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Cover illustration:

By Gerty Widerberg-Sonidsson

"Först Eftertanke och sedan Hårt Arbete"

I-or

Contents

List of publications

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

I. **Widerberg A**, Lundborg G, Dahlin LB. Nerve regeneration enhancement by tourniquet. J Hand Surg 26B, 347-351, 2001.

II. **Widerberg A**, Kanje M, Dahlin LB. Tourniquet compression: a non-invasive method to enhance nerve regeneration in nerve grafts (accepted for publication in NeuroReport (13) 4, 2002).

III. **Widerberg A**, Kanje M, Dahlin LB. C-terminal flanking peptide of neuropeptide Y in DRG following nerve compression. NeuroReport (12) 15: 3193-3196, 2001.

IV. Bergman S, **Widerberg A**, Danielsen N, Lundborg G, Dahlin LB. Nerve regeneration in nerve grafts conditioned by vibration exposure. Rest Neurol Neurosci 7: 165-169, 1995.

V. **Widerberg A**, Bergman S, Danielsen N, Lundborg G, Dahlin LB. Nerve injury induced by vibration: prevention of the effect of a conditioning lesion by D600, a Ca^{2+} channel blocker. Occup Environ Med 54: 312-315, 1997.

Permission to reprint the articles has been granted from the following publishers: Churchill Livingstone (I), Lippincott Williams & Wilkins (II-III), IOS Press (IV) and BMJ Publishing Group (V).

Thesis at a glance

Paper I

Question: *Can tourniquet compression act as a conditioning lesion, i.e. increase the regenerative capacity of the sciatic nerve?*

Method: *Unilateral compression (300 mmHg, 30 or 120 min) of the hindlimb.*

Conditioning intervals: *0, 3, 6 days.*

Surgery: *Bilateral test crush lesions (contralateral side as control).*

Evaluation time: *3 days post test crush lesion.*

Evaluation method: *Pinch reflex test.*

Conclusion: *Tourniquet compression induced a conditioning lesion effect measured as an increased regeneration length following the test crush lesion. The effect was most pronounced after compression for 120 min and a conditioning interval of 6 days.*

Paper II

Question: *Can sciatic nerve regeneration be enhanced in nerve grafts conditioned by tourniquet compression? Does tourniquet compression activate, i.e. induce proliferation, of non-neural cells?*

Method: *Unilateral compression (0,150, 200, 300 mmHg, 120 min) of the hindlimb.*

Conditioning interval: *6 days.*

Surgery: *A 10 mm long sciatic or tibial nerve graft, pre-conditioned by tourniquet compression, was used to bridge a freshly made nerve defect on the contralateral non-compressed side*

Evaluation time: *6 days post grafting, and for tibial grafts 8 days.*

Evaluation method: *Pinch reflex test, immunocytochemistry (BrdU and p75 receptor).*

Conclusion: *Tourniquet compression at 150 mmHg was sufficient to induce proliferation of non-neuronal cells and expression of the p75 receptor but did not increase regeneration distances into nerve grafts. Regeneration distances were significantly increased within nerve grafts taken from hindlimbs compressed with a tourniquet at 200 and 300 mmHg.*

Paper III

Question: *Can the expression of an injury related neuropeptide (CPON) in DRG neurons be altered by tourniquet compression?*

Method: *Unilateral compression (150, 300 mmHg, 120 min) of the hindlimb, or permanent sciatic nerve transection.*

Conditioning interval/Evaluation time: *3, 6,14, 28 days.*

Evaluation method: *Immunocytochemistry of CPON in L4 and L5 DRG.*

Conclusion: *CPON was transiently upregulated in DRG as a response to peripheral nerve compression, but to a lesser extent than after nerve transection.*

Paper IV

Question: *Can vibration exposure enhance regeneration into sciatic nerve grafts?*

Method: *Unilateral vibration exposure (80 Hz/32 m/s^2, 5 h/day, 2 or 5 days) of the hindlimb.*

Conditioning interval: *7 days.*

Surgery: *A 10 mm long sciatic nerve graft from the vibrated side was sutured into a freshly made defect of the sciatic nerve of the non-vibrated side and vice versa.*

