρειάζεται και για την ιατρική δηλ. αφιλοχρηματία, φιλότιμο, ερυθρίαση, μετριοφροσύνη, γνώση, κριτική ικανότητα, ηρεμία, ετυμολογία, ψυχική καθαρότητα, έγκυρη γνώμη, επίγνωση των ωφέλιμων και απαραίτητων για τη ζωή, αποφυγή ασχημιών, έλλειψη δεισιδαιμονίας, θεία υπεροχή.

Ιπποκράτης (Περί Ευσχημοσύνης, παρ.5)

When the doctor is also a philosopher, he becomes equal to God, simply because between these two attributes there is not much difference, considering whatever acquired through philosophy, is also a prerequisite for medicine; that is disregard for money, generosity, (blushing) humbleness, modesty, cognition, judgment, tranquillity, etymology, purity of the psyche, validity of opinion, awareness of the beneficial and necessities for life, avoidance of impropriety, lack of superstition, divine supremacy.

Hippocrates (For decency, par.5)

“Brains first and then Hard Work.”

Eeyore

INTRODUCTION

Peripheral nerve injuries are of significant social and economic importance, since they severely impede function and affect regular life. The etiology of peripheral nerves injuries is multiple and trauma to peripheral nerves account for around 3% of all hand and forelimb injuries [136, 137]. When an injury occurs to the nerve trunks of the upper extremity, with its unique and complex functional abilities, the consequences are severe and particularly if hand function is affected. The human hand is not only a working tool but also one that makes our daily activities and well being possible. It is an organ that connects us to the outer world by the sense of touch and one that makes our expression to the world heard via gestures or via the art of painting or music.

Although nerve injuries affecting the upper extremity are considered to be rare throughout Europe and USA, the impact that they have on work capacity, achievements of daily living and economy is considerable [19, 45, 51, 137, 138, 180]. It has been reported that the 11,000 Americans/year that are affected by nerve paralysis carries a costs of $7billion/year i.e nearly 5billion€ [48]. A more recent study from our department shows that the costs for an employed person who has sustained a median nerve injury is more than 51.000€. If this lesion is accompanied with tendon injuries then the total costs increase with 51% [136].

The severity of upper extremity nerve injuries varies from a simple cut of a digital nerve to extensive lacerations and avulsions of the roots of the brachial plexus. The conventional surgical methods to treat nerve injuries depend on the type of injury and could include simple neurolysis, end-to-end neurorrhaphy, nerve grafting up and procedures to neurotization. However, there are situations when the conventional repair methods are not applicable, these situations and the poor recovery after extended nerve lesions have prompted the development of alternative repair methods. To this end researchers have turned their attention to nerve conduits (see for example Lundborg [94], Doolabh [43], Strauch [152]) and during the last decade end-to-side nerve repair (see for example review by Zhang and Fisher [189]). End-to-side nerve repair, i.e. attachment of a single distal nerve segment (recipient nerve) end-to-side to an intact nerve trunk (donor nerve) when no proximal nerve segment is present, is used clinically [53, 54, 113], but its merits and mechanisms have been intensively debated. The end-to-side nerve repair method is the focus of this thesis, which covers investigations of the nerves in the forelimb of the rat as a model for studies of peripheral nerve regeneration with particular emphasis on end-to-side nerve repair and the mechanisms by which an end-to-side attached nerve segment can attract regenerating nerve fibers.

BACKGROUND

The nervous system

The nervous system is divided anatomically into the *central nervous system* (CNS), comprising the brain and the spinal cord, and the *peripheral nervous system* (PNS), which constitutes all nervous tissue outside CNS. The PNS is subdivided into the *somatic nervous system* and the autonomic nervous system. The PNS, which is dealt with in this thesis, is involved in voluntary functions and it consists of motor neurons that connect the brain and the spinal cord to skeletal muscles by efferent signals and of sensory neurons that bring afferent information from the body and the environment to CNS. The *autonomic nervous system* exerts control over many involuntary functions and consists of cells and axons that innervate smooth muscles, cardiac muscles and glands [177].

Neurons and non-neuronal cells

The cells of the nervous tissue can be divided into two broad categories: nerve cells (neurons) and supporting cells (neuroglia). The **neuron** is responsible for electrical activity in the nervous system. Neurons exist in a variety of shapes and morphologies. They can be divided into categories like motor neurons, sensory neurons and interneurons, the latter is the most abundant.

A typical neuron has four major functional domains: the cell body (the soma), a variable number of dendrites, the axon, commonly referred to as *nerve fiber*, and the presynaptic terminals. The *cell body* – the metabolic center of the cell - contains the nucleus, which stores the genes of the cell and the cytoplasm known as the perikaryon, which is filled with a variety of organelles [89]. The cell body usually gives rise to two kinds of processes: a single axon and one or more dendrites. *Dendrites* are highly branched out in a tree-like fashion and are the main conducting apparatus for receiving incoming signals from other nerve cells. Each neuron has a single, long tubular process, the *axon*, which is the main conducting unit for carrying signals - action potentials – to other neurons or the targets cells that are innervated by the neuron. The axon extends as a cylindrical process of variable length – up to a meter in humans, which end in small swellings called *terminal boutons*.

There are several types of glial cells. The supporting elements of the CNS are **neuroglia** from the Greek words neuro=nerve and glia=glue, since the main role of them was thought to be maintainance of the integrity of the neurons. In the CNS we find astrocytes (Greek astron=star and kytos=hollow vessel), the myelinating oligodendroglia (oligo=few and dendron=tree), microglia (mikros=small and glia=glue) and ependymal cells (upper garment) [1]. The functions of the glial

cells include myelination, secretion of trophic factors, maintenance of the ionic milieu of the neurons, scavenging of molecular and cellular debris, modulation of the rate of nerve signal propagation and of synaptic action and formation and maintenance of the blood-brain barrier [6, 64, 139]. In the PNS we find two types of glial cells – satellites cells that surrounds the neurons in ganglia and the Schwann cell which ensheaths axons and form myelin around large diameter nerve fibers. The axon-Schwann cellunits are surrounded by a basal lamina produced by the Schwann cells. The basal lamina forms a continuous tube along the entire length of the nerve. The Schwann cells envelop also small diameter axons; they are called non-myelinated nerve fibers. The myelin sheath is not continuous over the entire length of the axon but is interrupted at different intervals. Therefore, a gap exists between two adjacent Schwann cells referred to as a node of Ranvier [135]. This area of discontinuity is the site of voltage-gated sodium channels and ionic displacements involved in impulse conduction (action potential). The electric impulse travels along a myelinated axon by "jumping from node to node", the "saltatory" conduction, and increases the speed of the action potential conduction.

The peripheral nerve

The present thesis deals with injuries to the PNS and particularly nerves emanating from the brachial plexus. A nerve trunk is usually composed of an organized bundle of axons, with a variable mixture of motor and sensory axons. A condensed layer of collagenous connective tissue called the *perineurium* surrounds a bundle of axons with their endoneurial sheaths forming a fascicle. The perineurium is a mechanically strong multilammelar sheath composed of flattened cells with a basement membrane on both inner and outer aspects. It protects the contents of the endoneurial space, acting as a mechanical barrier to external trauma, and serves as a diffusion barrier as well. Inside the fascicle a delicate packing of loose connective tissue called *endoneurium*, consisting of basal laminae, capillaries, collagen fibers, endoneurial fibroblasts and macrophages, surrounds the nerve fibers with their associated Schwann cells. One or more fascicles are embedded in a loose layer of connective tissue, the *epineurium*. The epineurium cushions the fascicles during movement of the extremity and protects them against external trauma, providing a certain amount of gliding of the nerve. Superficial to the epineurium, a layer of loose connective tissue called 'adventitia' forms the nerve trunk and allows a great deal of neural gliding. Peripheral nerves are well-vascularized structures with separate but extensively interconnected microvascular systems (plexuses) in all the different layers. A peripheral nerve also contains autonomic nerve fibers, thus sympathetic unmyelinated nerve fibers innervate blood vessels of the nerve. They are called nervi nervorum, contain neuropeptides and are hypothesized to respond to stimuli during nerve damage [97, 160]. In

the present study, I used branches from the brachial plexus – the musculocutaneous, the radial, the median and the ulnar nerves as an experimental model. These nerves are mixed nerves containing both motor and sensory axons to a variable extent.

Sensory neurons

Ganglia are defined as collections of nerve cell bodies. The sensory neurons of the somatic nervous system have their cell bodies in the dorsal root ganglia (DRGs) observable as a swelling of the spinal nerve root. The neurons of DRG are pseudounipolar and send one branch to the spinal cord and one branch to the periphery [155, 156]. Size, neuropeptide content, and expression of trk-receptors or conduction velocity of their process can categorize these neurons. Large neurons transmit sensation and proprioception while small neurons deals with pain and temperature. In the rat the neurons are divided into small (<35μm) and large (>35μm) diameter cells, a size that correlates to the size of axon and thus the conduction velocity. Each ganglion is surrounded by connective tissue sheath and also contains abundant blood vessels. The satellite cells, which ensheaths the cell bodies in the ganglia, outnumber the neurons [59, 176]. In the present study, the DRG of the brachial plexus residing in the cervical region have been used together with the lumbar DRG of the sciatic nerve.

Motor neurons

The cell body of the motor neurons are found in the ventral horn of the spinal cord. There is a somatotopic representation within the ventral horn so that neurons innervating axial musculature is found more medially, while neurons innervating muscle groups in the limbs are located more laterally [78, 118, 121]. Motor neurons have a larger cell body than the sensory neurons and the processes of the motor neurons are myelinated. Typically, the sensory neurons outnumber the motor neurons as in the sciatic nerve of rats [146]. Recovery of motor function following nerve injury and repair is superior to that of sensory function.

The brachial plexus and its branches

For the reader, not familiar with the anatomy of the peripheral nerves of the upper extremity, a schematic drawing of the brachial plexus is shown in Fig 1.

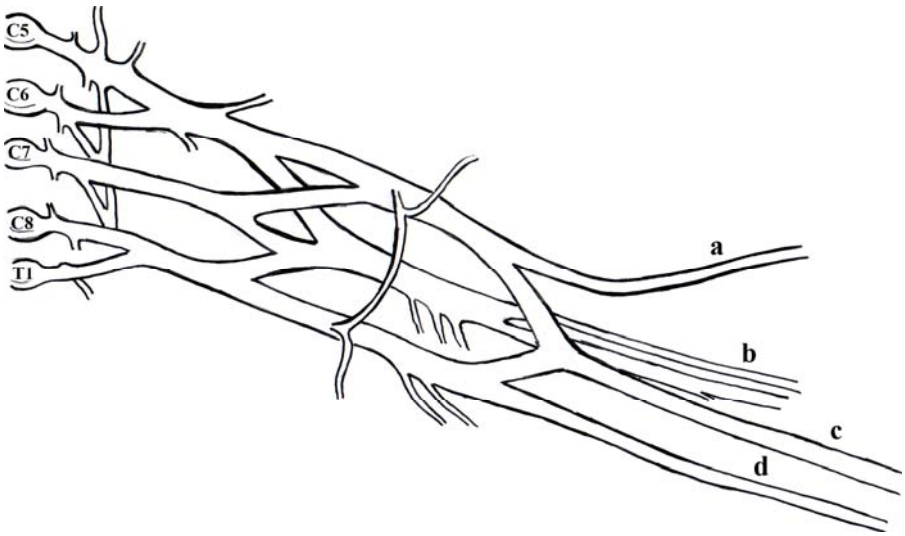

Figure 1

Schematic drawing of the brachial plexus. a: the musculocutaneous nerve, b: the radial nerve, c: the median nerve, d: the ulnar nerve.

The musculocutaneous nerve arises from C4-C7 level (mostly C5-C6) and innervates the biceps muscle, which is responsible for elbow flexion and supination. The median nerve emerges from C5-T1 (mostly C7) and innervates the flexor digitorum sublimis and profundus, the pronators, the flexor carpi radialis, and some intrinsic muscles of the hand. It controls wrist and fingers flexion, pronation and thumb movement. The ulnar nerve arises from C6-T1 (mostly C7) levels and innervates muscles in the thenar and hypothenar as well as the interosseous muscles of the hand that aim to adduct thumb and fingers as well as abduct fingers. The radial nerve arises from C5-T1 (mostly C7-C8) levels and innervates several muscles that result in forelimb supination as well as in wrist and fingers extension [15, 126, 183].

NERVE INJURY AND REGENERATION

The nerve cell body

When a nerve is injured so the continuity of the axon is disrupted, it results in extensive alterations along the whole nerve and the innervated targets but also in its projection areas in the

CNS [27, 95]. A nerve injury and the subsequent regenerative processes are unique since it is a cellular repair process and does not involve proliferation of the afflicted neurons. In many neurons the classical signs of an injury, consisting of swelling of the cell body, a shift of the nucleus from its central position to the periphery (eccentricity), dispersion of the Nissl substance (chromatolysis; Greek chroma=color and lysis=dissolution) and proliferation and swelling of other organelles [63, 89], occur. These changes reflect a modification in the metabolic priority from the maintenance to the production of materials needed for axonal repair and growth and involve alterations in the expression of hundreds of genes and proteins [56]. Collectively, these changes are referred as the cell body reaction [89]. The speed and the degree at which those events occur are dependent on the location of the injury, the type of injury, the type of neuron involved, the species and the age of the animal [44, 75, 87, 89]. The closer the injury to the cell body the more severe the reaction [186]. The outcome is poor in proximal lesions.

The profound changes that the cell body go through after an injury are triggered by signals, which are transported in neurons by axonal transport (anterograde - from the center to periphery - and retrograde - from the terminal organs to the central body). Axonal transport occurs along microtubules with motor proteins, such as kinesin and dynein, involved. The exact mechanisms by which the cell body response is initiated are not known, but the lack of return of target-derived factors by retrograde axonal transport is important. There are also signals emanating from the site of injury that could be picked up by the injured axons and transported retrogradely.

The injury-related signals may elicit the cell body reactions via classical signal transduction pathways involving cascades of mitogen activated protein kinase (MAPK) resulting in the activation of transcription factors [17, 27, 29, 40, 151]. One such transcription factor that responds to injury is the activating transcription factor 3 (ATF3), which belongs to the CREB (cAMP responsive element binding protein) family of transcription factors [69, 91, 167]. ATF3 is a stress-induced gene with its mRNA level greatly increasing upon the exposure of cells to stress signals within two to eight hours after the insult. The signaling pathway that this transcription factor is using is probably the JNK/SAPK one [90]. ATF3 has a role in protecting cells from apoptosis and can induce neurite elongation and outgrowth [80, 90, 128]. In *paper IV* I used ATF3 immunoreactivity as a marker for activation of neurons and Schwann cells in order to investigate the mechanism that underlies an end-to-side nerve repair.

NPY expression was also used to study the cell body reaction after a distal versus a proximal injury (*paper I*). Normally, NPY is not expressed in adult DRG neurons, which is different from other neuropeptides as galanin and PACAP, but its expression is dramatically increased after nerve injury [31, 79, 130, 131, 170] making it suitable as a marker for a neuronal injury. NPY expression

was studied indirectly by using antibodies to its C-terminal flanking peptide (CPON), which has the same distribution in the nervous system as NPY [10, 65].

Not all neurons reach to the stage of regeneration following axonal injury. It is estimated that up to 35% of the sensory neurons die after a nerve transection and sensory neurons are more susceptible than motor neurons [56, 74]. The pathways, which lead to neuronal death, are not well understood but it is thought that neurons die by apoptosis (Greek 'falling off'), the process of programmed cell death, due to deprivation of neurotrophic factors [132, 147, 176]. Apoptosis can be induced by a variety of conditions associated with injury, including oxidative stress [103, 144]. Apoptosis is involved in the regulation of the number of Schwann cells in nerves after injury [120].

The proximal nerve stump

Retrograde degeneration usually involves only a short segment of the axon (a few internodes). Regeneration begins with the formation of the growth cones at the nodes of Ranvier [134]. Materials required for elongation are delivered by axonal transport to the growth cone. The rate of growth cone advancement is therefore related to slow axonal transport. The growth cones have mobile filopodia enabling them to move actively and explore the environment [57, 58, 62]. The growth cones are endowed with integrin receptors, which interact with the extracellular matrix proteins of the Schwann cell basal lamina tubes to find their way back to the targets. Intracellular signaling cascades are known to regulate the advance of the growth cone [20, 41, 62, 150]. Axonal outgrowth cannot occur unless the regenerating axons are provided with a substratum (i.e. a matrix) with which they can interact.

The interstump zone

The gap between both ends of a transected nerve represents a biological factory or a construction area. We know the cellular processes that take place in different stages at the interstump zone, mostly from research around the field of silicone nerve conduits [33, 35, 99, 192]. At the early stages of regeneration, the gap is filled with an exudate containing different cells and neurotrophic factors and then a fibrin matrix is formed. This matrix contains fibronectin, macrophages and inflammatory cells. The matrix is utilized as a substrate for axonal outgrowth and it is invaded by blood vessels. Schwann cells migrate from both the proximal and the distal nerve segments. The axons grow in concert with the Schwann cells from the proximal nerve stump.

The distal nerve segment

The severed axon and its myelin sheath distal to the site of the injury undergo what is known as Wallerian degeneration, a phenomenon described in 1852 by Augustus Waller [84]. The axon distal to the injury begins to degenerate within hours and the myelin sheath fragments. Invading macrophages phagocyte the fragmented portions of the axon and the myelin [76, 77]. Macrophages play also an important role since they secret mitogenes for Schwann cells, including PDGF and FGF [36]. The degenerating process is accompanied by mitotic activity in the Schwann cells. The proliferating Schwann cells align longitudinally creating a continuous column of cells named bands of Büngner within the basal lamina tubes. These tubes play an important role in regeneration, since they consitute pathways for the regenerating axons to their targets. Schwann cells regulate the survival and growth of axons by regulating the synthesis of neurotrophic factors, like NGF, NT-4, BDNF [18]. The Schwann cells are also responsible for the production of basal lamina components.

ATF3 is up regulated in Schwann cells in the distal segment in response to nerve injury [80, 81] (Kataoka, Kanje and Dahlin, personal communication). Interestingly, if regeneration is prevented during a prolonged period of denervation the distal nerve segment looses its capacity to support regeneration [46, 55, 70-72, 154].

NERVE INJURIES AND REPAIR

History

Hippocrates of the 5th century BC stated that the brain influences muscles. Erasistratus in Alexandria, in the 3rd century BC demonstrated that nerves mediate this influence. In the 2nd century BC, Galen was the first physician to differentiate nerves from tendons and treated for the first time nerve injuries. He concluded that the function of nerves was mediated by what he called the animal spirits, formed in the brain. The first documented nerve repair though stems from Paul of Aegina in 600AD who used agglutination and sutures for nerve repair [124]. In the 13th century Roger of Parma used egg albumin as nerve suture material.

The surgical treatment of a nerve injury depends, as mentioned, on the severity of the lesion, and the severity also reflects the subsequent functional outcome after repair or reconstruction. Regeneration and recovery is excellent when we are dealing with a crush injury. In contrast, recovery following a nerve transection by an end-to-end approximation, which is the most common repair method in clinical practice, is dissatisfying [3, 21, 114, 166, 178]. In particularly, this concerns the sensory function.