Evaluation time: *4, 6, 8 days.*

Evaluation method: *Pinch reflex test.*

Conclusion: *Vibration for 5 days, but not 2 days, increased the axonal regeneration distances in the nerve grafts (4 and 6 days) probably by shortening the initial delay.*

Paper V

Question: *Can vibration induced injury/conditioning lesion be prevented by D600, a Ca^{2+}channel blocker?*

Method: *Unilateral vibration exposure (80 Hz/32 m/s^2, 5 h/day during 5 days) alone, or during which Ringer's solution or D600 + Ringer's solution was locally administered around the sciatic nerve by a miniosmotic pump.*

Surgery: *Bilateral test crush lesions on the 5th day of vibration (no conditioning interval).*

Evaluation time: *3 and 6 days.*

Evaluation method: *Pinch reflex test.*

Conclusion: *Local administration of D600 prevented the conditioning lesion effect induced by vibration.*

Abstract

The effect of short time vibration exposure and tourniquet compression on nerve regeneration in rats was studied with special reference to cell activation.

One of the hindlimbs was conditioned by either vibration exposure (5 hours/day-5 consecutive days) or compression (150-300 mmHg for 30-120 minutes), which was followed by a recovery period of 0-7 days. Test crush lesions or a transplantation of a conditioned nerve segment into a freshly made gap in the contralateral nerve (and vice versa after vibration), were performed. Axonal regeneration distances were measured after an additional 3-8 days. Furthermore the reaction of neuronal cellbodies, in the dorsal root ganglia, and non-neuronal cells was studied following compression.

Vibration exposure, and tourniquet compression in particular, increased axonal regeneration lengths after both test crush lesions and transplantation (up to 36%). This so called conditioning effect, which may be regarded as a sign of injury, was in one experiment prevented by treatment with D600, a calcium channel blocker.

The non-neuronal cells, which were mainly Schwann cells, increased their proliferation following compression along with an upregulation of the low affinity nerve growth factor receptor (p75). The neuronal cellbodies responded to compression by increased expression of the C-terminal flanking peptide of Neuropeptide Y (CPON).

Thus, tourniquet compression and vibration exposure, which are non-invasive methods, can increase the regenerative capacity of neurons. This requires activation of both Schwann cells and neuronal cellbodies. Conversely, the observed effect of such treatments may be regarded as a potentially harmful alarm reaction in the peripheral nervous system. Whether D600 prevents nerve injury caused by vibration exposure or compression remains to be investigated.

Introduction

Approximately 80.000 persons in Sweden suffer from injuries to their hands annually. Of these, 3% are nerve transection injuries [1]. They represent a major problem in hand surgery, since functional recovery after nerve transection in adults is poor. In spite of the introduction of refined microsurgical methods, the results are disappointing. Injuries to the hand cause great disability and costs for both the individual and the society since these persons often are young (20-25 years) [2] and employed at the time of the injury. The limited functional recovery after nerve transection injuries depends on several interdependent factors in both the peripheral and central nervous systems. Some of these factors are: slow axonal outgrowth, misdirection of the regrowing axons into the distal nerve stump, neuronal cell death/lesions in dorsal root ganglia and spinal cord together with an extensive re-organization of the cerebral motor and sensory cortex of the brain. Hence, surgery alone is insufficient to improve the outcome after nerve injury and further research on the mechanisms behind the events of degeneration and regeneration in the nervous system is needed.

Apart from nerve transection, there are a number of potential harmful threats to the peripheral nervous system (PNS), which can cause both reversible and irreversible damage to peripheral nerves. Neurotoxic drugs, stretch injuries, vibration injuries and chronic and acute compression injuries are some examples [3]. In that context, it is interesting that compression is used daily in connection to surgery on the extremities at various departments of hand surgery and orthopedics when a blood less field is induced by application of a pneumatic tourniquet [4]. According to recent data, 400.000 persons in Sweden are subjected to vibration exposure by hand held tools during their daily work (see review by Dahlin and Lundborg) [5]. Vibration exposure to hand by handheld tools may lead to considerable impairment of the hand function in the long-term perspective [6]. This thesis deals with two potential harmful injuries: acute compression and short-term vibration exposure and their possible effects on the PNS. The question, whether the injuries, or activation, induced by vibration and compression could be employed for the improvement of nerve regeneration within nerve grafts is also raised.