However, situations when an end-to-end nerve repair of the proximal and distal segments cannot be achieved are not uncommon. Such situation could arise after a direct trauma or as a

consequence of necessary resection of a nerve segment (i.e. neuroma, scar or tumor resection). Regeneration cannot occur unless a scaffold that supports regeneration bridges the nerve defect. Various methods have been proposed and used to bridge such nerve defects. If the gap is small, *nerve repair under tension* can be used but has several disadvantages because of the difficulty to determine the critical tension limits at which nerve stump blood supply is compromised. Stretching by *nerve mobilization,* by stripping mesothelial attachments, has the same disadvantages, but is now accepted that an amount of mobilization of less than 1:45 (diameter of the nerve:length mobilized) will not result in neural ischemia [166]. *Nerve rerouting and transposition, joint positioning and bone shortening* are other used techniques that make the end-to-end nerve repair possible in situations when defects are present [164].

For larger defects and for defects in the brachial plexus these methods are not applicable. For such defects *nerve grafting* has to be used. As early as 1863, the concept of nerve grafting emerged in the field of nerve repair by Philipeaux and Vulpian who performed the first nerve graft experiment in dogs [39, 133]. Albert, in 1876, performed the first human nerve graft. Hanno Millesi developed the present method for nerve grafting [115-117]. The gold standard for bridging nerve defects is the use of autologous nerve grafts. However, the resources of 'spare' nerves in the human body are however limited. The sural nerve is the most commonly used nerve graft in reconstruction of larger nerve trunks. Whichever the donor nerves, the morbidity of the area that these nerves innervate is a matter of concern. The results of nerve regeneration through a nerve graft depend on the defect length, the survival of the nerve graft itself, the ability of the regenerating fibers to cross two repair sites and the condition of the targets. Several attempts have been made to increase the regeneration potential of autologous nerve grafts. In 1976, Taylor and Ham introduced the concept of vascularized nerve grafts as a free tissue transfer in an attempt to improve circulation and graft survival [159]. The usefulness of this technique is, however, controversial. At the experimental level successful attempts have been done to improve regeneration in nerve grafts by predegeneration of the graft by transection or crush [30, 34], by vibration exposure of the donor limb [9] and treatment with hyperbaric oxygen [66] but the method have not been used clinically.

Considering the pitfalls of nerve grafting, great efforts have been made to develop alternative techniques including the concept of *conduits* made by non-neuronal tissue or artificial materials to bridge nerve defects [43]. A variety of different materials, like bone, artery, vein [105, 173, 189], gelatin, fascia, fat, epineurium [83], agar, trachea, casein, feather quill, rubber, dura, muscle [24, 60], parchment, tantalum, millipore [49] and silicone [28, 93, 98, 100], have been used with variable results. An ideal nerve conduit does not yet exist [47]. However, the use of nerve grafts and conduits are not applicable when the proximal nerve segment is missing due to a severe nerve

injury. In such situations alternative reconstruction methods are required. The possibility that end-to-side nerve repair could be a method of choice is considered in this thesis.

End-to-side nerve repair

End-to-side nerve repair is the procedure when the distal nerve segment of an injured nerve is attached end-to-side to an intact nerve. It is used when no proximal nerve segment is present. This method of repair is not new (see review by Al-Qattan [2]). Despres in 1876 repaired an injury of the median nerve by inserting the distal part between the fibers of the ulnar nerve. In 1899, Kennedy performed end-to-side in a patient with facial spasm. Ballance et al, in 1901, treated a facial palsy by suturing the facial nerve to the spinal accessory nerve and, in 1903, Harris and Low treated an injury to the superior trunk of the brachial plexus by suturing the distal part to the seventh cervical root (cited in Al-Qattan [2]). The technique was then abandoned for nearly 100 years, until Viterbo and colleagues [181] reintroduced it in experimental studies on animals but also in clinical trials with patients with facial palsy. Following Viterbo's report in 1992, numerous studies on end-to-side repair have been published (see for example review articles from Rowan et al. [141, 189]). There is little doubt that this method results in functional recovery and that a donor nerve can re-innervate the territory of a recipient nerve [52, 82, 129, 187]. However, the extent of motor and sensory reinnervation and the mechanisms by which end-to-side nerve repair attract axons in the donor nerve are still not clarified. Some of these issues are addressed in this thesis.

Technical aspects

End-to-side nerve repair is usually performed by suturing the recipient nerve to an intact donor nerve. It has been demonstrated that an epineurial window at the site of coaptation increases the number of axons growing out in the recipient nerve. In fact, Bertelli et al. claimed that regenerating axons could not transverse the connective tissue layers of the nerve unless a window was applied [11]. On the other hand, Yan et al. reported that sprouts were able to penetrate intact perineurium and epineurium [185]. Noah et al. designed a more complete model using the rat sciatic nerve model. They concluded that the connective nerve tissues are indeed some sort of barriers, since the amount of regenerating axons in the recipient nerve were more abundant in the group with an epi- and a perineurial window. The disadvantage was that a donor nerve injury was induced when an epineurial or a perineurial window were used [123]. In all my studies I performed the end-to-side nerve repair with the aid of an epineurial window (*papers II, III, IV*).

Motor versus sensory reinnervation

Several authors agree that end-to-side results in sensory regeneration, while motor regeneration is more limited [104, 106]. From studies with double labeling techniques and functional evaluation, the impression is that both sensory and motor regeneration occur but the sensory one seems to exhibit a more profound regeneration [158, 162].

The origin of the regenerating axons

Axonal sprouting out in the recipient nerve is believed to occur either by collateral sprouting or by terminal sprouting from axons in the donor nerve which have been damaged during the repair procedure [8, 86, 101, 122, 184]. Double retrograde labeling studies [4, 5, 67, 102, 142, 190] lend support to the idea that collateral sprouting may be one mechanism [26, 86]. It has also been claimed that the results in several experiments are void because the precautions to prevent outgrowth from the proximal part of the recipient nerve was not sufficiently rigorous and that in these studies nerve fibers could have grown out from the proximal stump and reached the end-to-side attached nerve segment [61, 108].

Evaluation method of functional recovery

Several evaluation methods have been proposed for investigation of the efficacy of end-to-side nerve repair apart from histology, morphometry and electron microscopy [119, 174]. Walking track analysis [38, 73, 161], grooming test [22], grasping test [12, 14], nerve conduction, electromyography [188], tetanic muscle force and muscle weight have been used to investigate the functional outcome after end-to-side nerve repair [127]. In the present thesis pawprints were used as a simple method to assess functional outcome after end-to-side nerve repair in the forelimb of the rat (*papers I, II, III*).

AIMS

The general aim of the study was to evaluate nerve regeneration after end-to-side nerve repair in an experimental model in the forelimb of the rat.

The specific aims were:

- To test if the brachial plexus and its branches is a suitable model to study nerve regeneration after different types of nerve injury and repair, including the cell body response after proximal versus distal nerve injury, and to evaluate if pawprints could be used to evaluate functional recovery.

- To investigate end-to-side nerve repair in the forelimb with special emphasis on functional recovery, source, type and extent of regenerating fibers from the donor nerve and to study if one donor nerve can support more than one recipient nerve.

- To study if there is any advantage of attaching also a proximal nerve segment end-to-side above the distal nerve segment as an extra source of axons.

- To examine what type of manipulations that activate neurons and non-neuronal cells of relevance for end-to-side nerve repair.

"The main reason for healing is love"

Paracelsus

MATERIALS AND METHODS

This section gives a brief description of the methods and procedures used. For further details, the reader is referred to the individual papers.

Experimental animals, anaesthesia and ethics

Adult female Wistar rats (Mollegaard, Denmark) with an initial body weight of 180-200g were used throughout the study. An intraperitoneal injection of a 1:10 solution of pentobarbital (60mg/ml) and physiological saline was used to anaesthetize the animals. Additional doses of the same solution were used during the surgical procedures, if needed. The local animal ethics committee at Lund University, Sweden approved all experiments.

Surgical procedures

In all surgical procedures the nerves of the forelimb of the rats were approached via a hockey stick incision extended distally if needed.

In *paper I*, *a crush lesion* was made twice for 40s each using a fine watchmaker's forceps to the median or ulnar nerves, or both, or to the radial nerve, approximately three mm above the elbow. At the same level, a transection was performed to the median and ulnar nerves or the radial nerve in other rats and an *end-to-end* nerve repair was made with three epineurial stitches. In a third set of experiments, a 10 mm long nerve segment was harvested from each nerve (median, ulnar or radial or both median and ulnar nerves) and inserted as a reversed *graft*, at the same level, following 180° rotation. In a fourth set of experiments, a *conditioning* crush lesion was performed to the preferentially sensory part distal to the elbow or to the mixed part of the median and ulnar nerves, proximal to the elbow, three days prior to the main crush injury.

In *paper II and III*, the median, ulnar, radial and musculocutaneous nerves were exposed and a three mm long epineurial window was made in the musculocutaneous nerve. The median and ulnar nerves or the radial nerve were transected and their distal segments were attached end-to-side to the epineurial window of the musculocutaneous nerve, while their proximal segments were ligated and buried into muscle.

In *paper III,* the proximal segment of the transected radial nerve in another set of animals was also attached to an epineurial window on the musculocutaneous nerve in an end-to-side fashion, along with the distal radial nerve segment (ten mm distance).