Background

The peripheral nervous system

The nervous system is divided into a central and a peripheral part. The central nervous system (CNS) comprises the brain and spinal cord. The peripheral nervous system (PNS) refers to nervous tissue outside the CNS and contains two broad classes of somatic neurons: motor neurons with the nerve cell body located in the anterior horn of the spinal cord and sensory neurons with the nerve cell body in the dorsal root ganglia (DRG). Motor neurons carry efferent signals from the CNS to the effector organs (muscles) and the sensory neurons lead afferent information from the inside of the body and the environment to the CNS. The autonomic nervous system (ANS) refers to the nervous tissue concerned with visceral functions (glands, smooth muscles) and is composed of sympathetic and parasympathetic nerve fibers. The ANS is by definition a motor system, but autonomic fibers with afferent sensory information accompany the motor fibers of the autonomic system [7]. The nervous system also contains glial cells that occupy the spaces between neurons and modulate their functions. The principal type of glia cell in the PNS is the Schwann cell, which has many important functions, but the main function is to house, support and interact with the axons. This thesis is mainly focused on the somatosensory neurons in the sciatic nerve of the rat.

The dorsal root ganglia and the sensory neurons

The sensory neurons are pseudo-unipolar cells which have their cell bodies clustered in the DRG, which are masses of nerve tissue that lie just outside the spinal cord. The neurons have a long peripheral process, the axon, which receives the information from the different sensory receptors, and one shorter central process, which conveys the information to the CNS. Approximately 98-99% of the estimated number of 11.000 cell bodies of rat sciatic sensory neurons resides in the L4 and L5 DRG [8]. The cell bodies are surrounded by numerous satellite cells. The cell bodies of the sensory neurons within the DRG can be divided into different classes according to their size [8] or properties, like immunocytochemical labeling for lectins [9], metabolic properties [10], neuropeptide content and expression of neurotrophin

receptors [11]. They can also be divided into large, lightly stained, neurons (>35μm), which are rich in neurofilaments, or small, darkly stained, neurons (<35 μm) [12], depending on their appearance in methylene blue stained sections [13]. There is a general correlation between the size of the DRG cell body and the size of its axon and thus the conduction velocity [13-15]. The large neurons have in general thick fast-conducting myelinated fibers that transfer information from mechano-receptors and some nociceptive end organs whereas small neurons have non-myelinated fibers [16], which mainly carry impulses from thermo- and nociceptors.

Neuropeptides in sensory neurons

Many intact small and medium sized DRG neurons express a variety of neuropeptides under normal circumstances, such as the excitatory peptides, Substance P (SP) and calcitonin-gene related peptide (CGRP), which facilitate transmission in the dorsal horn. In contrast, large DRG neurons seem to be devoid of neuropeptides. Other neuropeptides of the DRG are somatostatin (SOM), vasointestinal peptide (VIP), galanin (GAL) and neuropeptide Y (NPY). NPY is a 36 amino-acid peptide, which normally is not expressed in adult DRG neurons although it is one of the most abundant neuropeptides in the nervous system [17]. NPY is involved in numerous homeostatic mechanisms as the control of the systemic blood pressure, in memory, feeding and anxiety [18].

After axotomy, mRNA expression of CGRP and SP is down regulated [19] while the expression of NPY, GAL and VIP are dramatically increased in the affected neurons [20, 21]. NPY is thought to attenuate signal transmission to the dorsal horn in the spinal cord after nerve injury [22]. It has also been suggested that NPY has a role in mediating hyperalgesia and analgesia in response to nerve injury [23]. Since NPY is expressed in injured neurons, it makes it a suitable marker for nerve injury. In *paper III*, the expression of C-terminal flanking peptide of neuropeptide Y (CPON), which is the C-terminal of NPY and has the same distribution as NPY in the nervous system [24], was studied in relation to nerve compression and nerve transection.