In *paper IV*, three different main surgical procedures were performed to the fore- or hind limb of the rats. 1) "No injury groups": a 20 mm long piece of muscle (gastrocnemius) or nerve (tibial or

peroneal nerve) was harvested and placed alongside the musculocutaneous or sciatic nerves. 2) "Nerve manipulation groups": In other rats a three mm long epineurial window, with or without two epineurial sutures, was made to the musculocutaneous nerve with the tip of a scalpel blade, care taken not to damage the perineurium. In a separate group, two epineurial sutures were placed on the sciatic nerve. 3) In a third group of rats – "End-to-side nerve repair groups" - a three-mm long epineurial window were made on the musculocutaneous or sciatic nerves and a 20 mm long tibial or peroneal nerve graft was attached in an end-to-side fashion to the musculocutaneous or the sciatic nerves, respectively.

In all groups, where an end-to-side nerve repair was performed (except in half of the experiments in paper III), the proximal stump of the recipient nerve was ligated, rotated and buried in the surrounding tissues. The wounds were closed and the animals were allowed to recover for 7 (paper IV), 30 - 60 (paper I), 180 (paper II) or 240 (paper III) days.

Functional Evaluation

Pawprints

Functional assessment after different nerve injuries and repair was investigated in *papers I, II and III* using measurements of *pawprints* of toes 1^{st}-4^{th} and 2^{nd}-3^{rd} at various time intervals. To obtain records during walking, the forepaw of the rats was marked with ink. The animals were allowed to walk freely across a 'corridor' on a sheet of paper. Toe spread during walking, defined as the distance between the first and the fourth digits or between the second and the third digits, was measured using a caliper. The pawprint measured was the one obtained from a non-interrupted step.

Tetanic muscle force and muscle weight

In *paper II*, tetanic muscle force was measured in the extensor carpi radialis brevis and longus or the flexor carpi radialis muscles in the rats where the radial or the median and ulnar nerves were attached end-to-side to the musculocutaneous nerve. Six months postoperatively the muscles were exposed and their tendon was cut as distally as possible and ligated. After transfixation of the shoulder, elbow and wrist joints with Kirschner wires, the corresponding tendon was attached to a force transducer (Grass Medical Instruments, USA). Supramaximal stimulation (6V at 100Hz) was delivered to the end-to-side attached nerves through platinum wire electrodes. Muscle response was recorded on a Macintosh computer and analyzed using Lab View software. The same procedure was applied to the right control side of all the animals and the muscle force was expressed in percentage of the control side. The wet weight of the muscles was registered using an electronic balance and expressed in percentage of the uninjured contralateral side.

Evaluation of nerve regeneration and cell body response

Histology

Routine histology was used to examine the regenerated nerve structures. Distal nerve segments of the median, ulnar, radial and musculocutaneous nerves from the animal groups in *papers I, II and III* were harvested and fixed in 2.5% glutaraldehyde and transferred to 0.1M Na-cacodylate buffer. They were postfixed with 2% osmium tetroxide, soaked again in Na-cacodylate buffer and dehydrated in serial alcohol solutions. The preparations were then immersed in propylenoxide, followed by propylenoxide Agar Resin 1:1 solution and embedded in Agar Resin and polymerized. One-micrometer-thick cross sections were cut with a microtome. The sections were stained with methylene blue and Azure II (Richardson's solution) or paraphenylene-diamidine (*paper II and paper III*) and examined by light microscopy.

Morphometry

Digital photomicrographs of each nerve section were processed with Adobe Photoshop® software and analyzed with the public domain NIH-Image software (Wayne Rasband, National Institutes of Health, USA). Using the 10x objective, the whole cross-section nerve area was outlined and measured. A grid in which each field corresponds to the area covered by the 100x objective was applied and a minimum of one out of four fields were selected and photographed in 100x objective. The micrographs were analyzed by NIH-Image and the Total Number of Fibers (TNFe;extrapolated), Mean Myelinated Area (MMA), Myelination ratio (M-ratio) and axonal diameter (d) were measured [145].

Immunocytochemistry and histochemistry

Neurofilament staining

On the 30^{th}, 60^{th} (*paper I)* or 240^{th} days (*papers II and III*), the rats were killed with a lethal dose of pentobarbital. The previously injured nerves, including 5 mm proximal and distal to the lesion or repair sides, were harvested. The nerve segments were fixed in Stefanini fixation (2% paraformaldehyde and 1.9% picric acid in 0.1M phosphate buffer (pH 7.2) for two hours and then washed and cryoprotected in 20% sucrose phosphate buffered saline (PBS). They were mounted in Tissue Tek® and cryosectioned longitudinally in 8μm thick sections. The nerve sections were incubated in methanol and 3% H_2O_2 for 30 min, washed in PBS for 15 min and soaked in horse serum for 20 min. The sections were incubated with primary antibody against neurofilament (NF 70kDa, DAKO, Denmark) (1:80) for 2h. For visualization, biotinylated, peroxidase conjugated

avidin-biotin complex (ABC; Vectastain, Vector Laboratories, USA) was used. The sections were stained with carbazole and treated with Mayer's Hematoxylin (HTX) and washed in tap water. At the end, they were mounted in Kaiser's glycerin and examined in light microscopy.

Cell bodies

At different time intervals for each project, a laminectomy was performed and dorsal root ganglia (DRGs) from C5 to T1 [in details: C7 –T1 (*paper I)*, C6-T1 (*paper II)*, C5-C8 (*paper III)* and C4-C7 and L4-L5 (*paper IV*)] as well as spinal cord levels from C3-T3 [*papers II, III and IV*] and the lumbar enlargement sections [L4-L5: *paper IV*] were harvested and prepared for different types of immunocytochemistry.

CPON

In *paper I*, DRGs from C7 to T1 were removed at days 6, 15, 21 and 30 from both the experimental and control sides. The dissected DRGs were immersed in Stefanini's fixative (see description above) for two hours, followed by washing and cryoprotection in 20% sucrose PBS. After mounting in Tissue Tek® (Sakura, Torence, USA), the DRGs were cut in 10µm thick sections. They were washed in PBS and exposed to a primary rabbit antibody against CPON (DAKO, Denmark), diluted 1:280 in PBS containing 0.25% Triton-X (Packard, Meridian, MS, USA) and 0.25% bovine serum albumin (BSA; Sigma-Aldrich). The sections were incubated at 4° C overnight. The sections were washed (3x5min in PBS) and exposed to fluorescein isothiocyanate (FITC) conjugated swine anti-rabbit immunoglobulins (DAKO) at a dilution of 1:80 in PBS for 1h at room temperature and in darkness. The sections were finally washed in PBS and mounted in 50% (v/v) glycerol in PBS for fluorescence microscopy.

ATF3

On day 7 all animals in *paper IV* were killed with a lethal dose of pentobarbital. For the forelimb, the musculocutaneous nerve, DRGs from C4 to C7 and spinal cord levels from C3-T3, from ipsi- and contralateral sides, were harvested. For the hind limb, sciatic nerves, DRGs L4 and L5 and lumbar enlargement sections (L4-L5) of the spinal cord were removed. All samples were fixed in Stefanini fixative for two hours. There were washed with 0.01M PBS (pH 7.4) and kept in 20% sucrose in 0.01M PBS at 4° C. Specimens were embedded in O.C.T. compound (Sakura Finetek Europe, Leidens, Netherlands) and frozen. Samples were cut longitudinally using a cryostat at 10µm thickness and mounted on glass slides. Segments were rinsed in PBS for 15min and blocked in 0.25 % Triton-X and 0.25 % BSA in PBS for 20 min. The sections incubated with

polyclonal rabbit anti-ATF3 antibody (1:200; Santa Cruz Biotechnology, CA, USA) in 0.25 % BSA and 0.25 % Triton-X in 0.01M PBS for 24 hours at 4° C. After washing with PBS, sections were incubated in Alexa Fluoro (488 conjugated goat anti-rabbit IgG (H+C)) antibody (2mg/ml, 1:500; Molecular Probes, OR, USA) in 0.01M PBS for one hour at room temperature. After washing with PBS, sections from the forelimb were mounted in glycerol/PBS (1:1), while sections from the hind limb group were incubated with bisbenzimid (Hoecht 33258 dye in 0.01M PBS, 60mg/ml) for 1min to detect nucleus. They were also mounted in glycerol/PBS.

Retrograde labeling

On the end point evaluation day for all groups in *papers II and III* (180^{th} and 240^{th}, accordingly) animals were re-anesthetized and the most distal parts of the median, ulnar, radial and musculocutaneous nerves were exposed. The distal part was cut, removed and prepared for histology and the cut ends were prepared for retrograde tracing. Thus, the nerve ends were isolated from the surroundings with a piece of parafilm. Crystals of Diamidino Yellow (DY) were carefully placed on the cut end of the median, ulnar and radial nerves, while, with a different set of instruments, crystals of Fast Blue (FB) were placed on the cut end of the musculocutaneous nerve. The parafilm was removed, and the labeled ends were isolated and buried in the surrounding tissues. The skin was sutured and the animals were allowed to recover. After another seven days, the rats were sacrificed with a lethal dose of pentobarbital and a laminectomy was performed. DRGs from C6-T1 (*paper II)* and from C5-C8 (*paper III)* were harvested along with spinal cord levels from C3-T3. The preparations were fixed in Stefanini fixation for two hours and cryoprotected in 20% sucrose PBS buffer. The spinal cords and the DRGs were mounted in Tissue Tek® (Sakura, Torrence, USA), and sections, 10µm and 8µm thick, respectively, were prepared. Labeled DY, FB and double labeled neuronal cell profiles in every 12^{th} spinal cord section (*paper II)* or every 4^{th} spinal cord and DRGs sections (*paper III*) for each animal were counted. Only neuronal profiles with a clearly stained nucleus were counted.

Image Analysis

All sections subjected to histology, immunocytochemistry, and retrograde labeling were studied in a Nikon FXA microscope equipped with fluorescence optics. Sections were photographed using Kodak DCS-100 or SPOT RT color (Diagnostic Instruments, Sterling Heights, USA) digital camera connected to a Power Macintosh computer equipped with Adobe Photoshop® software.

Statistical analyses

All values of pawprints (*papers I, II and III*) are presented as percentage of pre-operative value and expressed as mean (SEM; standard error of the mean). Repeated ANOVA, followed by Bonnferroni-Dunn post-hoc test, were used to compare the data from the contralateral and ipsilateral side and the time pattern within and between the experimental groups. End point values (*paper III*) were compared using un-paired t-test.

The number of CPON positive (*paper I*) and retrograde labeled (*papers II and III*) sensory neuronal profiles are presented in percentage of the total number of sensory neuronal profiles with a clear nucleus and expressed as mean (SEM). A two-factor ANOVA (factors: injury and time) was used in *paper I* to compare the CPON-positive sensory neurons after distal versus proximal injury over time. Chi^2-test was used to detect any difference regarding the distribution of retrograde labeled cell profiles in *paper III*.

For tetanic muscle force, muscle weight (*paper II*), the morphometry (*papers II and III*) and ATF3 induction (*paper IV*) in DRGs and in spinal cord, the values are presented as median (IQR=interquartile range) or median (min-max) for each group. The various groups in *paper IV* were pooled into the above mentioned three groups: 1) "No injury"; 2) "Nerve injury" and 3) "End-to side nerve repair". Mann-Whitney was used to detect differences between two groups (*paper II* and *III*) and the Kruskal-Wallis with subsequent Bonferroni correction for three groups [153] (*paper IV*) concerning these values. A p-value of less than 0.05 was considered as significant in all papers.

RESULTS AND COMMENTS

The forelimb of the rat as an experimental model for nerve regeneration after different nerve injuries (*paper I*)

In order to evaluate the possible use of the forelimb of the rat as an experimental model for studies of the nerve injuries at the brachial plexus and its branches, including the reaction of the cell body (CPON expression), a series of different types of nerve injuries were performed and the basic properties of regeneration in that system were investigated.

I used a simple method for evaluation - pawprints. A single lesion to the median or the ulnar nerves showed no effect on the pawprints. In all other experiments there was a decrease in toe spreading to a variable extent and thereafter a recovery depending on the severity of the lesion. Pawprints (all values for 1^{st}-4^{th} toes, but 2^{nd}-3^{rd} toes showed essentially the same pattern) after *a crush injury* exhibited normal values in 21 days for both median and ulnar nerves and in 14 days for the radial nerve. *Transection and end-to-end repair* showed an improvement up to 88% of preoperative control value and almost full recovery when the median and ulnar nerves or the radial nerve, respectively, were injured and repaired. A *graft interposition* showed a return in pawprint function up to 75% and 84% for the median/ulnar and the radial nerves, respectively. The pawprints were also a useful tool in evaluating the effect of *a conditioning lesion* to both motor and sensory axons where the recovery of pawprint values exhibited the classical conditioning lesion effect, i.e. an improved regenerating capability following a test crush lesion in all the tested nerves [50, 92, 111]. Surprisingly, a conditioning lesion of preferential sensory axons of the median and ulnar nerves distal to the elbow had almost the same effect.

By immunocytochemistry and conventional histology all nerves showed the same pattern of nerve regeneration as in any other similar experiment in the hind limb of the rat (see for example [37, 88, 165, 175, 182, 191], i.e. a crush lesion revealed almost no difference from an uninjured nerve; a transected and repaired nerve showed misdirection of axons; in a nerve graft the fascicular organization was disturbed with less axons and many axons grow also on the outside of the graft.

CPON was used as a marker of the cell body reaction in DRGs C7-T1. A number of CPON-immunoreactive sensory neurons (up to 23% on day 14) were found in the DRGs corresponding to the inflicted nerves after a crush injury. In contrast, few cells exhibited positive staining in the contralateral uninjured side. The CPON reaction was also dependent on the distance of the lesion to the cell body with a significant more profound effect after a more proximal lesion (14% versus 6% for the more distally crushed median and ulnar nerves on day 14^{th}; pooled data).

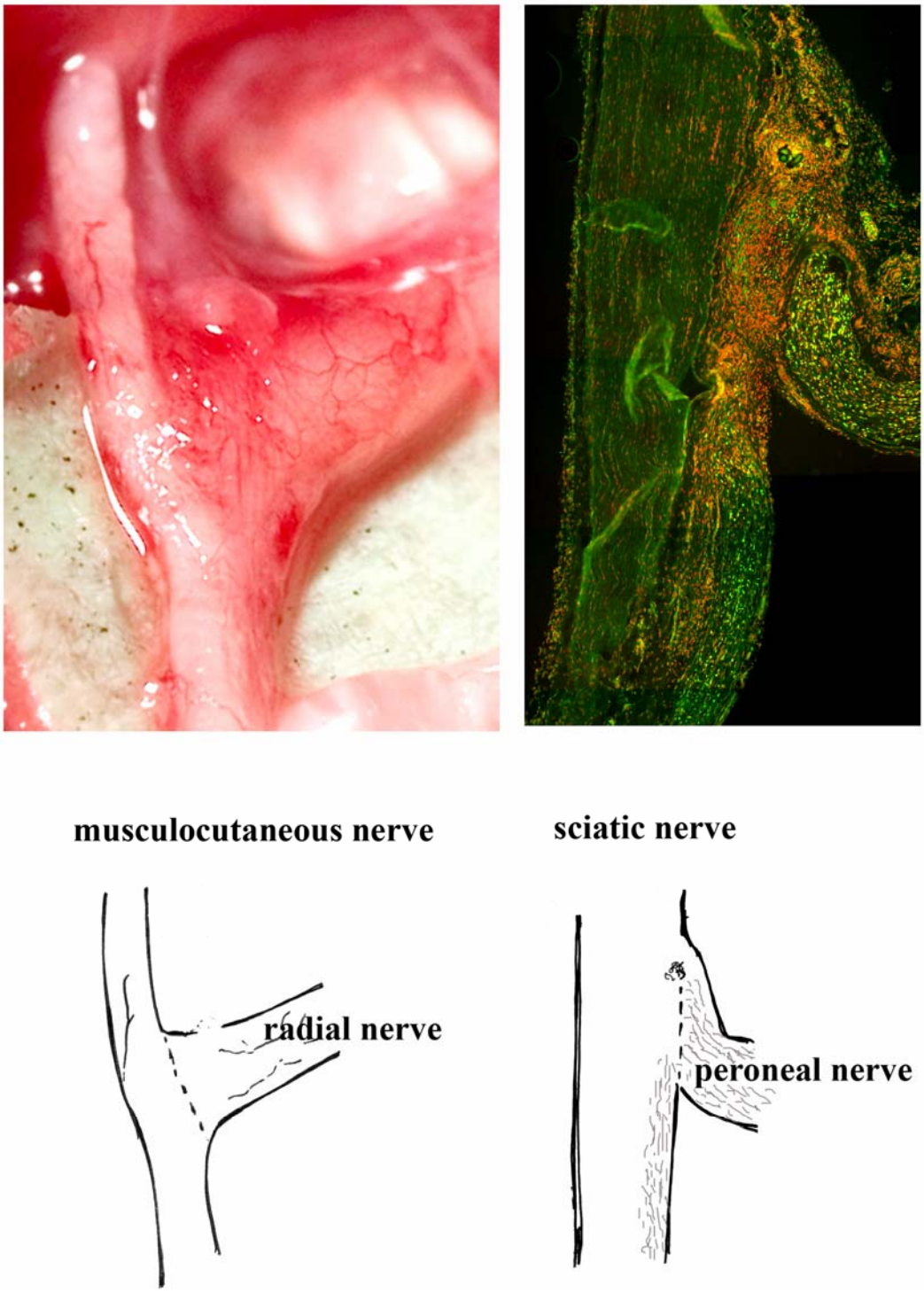

Figure 2

End-to-side nerve repair: a: macroscopic picture of a distal radial nerve attached end-to-side to the musculocutaneous nerve in a rat (upper left) and a schematic drawing of the reconstruction (lower left), b: microscopical picture of a longitudinally sectioned sciatic nerve (donor) with a segment of the peroneal nerve (recipient) attached end-to-side (right side), stained for the transcription factor ATF3 (upper right) and a schematic drawing of the reconstruction (lower right). Note the positive staining (green) for ATF3 in the peroneal nerve segment and the adjacent part of the sciatic nerve.

End-to-side nerve repair in the forelimb of the rat (p*aper II*)

The rapid recovery of forelimb function was taken advantage of to study the source, type and extent of regenerating fibers after end-to-side nerve repair. To this end the musculocutaneous nerve was used as the donor nerve and the radial or both the median and ulnar nerves served as the recipients that were attached end-to-side on a three-mm long epineurial window on the donor nerve. The pawprints (1^{st}-4^{th}) revealed the lowest function on day 45 for the radial nerve group with a value of 46% of preoperative control value and a recovery to 72% of preoperative control values at the end evaluation point on day 180. The corresponding values for the median/ulnar nerves were 48% on day 14 and 60% at the end. The results for 2^{nd}-3^{rd} toes showed essentially the same pattern. Injured but non-repaired rats showed a slight compensation in walking tracks, but no real recovery.

Morphology of the distal nerve segment harvested at the time of retrograde labeling showed evidence of axonal outgrowth in both the radial and the median/ulnar nerves. The most distal parts of the attached nerves were predominated by smaller axons that were assembled into clusters. The morphometrical analysis showed that the TNFe in the median/ulnar group was significantly higher than that in the radial nerve, while the MMA was higher in the later group. The myelination ratio and the axonal diameter revealed no statistically significant difference between the end-to-side attached nerves.