The peripheral nerve

Each peripheral nerve, like the rat sciatic nerve, is actually a bundle of mixed somatic sensory and motor axons and autonomic nerve fibers. Each single myelinated axon is surrounded by a chain of Schwann cells of which the Schwann cell plasma membrane is wrapped in many layers around the axon creating an insulating myelin sheath. This axon-Schwann cell unit is surrounded by a basal lamina tube, consisting of type IV collagen, laminin, heparan sulphate, proteoglycan and fibronectin [25, 26], mainly produced by the Schwann cells. The non-myelinated spaces between the Schwann cells along the axon are termed nodes of Ranvier. This segmental constitution of the myelinated axon permits the saltatory propagation of the nerve impulse, speeding up the conduction velocity as much as hundred times. Non-myelinated axons, on the other hand, share Schwann cells with up to 15 other non-myelinated axons. These axons are thin, which leads to a slow conduction velocity. Bundles of axons are grouped together forming fascicles surrounded by a strong multilaminated connective tissue membrane the perineurium, which also constitutes a diffusion barrier. The intrafascicular space between the axons, the endoneurial space, is filled out with fibroblasts, macrophages and components of extracellular matrix. The fascicles are embedded in a protective and supporting connective tissue, the epineurium. The nerves are well vascularized with segmental vessels approaching the nerve. Blood vessels anastomose through a vascular network between the different compartments. The vessels passing through the perineurium run obliquely, thereby constituting a possible valve mechanism making the nerve vulnerable to compression [27]. The endothelial cells of the endoneurial capillaries form tight junctions, thus creating a blood-nerve-barrier that is designed to control the nerves´ internal milieu, an equivalent to the blood-brain-barrier [28, 29].

Nerve injury

Nerve lesions may have various causes, such as mechanical (compression, vibration, stretching, transection), ischemic, thermal, or chemical trauma. Axonal injury (transection or crush), which disrupts the contact between the nerve cell body and the peripheral target organ, may cause death of up to 35% of the neurons [30, 31]. It is not known why so many cells die, but events that

lead to programmed cell death (apoptosis), partly induced by deprivation of neurotrophic factors, is probably involved [32]. It also seems that sensory neurons are more susceptible to axonal injury than motor neurons [33]. As a reaction to a peripheral axotomy, the nerve cell body of surviving neurons displays several changes that are characteristic of a switch from a "signaling mode" to a "growing mode" [34]. Typical morphological changes are swelling of the soma, dissolution of Nissl bodies (chromatolythic changes) and peripheral displacement of the nucleus [35]. These alterations are accompanied by metabolic changes, which comprises alteration in the synthesis of many neuropeptides [36] (see above), growth associated proteins [37], and cytoskeletal proteins [38]. The phenotypic changes are preceded by the expression of immediate early genes and transcription factors, as for example c-Jun and activating transcription factor 3 (ATF 3). These factors are involved in the cascade of events leading to regeneration and they are probably induced via an injury provoked activation of various signal transduction mechanisms [39, 40]. Injury to the central axon does not lead to these profound changes in the nerve cell body, suggesting major differences in regeneration capacity between the central and peripheral axon [39].

The distal part of the nerve undergoes a process termed Wallerian degeneration following axonal injury in which the axons and the myelin sheaths distal to the injury undergo degeneration. Hematogenously invading macrophages [41], are predominantly responsible for the digestion of myelin. Macrophages are also important for the regeneration process [42] being a source of cytokines and growth factors. These cells also contribute to induction of proliferation of Schwann cells [34]. The synthesis of nerve growth factor (NGF) [43] and its low affinity receptor (p75) by Schwann cells may also be induced by macrophages [44]. The Schwann cells play a dominant role in creating a regeneration supportive microenvironment in the distal nerve stump. The loss of axon-Schwann cell contact is a signal that causes the Schwann cells to undergo mitosis and line up in typical cell columns (bands of Büngner) in the basal lamina tubes of the distal nerve segment. The basal lamina tubes of the Schwann cells constitute an outgrowth channel for regenerating axons. It has been shown that elongation of axons is markedly stimulated when the axons enter a tube and contact growth

promoting molecules of the inside of the basal lamina tubes [45]. The proliferative Schwann cells (which can be detected by BrdU technique, see *paper II*) are trophic sources for regenerating axons by providing with increased production of extracellular matrix and adhesion molecules [46]. They also produce and present neurotrophins, as NGF [47], brain derived neurotrophic factor (BDNF), and the neurotrophins NT-3, NT-4/5 and NT-6, to the outgrowing axons [48]. The neurotrophins exert their influence by binding to the high-affinity tyrosine kinase-receptors (TrkA, TrkB and TrkC), which mainly bind to NGF, BDNF and NT-3; and the low-affinity receptor, termed p75 receptor, which binds all of the neurotrophins [48]. The p75 receptor has attracted a lot of attention lately. It is expressed by the entire population of Schwann cells in the bands of Büngner in the distal stump of axotomized nerves [49]. The expression of the p75 receptor is suggested to be induced by loss of axonal contact or early axonopathy [50, 51]. As opposed to the trophic effects elicited by the high affinity neurotrophin receptors, signals emanating from p75 receptor have been associated with increased cell death by apoptosis [52] or modulation of cell stress/injuries responses. The precise signaling mechanism of p75 receptor is not understood but it may serve as a general mechanism for the transduction and modulation of stress signals by neurotrophins [53]. In *paper II*, the upregulation of p75 receptor in the sciatic nerve following tourniquet compression was studied.

Conditioning lesions

The regenerative capacity in a peripheral nerve can be markedly improved if the nerve has been injured or stimulated previously; such a lesion is called a "conditioning lesion" [54]. If a test crush lesion (all axons are disrupted, but the basal lamina tubes of the axons are preserved) is applied to a nerve some days after a conditioning lesion, the axons start to grow earlier with an increased rate as compared to a nerve which has not been conditioned. This phenomenon is called the conditioning lesion effect. The time between the two injuries is called the conditioning interval. The conditioning lesion effect has been observed after different types of conditioning lesions such as crush and transection [55], acute and chronic nerve compression [56], vibration exposure [57], and the application of an electromagnetic field [58]. The

optimal conditioning interval period has been studied experimentally and it seems that a conditioning interval of at least 3-6 days is needed for enhanced regeneration in nerve grafts [59]. The precise mechanism behind the conditioning lesion effect is not clear but a complex series of changes takes place both locally and distal to the conditioning site such as activation of non-neural cells (mainly Schwann cells) [60, 61]. The nerve cell body is most probably involved in this reaction and thus, the entire neuron is activated by the conditioning lesion. It was tested if tourniquet compression and vibration exposure could act as conditioning lesions in *paper I-II* and *IV-V*, respectively.

Compression injury

Compression injuries are either acute or chronic. The thesis will primarily discuss acute compression and its effects on peripheral nerves and possible clinical implications.

In upper and lower extremity surgery, pneumatic tourniquets are often used in order to create a blood less operative field. The occurrence of mechanical nerve injury in connection with anesthesia and surgery is probably more common than generally believed [62]. Prevention of injury as well as understanding the pathophysiological mechanisms behind the injury is of great importance.

Compression injuries can be classified according to the functional and structural consequences of the induced lesions. As, for example, when a leg or an arm "go to sleep", due to a slight acute compression, it is caused by a transient ischemic nerve block. The defect is immediately is reversible after removal of the compression and no structural damage is recognizable [3]. When compression of higher magnitude is applied to a nerve trunk, it can cause a local conduction block (neurapraxia), which may last for weeks or months, due to local myelin damage. The axonal continuity is still preserved and thus the nerve injury is reversible. A clinical example of neurapraxia is the "Saturday night palsy" which may occur after high radial nerve compression. With more severe compression lesions, the axonal continuity may be lost (axonotmesis), but the continuity of the basal lamina tubes of the axons is preserved. Wallerian degeneration occurs in the distal stump and the axon has

to regenerate to restore function. The prognosis after this type of injury is fairly good. The most severe compression injury corresponds to a nerve transection; all parts of the nerve loose their continuity (neurotmesis) and surgical repair is required to recover some function but the outcome is often poor [63].

The severity of compression injury depends not only on the magnitude of pressure; the duration and the mode of compression are also of importance [56, 64]. It has been demonstrated experimentally that wider cuffs can reduce mechanical nerve injury [64, 65]. Experimental studies have revealed microscopical and ultrastructural changes in peripheral nerves after tourniquet compression at various durations and magnitudes [66-68]. Nitz et al. demonstrated that tourniquet compression at 200 and 300 mmHg for 2 hours induced local structural changes at the compression site [67]. Demyelination and axonal degeneration was observed in rat sciatic nerves compressed at 300 mmHg for 2 hours, while compression at 200 mmHg, same duration, induced minor reversible changes, such as edema.