Retrograde labeling was used to detect the origin of the regenerating fibers into the recipient nerves. The number of double labeled motor neuronal cell profiles, corresponding to collateral sprouting, was very low irrespective if a radial or a median/ulnar nerves were attached end-to-side. When the radial nerve was attached end-to-side to the musculocutaneous the number of DY positive neuronal profiles were 146 in total of five rats, the FB positive cells 234 and only three neuronal profiles were double labeled. In the group, where the median and ulnar nerves were end-to-side repaired to the musculocutaneous, the corresponding values were 199 DY positive, 179 FB positive and again only nine double labeled motor neuronal profiles. In the sensory neuronal pool the results were about the same with mainly cell profiles labeled with only one dye and only 1.9% and 3.3% of the total number of cells double labeled in the radial and median/ulnar nerve group, respectively. The data suggest that both motor and sensory neurons send axons into the end-to-side attached nerves. However, the few double-labeled cells indicate either that collateral sprouting is not the only mechanism by which fibers could be recruited or that pruning has occurred.

Electrical stimulation of the end-to-side attached nerve/s elicited contractions in the muscles normally innervated by these recipient nerves with a median tetanic muscle force of 63-70% of the contralateral side. There was no difference in percent of contralateral side between a radial or a

median/ulnar nerve as a recipient with respect to tetanic muscle force or muscle weight. In the non-repaired group of animals, no response could be elicited.

A novel finding in my study was that, with end-to-side nerve repair, the donor nerve can reinnervate the territory of more than one recipient nerve.

…could the proximal nerve segment of an injured nerve be a second source of regenerating nerve fibers? (*paper III*)

A group of rats, where only the distal segment of the radial nerve was attached end-to-side to an epineurial window of the musculocutaneous nerve, was compared with another group, where also the proximal radial nerve segment was attached end-to-side to the musculocutaneous as an extra source of axons. The values of the distance between the 1st and the 4th toes dropped down to 47% and 41% in the classical end-to-side and the double attached end-to-side group, respectively. Function improved gradually with the end point value on day 240th reaching 69% and 54%, accordingly. As expressed statistically the pawprint evaluation showed no time independent effect (repeated ANOVA) of the surgical procedure but there was a significant time effect. However, the repeated ANOVA showed a significant interaction between the repair method and time, i.e. the effect of time was different between the two groups. The end-point values showed only a statistically significant difference regarding toe distances between 2nd – 3rd toes, in favor of a single attached distal nerve segment.

Using retrograde labeling, only few double-labeled motor neuronal cell profiles were counted in the group where only the distal radial nerve segment was attached and no double-labeled cells at all were detected in the group where both the distal and the proximal radial nerve segments were attached. As for the sensory neuronal pool very few double-labeled cells were seen in either group of experiments. However, the distribution of the DY and FB positive neuronal cell profiles was different (except for FB and DRGs) between the two groups as well as in the sensory and the motor neuronal pool. The phenomenon that there was a shift of DY labeled neurons towards higher levels when a proximal radial nerve was attached; I have no reasonable explanation to offer for this finding, but the same pattern, with mainly DY labeled cell profiles in lower segments when only a single distal nerve segment was attached, was also seen in *paper II* and *paper III*.

In morphometrical analyses the two groups revealed no statistically significant difference concerning TNFe, MMA or axonal diameter, while a larger axonal area (higher M-ratio) was seen for the group where both a proximal and a distal segment was attached. Furthermore, morphometrically, there was no difference of the donor musculocutaneous nerve.

Considering our results, it seems that the attachment of a proximal nerve segment did not improve recovery as compared to an end-to-side nerve repair with a single distal nerve segment.

Nuclear translocation of ATF3 as a marker for cell activation and injury after different manipulations (*paper IV*)

In *paper IV* I rose the question what types of manipulations associated with end-to-side nerve repair could result in activation of neurons and non-neuronal cells. Different types of injuries and manipulations to the musculocutaneous and sciatic nerves of the rat and their effect on ATF3, a trancription factor, were investigated. In the *cervical DRGs* ATF3 immunoreactive sensory neurons were rare when a piece of muscle or nerve was placed alongside the musculocutaneous nerve. When any type of injury (epineurial window with or without sutures) was performed to the musculocutaneous nerve, the ATF3 positive sensory neuronal profiles increased significantly. This was true also when an end-to-side nerve repair was done. In the lumbar DRGs the observed pattern was similar, where there was a significant difference with respect to severity of the manipulation. In the *spinal cord levels*, ATF3 positive neuronal cell profiles were found in the ventral horn to a variable extent in the different groups. In general, there was a significant profound response when any type of injury (epineurial window with or without sutures and end-to-side nerve repair) was performed to the musculocutaneous nerve as compared to "no injury" (piece of muscle or nerve alongside). At the lumbar level, ATF3 was also expressed but there was no difference between the three groups.

No induction of ATF3 in non-neuronal cell nuclei was found in the endoneurium from the "no injury group", while application of an epineurial window with or without sutures induced ATF3 induction at the lateral part of these nerves (Fig. 2). In accordance, end-to-side nerve repair induced ATF3 in non-neuronal cell profiles at the side of attachment indicating cell activation.

A certain amount of manipulation, i.e. application of an epineurial window/sutures with or without a nerve segment attached end-to-side, was needed to activate neurons in DRG and spinal cord and locally in the nerve, but a piece of nerve or muscle applied alongside the nerve was not enough to activate the neurons and the non-neuronal cells.

GENERAL DISCUSSION

The importance of the hand as a useful tool for work, well being, art and expression is pin pointed to every single moment in our everyday life, as well as in a plethora of research, clinical and epidemiological studies. Nerve injuries, depending on their severity, have from mild to dramatic consequences for the patient as well as costs for the community.

The forelimb as an experimental model to study nerve regeneration

The sciatic nerve of the rat is the dominant model for studies of peripheral nerve regeneration. However, with respect to that the majority of human peripheral nerve injuries affect the upper extremity, the necessity of an experimental model closer to my interest as a hand surgeon emerged. Few reports are available describing the anatomy of the rat brachial plexus [15]. I speculated that since the distances in the forelimb of rats are much shorter, a fact that makes any study quicker and the complexity of the intercommunicating nerve trunks gives us the possibility of studying selective injuries to motor or sensory branches. The aim of my first study was to delineate basic regenerative measures following different types of nerve injuries. Meanwhile, the evaluation systems available for the forelimb of the rat are complex, require extensive training or manipulation of animals and are not always objective [13, 14, 42, 68, 169]. To this point *pawprint* evaluation was used to investigate functional return after different types of injuries and repair. Pawprints are a simplification of the more complex walking track mainly used for evaluation of function after injury to the sciatic nerve in the hind limb. I could demonstrate the feasibility of this method to evaluate function in the forelimb. Pawprints has proved to be a simple, reproducible and accurate method for functional evaluation. One weak point of the method is that injury to either the median or the ulnar nerve had little or no effect on toe spreading, indicating the presence of unique compensatory mechanisms for toe spreading in the rat, or the existence of branching between the two nerves. In the first set of experiments, recovery of function assessed by pawprints was much faster and better after crush injuries than after transection and direct repair or after nerve grafting as anticipated. The latter repair methods resulted in an incomplete functional recovery. Conventional histology of the nerves sustained a crush injury revealed no misdirected fibers while an increasing amount of them were observed in the transection/repair and the nerve graft groups. The results are similar to the ones obtained from other studies on the rat sciatic nerve model. The only differences are the advantage of the forelimb model with respect to time – the recovery was faster – and the absence of articular contractures or autotomy [16, 171, 172].

Injuries to the nerve of the forelimb exhibited the classical *conditioning lesion* effect, i.e. an increased regenerative capability following a crush test lesion, as seen in the sciatic model [32, 110, 111, 148, 149]. A surprising outcome was that a conditioning lesion to the preferentially sensory part of the nerves resulted also in a faster recovery of pawprints after a crush injury. This observation can be explained by the influence of releasing substances from the conditioned sensory neurons to the injured motor neurons at any level of the neural pathway.

There are reports about the distance-dependent neuronal activation and cell death after a nerve injury [157, 186]. As a marker of neuronal activation and injury in the DRGs, I used CPON and I found a massive increase of CPON-positive sensory neurons after a crush injury. The reaction was even more dramatic when the crush was performed at a proximal level, closer to the cell bodies. These results are similar to those of other authors using nerve transection [179]. In spite of that the branches of the brachial plexus are smaller than the sciatic nerve, the basic reactions and regenerative properties after injury and repair were similar to the sciatic nerve model.

End-to-side nerve repair

The effectiveness of end-to-side nerve repair has been the subject of discussions. Those who favor the technique describe both sensory and motor recovery [53, 85, 101, 125] while opponents talk about poor or no recovery at all [11, 109]. In *papers II and III* I clearly demonstrated that integrated function, assayed by pawprints, does return after end-to-side to 60-72% of preoperative value, a percentage that merits attention to the technique if no other alternative repair method is available. The same results were obtained for tetanic muscle force of the extensor carpi radialis brevis/longus andor the flexor carpi radialis muscles in the rats, where the radial or the median and ulnar nerves were attached end-to-side to the musculocutaneous nerve, respectively. Thus, motor fibers from the donor nerve must have entered the end-to-side attached nerve segment and they have reached the target organs previously innervated by the recipient nerves. Using retrograde labeling, I also found that both sensory and motor neurons send axons that remyelinated into the end-to-side attached nerves.

Retrograde labeling for evaluating the origin of the regenerating fibers

It has been suggested that the main explanation for the regeneration into the end-to-side attached nerve is collateral sprouting [8, 86]**.** Using the retrograde labeling technique, I observed a really small amount of double-labeled cells both in the DRGs and in the spinal cord. Six months after end-to-side nerve repair, only 2-7% of sensory neurons were double labeled (*paper II*). At eight months (*paper III*), the percentage was even lower reaching 1-2% of the total amount of

sensory neurons in the DRGs. Similar results were obtained from the measurements in the spinal cord where very few double labeled motor neuronal cell profiles were found. Retrograde labeling, thus, demonstrated that collateral sprouting does occur to some extent and that both motor and sensory neurons send axons to an end-to-side attached nerve. A crude estimate of the extent of motor and sensory reinnervation showed an advantage of sensory above motor, results that are in accordance with those of others [106, 142]. As for the low number of double labeled cells a couple of explanations can be given. The data suggests that either collateral sprouting is not the only mechanism or that pruning of the axons in the donor nerve has occurred [140]. The results from *paper IV* indicate that an epineurial window with or without sutures and attachment of a nerve segment activate neurons in DRG and spinal cord as well as non-neuronal cells at the side where the injury was applied. This indicates that terminal sprouting may occur directly from the level of attachment. The pruning theory is supported by my other findings since the number of double labeled cells in the DRGs decreased by time from 2-7% to 1-2% after six and eight months observation, respectively.

Two recipients

A novel finding in *paper II* is that one donor nerve could nurture two end-to-side attached nerves. When suturing both median and ulnar nerves to the musculocutaneous nerve the functional recovery assessed by pawprints and tetanic muscle force was similar to the one obtained from the group where the musculocutaneous nerve served as the donor only to the radial nerve. In addition, no obvious differences were seen regarding the sensory and motor reinnervation, evaluated by double labeling. Again, reinnervation of both motor and sensory neurons occurred, with collateral sprouting still being apparently one but not the main mechanism for regeneration. The only differences between the two groups (one or two recipient nerves) were found in morphometrical analysis. More nerve fibers in the recipient nerve were measured in the median/ulnar group but in a lower stage of maturation, since the myelin area per fiber was significantly lower than in the radial nerve. Comparing those results with the functional outcome we can keep pace with the findings of other researchers that have stated that functional recovery does not always reflect the number of regenerating fibers [145, 168].

Proximal nerve segment

In end-to-side nerve repair experiments, unintended recruitment of axons from the proximal segment of an injured nerve, the distal segment of which is attached end-to-side to a donor nerve, cannot be ruled out. Having this in mind, I speculated of the potential benefit of the use of this

proximal segment if it was attached on the same donor nerve as well. However, in *paper III*, I found that the attachment of the proximal segment did not improve recovery as compared to an end-to-side nerve repair with only the distal nerve segment attached [107, 108]. From pawprints, I found that there was no significant time-dependent effect of surgical procedure per se but only a significant interaction between the surgical procedure and time. Regarding morphometry, no advantage could be gained from the hypothesized recruitment of axons from the proximal nerve segment. An interesting finding though, from double labeling, is a shift towards higher levels of the distribution of DY labeled sensory and motor neurons when the proximal nerve segment was also attached. For this observation I have no reasonable explanation to offer. The distance between the proximal and distal nerve segments attached to the donor nerve may also be important [107].

Origin of regenerating fibers

Based on the results in this thesis, one may suggest that end-to-side nerve repair is an alternative in nerve reconstruction but the mechanisms by which neurons and non-neuronal cells are activated and thereby initiate axonal outgrowth into the attached nerve segment is still not clarified. Most likely collateral sprouting is not only the mechanism by which axons advance into the distal nerve segment. Various authors have suggested that a type of "manipulation", such as incision to the donor nerve [125], application of sutures [162], induction of a window [123, 140, 185] or factors emanating from a denervated nerve [163], is needed on the donor nerve in order for axons to be recruited into the attached nerve segment. To shed light on the mechanism, I investigated what type of end-to-side related stimuli is required to initiate cell activation, such as nuclear translocation of ATF3 (*paper IV*). I showed that creation of an epineurial window and/or sutures with or without attachment of a piece of mixed or sensory nerve induced ATF3 in neurons and non-neuronal cells in DRGs, spinal cord and locally in the donor nerve trunk. In contrast, application of a piece of muscle or nerve alongside a 'donor' nerve did not induce any type of ATF3 activation in the cell nuclei. Diffusible factors released from the denervated nerve segment or muscle could potentially affect the donor nerve. However, loss of target-derived factors, like NGF and GDNF [7]**,** can induce activation of ATF3 and therefore application of a piece of muscle close to the donor nerve has no reason to induce ATF3. One cannot exclude that factors released from a denervated piece of nerve applied end-to-side to a donor nerve may induce ATF3. The latter seems to have increased the number of positive ATF3 nuclei in some experimental settings (*paper IV*). In the same study the number of positive ATF3 neuronal profiles in the DRGs and the spinal cord were quantified and, compared with the double labeled nuclei from our previous experiments, I can say that there seems to be a different sensitivity of motor and sensory neurons [85, 141]. The induction of ATF3 in some

parts of the nerve trunk may also be an indication of Wallerian degeneration of fibers in the donor nerve [23]. It is therefore reasonable to suggest that an injury to the donor nerve in an end-to-side nerve repair is a prerequisite for activation of neurons and non-neuronal cells leading to terminal sprouting of injured axons into the recipient nerve.

Clinical applications

The need for alternative nerve repair methods after extensive nerve injuries has since long been expressed. The merits of the end-to-side nerve repair have been extensively discussed. In this thesis, I have tried to give some answers to the question that emerge from the use of this technique. Since the ultimate goal for any nerve repair is the return of function, I can say from the present results that end-to-side nerve repair is indeed an alternative method in our quiver when no other repair options are possible in the upper extremity. During the last years, from Viterbo's studies until now, an increasing number of surgeons has started to use end-to-side in the clinical practice [52-54, 85, 97, 112, 129]. The present results lend support to the clinical use of end-to-side nerve repair during some circumstances. It is possible that the technique may be more appropriate in reconstruction of some specific nerves in the upper extremity.

The Future

A better understanding of the mechanisms by which end-to-side nerve repair is based on is important. It is also essential that any loss of function in the donor nerve is diminished if the method should be used. Furthermore, in end-to-side nerve repair, but also in conventional nerve repair and reconstruction, the cortical reorganization that occurs in humans after peripheral nerve injuries play a pivotal role during the early and late phase of the rehabilitation of the injury. Plasticity changes do occur at multiple levels [25, 96]. Cortical input-output relationships are continually reshaped throughout life [143] and there is a challenge to the brain to handle these phenomena when a collateral sprouting mechanism is involved. The events taking place in CNS after different types of nerve repair and reconstruction is a new road for research.

"I don't ponder, I don't measure, I don't find a snug berth. I follow my deep heartbeat"

N.Kazantzakis

CONCLUSIONS

- The forelimb of the rat with the brachial plexus and its branches is an excellent experimental model to study nerve regeneration. Functional recovery is rapid and can be evaluated by pawprints. The proximity of the nerve trunks in the forelimb also makes it a suitable model for studies of end-to-side nerve repair. As anticipated functional recovery was dependent on the severity and the level of the lesion and a conditioning lesion lead to quicker recovery. Interestingly, both an injury, preceding the nerve lesion, to motor and sensory axons or preferentially to sensory axons acted as a conditioning lesion. CPON expression could be used for studies of the cell body reaction and its dependence on the level of the lesion from the cell bodies.

- End-to-side nerve repair results in functional recovery, up to around 60-70% of preoperative value with respect to pawprints and tetanic muscle force. Myelinated motor and sensory fibers were recruited from the donor nerve and present in the end-to-side attached recipient nerve. Collateral sprouting is one but probably not the only mechanism underlying end-to-side nerve repair efficacy. One donor nerve can reinnervate the territory of two recipient nerves.

- Attachment of the proximal nerve segment of an injured nerve to the same donor nerve as the distal recipient nerve segment as an extra source of axons resulted also in axonal outgrowth and some functional recovery but did not improve recovery. There was a difference in the distribution of retrograde labeled cell profiles after attachment of a proximal and a distal nerve segment vs. only a distal nerve segment, which indicates different sources of axons in the two models.

- A limited injury to a donor nerve, inflicted during the repair method or unintentionally, seems to be a prerequisite for activation of neurons and non-neuronal cells, leading to sprouting of axons in an end-to-side attached nerve.

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«Νόσημα γάρ αίσχιστον είναι φημι συνθέτους λόγους»
'There is nothing worse than the beautiful words that deceive you'

AESCHELUS (Prom. Desm., 685-686)

SVENSK SAMMANFATTNING (SUMMARY IN SWEDISH)

De perifera nervstammarna i extremiteterna förmedlar signaler, dels till muskler, dels från känselkroppar som är belägna i bl a huden. Nervstammarna kan skadas av vassa eller skärande föremål och genom olika typer av slitvåld. Dessa skador ger ett mycket stort handikapp för den enskilde patienten, som förlorar muskelfunktion och känsel i extremiteten. Dessutom förorsakar skadorna också kostnader för samhället. Ett särskilt problem är de skador som drabbar de olika delarna i armens nervfläta uppe runt skulderregionen med mycket omfattande funktionspåverkan som följd.

Efter en nervskada försvinner (degenererar) den del av nervtråden som ligger bortom nervskadan medan bindvävskomponenterna (stommen) i själva nervstammen finns kvar. Vid de enklare avskärningsskadorna kan nervstammens hölje sys ihop ända mot ända varefter de skadade nervtrådarna växer ut på nytt. Utväxten blir oftast ofullständig pga att många nervtrådar växer fel och att många nervceller dör av skadan.