When the tissues beneath a pneumatic tourniquet are subjected to pressure, they deform and create a pressure gradient, redistributing compressed tissue toward non-compressed areas. The pressure gradient will thereby be greatest under the edges of the cuff. Intraneural blood vessels, as well as nerve fibers, are displaced longitudinally by shear forces, which may cause disruption or invaginations of the myelin sheaths locally; an effect termed the "edge effect" [69, 70]. Intraneural microcirculation and axonal transport are disturbed already after local application of a low pressure of 20–30 mmHg [71, 72]. Compression at higher pressure (400 mmHg, 2 hours) may interfere with the microcirculation up to a week [72] after removal of the compression as a consequence to damage to the endothelial cells causing endoneurial edema which in turn leads to a "no reflow phenomenon" i.e. prolonged ischemia. It seems, however, that compression is of greater importance for the severity of nerve injury than ischemia per se, since tibial nerves (distal to tourniquet) are less damaged than sciatic nerves [73] Nerve compression not only affects the nerve trunk peripherally. Structural and biochemical changes reminding of those seen after nerve transection injuries in the corresponding nerve cell

bodies are also induced [74] which probably reflects a reaction to cell stress / trauma. In *paper I-III*, the effects of tourniquet compression on nerve regeneration and nerve cell bodies are studied.

Vibration injury

Vibration represents a type of rapidly iterated compression applied to the skin and the vibration energy may be transmitted over various distances in an extremity depending on the frequency of the vibration. Low frequency vibration energy is transmitted more proximally in the arm than high frequency vibration [75]. The development of a "hand-arm vibration syndrome" is also dependent on the duration and acceleration of the vibration.

Long-term work with handheld vibrating tools may cause a complex of peripheral neurological, circulatory and musculoskeletal dysfunctions as paresthesia, white fingers, sensory disturbance, impaired dexterity, [76] muscle weakness [77] and pain. The neurological symtoms may sometimes wrongly be interpreted as signs of nerve entrapment. Symptoms that are associated with vibration exposure have been extensively studied, but only a limited number of experimental studies have examined the changes seen in nerves after vibration exposure. The precise pathophysological mechanism behind the vibration-induced neuropathy remains unclear [5]. The changes seen in nerves after short-term vibration of a rat hindlimb span from edema formation to structural changes in both non-myelinated and myelinated nerve fibers [78-80]. Furthermore, it is demonstrated that vibration exposure can act as a conditioning lesion [57]. This has been observed as an increased regenerative capacity after a test crush lesion in a nerve previously exposed to vibration. The vibration-induced conditioning lesion effect takes some time to develop. The most pronounced effect is reached a week after the end of exposure period, which probably reflects the time needed to activate the Schwann cells. The observed effect probably indicates an alarm reaction of the neuron which may be accompanied by central changes in the DRG cellbodies reminding of those seen after more severe trauma, but less extensive. The testing of this hypothesis is in progress. In *paper IV* it was investigated if short time vibration can act as a conditioning lesion.

It has been observed that regeneration of the rat sciatic nerve can be stimulated (conditioned) when rats are exposed to a 50 Hz sinusoidal magnetic field [58, 81]. The use of a L-type calcium channel blocker D600 (methoxyverapamil) inhibited this stimulation [82] implying that Ca^{2+} fluxes are involved in the stimulatory effect of magnetic fields on nerve regeneration. It is not known if vibration exposure affects Ca^{2+} fluxes in the same manner. The calcium ion is involved in neural plasticity in many ways. It is suggested that peripheral nerve injury disrupts the permeability barrier function of the plasma membrane allowing influx of Ca^{2+}. The intra axonal increase in Ca^{2+} concentration may be detrimental for the axon by starting a series of events that leads to axonal degeneration [83, 84]. However, calcium ions are also involved in the events of axonal regeneration and growth cone behavior. Growth cone motility and neurite extension is regulated by intra- and extracellular calcium concentrations in a complex manner [85, 86]. In this context, various calcium channel blockers have been tested and they seem to have effect on both neurite elongation and growth cone motility in a dose response mode. High concentrations of a L-type calcium channel blocker have been reported to decrease neurite elongation and conversely low concentrations may accelerate elongation in vitro studies [87]. Furthermore, Nimodipine (also a L-type calcium channel blocker) given systemically in low concentrations accelerates axonal sprouting after surgical repair of rat facial nerve [84]. In *paper V* it was tested if D600 could inhibit the conditioning lesion effect of vibration.

Aims

The general aim of this thesis was to investigate nerve regeneration following two experimentally induced injuries to rat hindlimb - tourniquet compression and vibration exposure - with special reference to cell activation.

More specific aims were to:

- evaluate if a non-invasive method, tourniquet compression of a nerve with an inflatable tourniquet, can act as a conditioning lesion.

- examine the local non-neuronal cell reaction to compression and if tourniquet compression could be used to stimulate nerve regeneration in selected situations, like nerve grafting procedures.

- investigate whether tourniquet compression induces expression of an injury-related neuropeptide (CPON) in the nerve cell bodies of the dorsal root ganglia (DRG) of the rat sciatic nerve.

- study if nerve regeneration is altered in nerve grafts conditioned by vibration exposure and to delineate possible evoked mechanism(s) responsible for the increased regenerative capacity after vibration exposure.

- investigate whether the effect of a conditioning lesion induced by vibration can be prevented by local treatment with a L-type Ca^{2+} channel blocker, D600.

Materials and methods

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

Animals, anesthesia and ethics

Adult female Wistar rats weighing 180-200 g (Møllegaard, Denmark) were used throughout the study. During surgery and tourniquet compression, the rats were anesthetized by an intraperitoneal (i.p.) injection of a mixture of pentobarbital (60 mg/ml) and saline in 1:10 volume proportions. During vibration exposure, the rats were anesthetized by an i.p. injection of a mixture of pentobarbital (60 mg/ml), diazepam (5 mg/ml) and saline in 1:2:1 volume proportions. The local animal ethics committee of Lund University approved all experiments.

Tourniquet compression

In *paper I-III*, the hindlimbs of each rat were shaved and one hindlimb was compressed with a 1.6 cm wide inflatable tourniquet (Hokanson, Blidö, Sweden, Fig 1), which was applied at thigh level, at different durations and magnitudes as follows:

Paper I: 300 mmHg, for 30 or 120 minutes

Paper II: 0, 150, 200 and 300 mmHg, for 120 minutes

Paper III: 150 or 300 mmHg, for 120 minutes

The pressure was controlled by a calibrated inflator (Stille-Werner, Sweden). After removal of the tourniquets, the animals were allowed to recover from anesthesia.
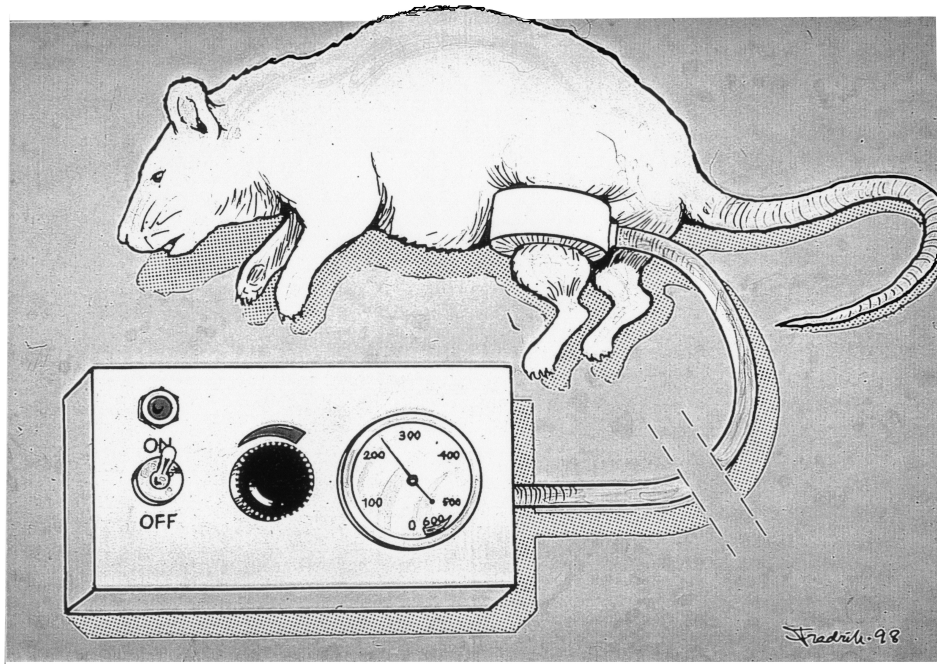
Figure 1. *The tourniquet model in paper I-III. A mini-tourniquet was applied to the hindlimb of the anesthetized rat for 30-120 minutes at a pressure level of 150-300 mmHg.*