Vid mer omfattande nervskador uppstår en defekt i den skadade nervstammen. Detta är särskilt vanligt vid nervskador i armens nervfläta. Alternativet är då att använda en mindre viktig nerv, exempelvis från baksidan av underbenet, som förflyttas (transplanteras) och läggs som en kabel (nervgraft) mellan den skadade nervstammens ändar. Nervtrådarna använder sedan nervgraftet som en bro för att nå den nedre delen av nervstammen. Ibland kan skadan vara så omfattande att den övre nervstammen saknas. Den föreliggande avhandlingen fokuserar på möjligheten att i sådana fall koppla den nedre nervstammen ända mot sida till en annan och helt intakt nervstam för att på så sätt ”locka ut” eller ”låna” nervfibrer och därmed få viss funktion i den skadade nervstammens försörjningsområde.

I den första delen av avhandlingen studerades återkomst av funktion efter nervskador på råttans framben. Tre olika nerver i frambenet (sk medianus, ulnaris och radialisnerverna) skadades på olika sätt (krosskada, avskärning som reparerats traditionellt ända mot ända, avskärning som reparerats med nervgraft). Återkomsten av funktionen avspeglades i råttans gångförmåga, särkilt tassavtrycken, och var beroende av skadans omfattning. Nervcellskropparna till de nervceller som förmedlar känselimpulser påverkades mer om skadan var lokaliserad nära nervcellskroppen än om den var belägen mer perifert. Detta kunde visas med hjälp av antikroppsteknik för att åskådliggöra förekomst av en nervskademarkör (CPON). Om en nervstam utsätts för en skada vid ett tillfälle samt efter några dagar för ytterligare en skada har nerven ”konditionerats” varefter funktionsåterkomsten är mycket snabbare. Den första delen av avhandlingen visar att nervflätan och dess nervgrenar hos råtta är en lämplig modell att studera andra typer av reparationsmetoder.

I den andra, och mer omfattande delen, av avhandlingen fokuserades på möjligheten att rekonstruera en nervskada genom att använda den nedre nervändan (av exv. radialis, medianus och ulnarisnerv), där den övre nervändan saknas, och koppla denna ända mot sida till en intakt för övrigt oskadad nerv (i modellen användes den sk muskulocutaneusnerven). Detta kallas ”end-to-side” nervreparation.

Efter reparationen ända mot sida (end-to-side) noterades att såväl känsel- som muskelnervceller från den intakta donatornerven skickade nervtrådar ner i den nervstam som kopplades ända mot sida. Funktionen i frambenet och framfoten återkom till viss del vilket kunde ses på tassavtrycken. Det var möjligt att koppla två stycken skadade nervstammar till en intakt donatornerv. Nervtrådar växte ut i båda de kopplade nervstammarna.

Olika färgämnen användes för att studera ursprunget till de nervtrådar som växte ner i den kopplade nervstammen. Resultatet visade att få nervceller i donatornerven skickade ut nervtrådar i den kopplade nervstammen samtidigt som den ursprungliga nervtråden var intakt (sk kollateral sprouting eller utväxt). Andra mekanismer än kollateral utväxt ligger bakom funktionsåterkomsten. Det fanns inga fördelar med att också koppla en nervfiberinnehållande nervstam ända mot sida som en extra resurs av nervtrådar mer centralt på donatornerven. Den använda donatornerven fungerade därmed som en nervbro (nervgraft).

En speciell nervskademarkör (sk transkriptionsfaktor ATF3) användes för att studera vilken typ av behandling av den donatornerven som krävs för att aktivera denna till nervfiberutväxt. Det krävs någon form av ”skada” på donatornervstammen, som exempelvis skapande av ett litet fönster i nervens bindväv och/eller tillsats av små suturtrådar med eller utan en kopplad nervstam ända mot sida för att aktivera donatornerven. Att bara lägga en bit muskel eller sena (utan annan nervmanipulation) längs med donatornerven ger ingen cellaktivering i donatornerven eller i dess nervcellskroppar.

Sammanfattningsvis visar resultaten i avhandlingen, som är ett samarbete mellan Sverige och Grekland, att koppling av en skadad nervstam, som saknar sin övre del, ända mot sida (end-to-side) till en intakt sk donatornerv är en användbar metod att rekonstruera omfattande nervskador, särskilt inom armens nervfläta.

ABSTRACT IN GREEK (ΠΕΡΙΛΗΨΗ)

Οι νευρικές κακώσεις έχουν σημαντικό αντίκτυπο όχι μόνο στη ζωή του ατόμου, αλλά και στην κοινωνία. Ο 'ιδανικός' στόχος για κάθε μέθοδο νευρικής αποκατάστασης είναι η επιστροφή της λειτουργικότητας. Όταν αντιμετωπίζουμε σοβαρές νευρικές βλάβες, που δημιουργούν κενό μεταξύ των νευρικών τμημάτων, ή όταν η κάκωση αφορά κλάδους του βραχιονίου πλέγματος, οι εναλλακτικές μέθοδοι θεραπείας που διαθέτουμε σαν κλινικοί γιατροί είναι περιορισμένες. Στην παρούσα διατριβή, χρησιμοποιήθηκε ένα πειραματικό μοντέλο που αντιστοιχεί στα ιδιαίτερα χαρακτηριστικά του βραχιονίου πλέγματος. Διαφορετικού είδους κακώσεις και μέθοδοι θεραπείας πραγματοποιήθηκαν στο άνω άκρο του ποντικού και ιδιαίτερη έμφαση δόθηκε στην τελικοπλαγία νευροραφή, δηλ. όταν το περιφερεικό τμήμα ενός διατμιθέντος νεύρου συνδέεται τελικοπλάγια σε ένα ακέραιο νευρικό κορμό. Το κερκιδικό ή αμφότερα το μέσο και το ωλένιο νεύρο υποβλήθηκαν σε διάφορες βλάβες: συνθλιπτική κάκωση, διατομή και κλασσική τελικοτελική συρραφή, διατομή και παρεμβολή νευρικού μοσχεύματος και διατομή και τελικοπλαγία νευρορραφή. Στο τελευταίο πρωτόκολο, το περιφερεικό τμήμα του τραυματισμένου νεύρου ενώθηκε τελικοπλάγια στο σύστοιχο ακέραιο μυοδερματικό νεύρο. Αποτυπώματα ΄παλάμης' (pawprints) και μυική τετανική ισχύς, αναλύθηκαν, έξι μήνες μετεγχειρητικά, για την εκτίμηση της λειτουργικής αποκατάστασης. Ανοσοιστοχημεία του C- τερματικού πλαγίου πεπτιδίου του νευροπεπτιδίου Y (CPON; C-terminal flanking peptide of NPY), ενεργοποίηση της εγγραφής του παράγοντα 3 (ATF3; Activating transcription factor 3; Δείκτης κυτταρικής δραστηριότητας), και χρώση για λεπτές πρωτεινικές ίνες νευρικού κυττάρου (neurofilaments), χρησιμοποιήθηκαν για εξέταση της αντίδρασης της βασικής οργανικής μονάδας και για την εκτίμηση της νευρικής αναπαραγωγής. Ανάδρομος ισοτοπικός προσδιορισμός χρησιμοποιήθηκε για τον εντοπισμό της πηγής των ανανευρούμενων νευρικών ινών και μορφομετρία για την ανάλυση της ποιότητας των αναγεννημένων νευρικών ινών στα νεύρα δέκτες. Το μοτίβο της νευρικής αναγέννησης ήταν ανάλογο με την σοβαρότητα της νευρικής βλάβης και την μέθοδο αποκατάστασης αυτής. Μια 'βλάβη προετοιμασίας' είχε το κλασσικό αποτέλεσμα της βράχυνσης του χρόνου ανάνηψης. Στην τελικοπλαγία νευρική συρραφή αμφότερες μυικές και αισθητικές νευρικές ίνες έστειλαν νευράξονες στα νεύρα δέκτες. Ο ανάδρομος ισοτοπικός προσδιορισμός έδειξε ελάχιστα διπλά ισότοπα, γεγονός που υποδεικνύει ότι η έμμεση και παράλληλη ανάπτυξη (collateral sprouting) είναι ένας μόνο μηχανισμός με τον οποίον η νευρική αναγέννηση συμβαίνει. Δύο νεύρα δέκτες μπορούν να υποστηρικτούν από ένα νεύρο δότη. Δεν υπήρξε κανένα πλεονέκτημα στην ανανευρωτική ικανότητα μετά από την συρραφή και του κεντρικού νευρικού τμήματος στο ίδιο νεύρο δότη, ως προσπάθεια επιπρόσθετης πηγής νευραξόνων. Η έκφραση του CPON στις κεντρικές νευρικές μονάδες, είναι ανάλογη του επιπέδου της βλάβης. Η επαγωγή του ATF3 στους νευρώνες, στα ραχιαία γάγγλια και στην σπονδυλική δτήλη ανέδειξε ότι η εφαρμογή ενός επινευρικού παραθύρου και/ή ραμμάτων, με ή χωρις την σύγχρονη συρραφή νεύρου σε ένα δυνητικόνεύρο δότη είναι απαραίτητα για την ενεργοποίηση του ATF3, ενώ η εναπόθεση τμήματος μυός ή νεύρου παραπλεύρως ενός δυνητικού νεύρου δότη δεν είχε καμία επίδραση. Το γεγονός αυτό υποδηλώνει ότι κάποιου βαθμού κάκωση στο νεύρο δότη είναι προυπόθεση για την ενεργοποίηση των νευρικών και μη κυττάρων που θα έχει ως επακόλουθο την 'βλάστηση' νευρικών ινών στα νεύρα δέκτες. Η τελικοπλαγία νευρική συρραφή είναι μια εναλλακτική μέθοδος αποκατάστασης σοβαρών νευρικών κακώσεων, σε περίπτωση που το κεντρικό νευρικό κομμάτι δεν είναι διαθέσιμο ως πηγή νευρικής αναγέννησης.

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