Vibration exposure

In *paper IV* and *V,* the animals were exposed to vibration. The rats were placed in a soft network support and one of the hind paws was attached to a plate horizontally, which was fixated on the top of a vibration exciter. In this way the paw could be exposed to a sinus waved vibration for 5 hours/day during 2 or 5 consecutive days with a defined frequency and acceleration level (80 Hz, 32m/s^2) [57, 88]. This frequency corresponds to the frequency of many vibratory handheld tools used in the industry.

Surgical procedures

Transection

In *paper III,* the sciatic nerve was transected as comparison to tourniquet compression. In deep anesthesia, the sciatic nerve was unilaterally exposed and transected at thigh level. Approximately 4 mm of the nerve was resected to prevent regeneration. After wound closure, the rats were allowed to recover before evaluation 3, 6, 14 or 28 days later.

Nerve grafting

In *paper II* and *IV,* nerve grafting procedures were carried out. Following tourniquet compression or vibration exposure, the rats were re-anaesthetized after a conditioning interval of 6 or 7 days, respectively. A 10 mm nerve graft from the compressed sciatic nerve was excised and transplanted to bridge a freshly made nerve defect at midthigh level of the corresponding contralateral non-exposed nerve. In the vibrated animals the vibrated nerve graft was sutured into a corresponding contralateral defect of the non-vibrated contralateral sciatic nerve and vice versa, thereby creating two models within the same animal (Fig 2). In another group of rats the hindlimb was compressed as above (300 mmHg), but a tibial nerve graft from distal to the tourniquet was used to bridge the defect of the contralateral sciatic nerve. The sciatic and tibial nerve grafts were sutured using three single 9-0 epineurial sutures at each end of the graft. The wounds were closed and the animals were allowed to recover for 6 and 8 days *(paper II)* or 4, 6 and 8 days *(paper IV)*. The length of regenerating sensory axons in the two different nerve grafts was evaluated by the pinch reflex (see below).

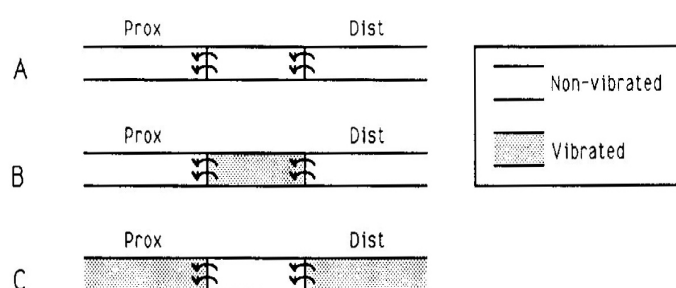*Figure 2. Schematic drawing of the experimental models in paper IV. A 10 mm long nerve segment was sutured as a nerve graft between transected nerve ends at the contralateral side (A). In model B, a nerve segment from a vibrated nerve was sutured as a graft (vibrated graft) into the non-vibrated nerve. Finally, in model C, a non-vibrated nerve graft was sutured into a vibration-exposed transected nerve.*

Miniosmotic pumps

In *paper V,* miniosmotic pumps (2002, 0.5μm/h, Alzet, USA) were used to deliver a L-type Ca^{2+} channel blocker (D600, methoxyverapamil, Sigma, Sweden), in Ringer's solution (0.2 mg/ml), or Ringer's solution only, to the sciatic nerve during the vibration exposure. Three days before start of vibration exposure, the rats were anaesthetized and a pre-activated miniosmotic pump was implanted subcutaneously in the back of the rat. One of the sciatic nerves was exposed in the midthigh region and a 6 cm long silicone catheter with an inner diameter of 0.7 mm (4 FR Mentor Corporation, Goleta, CA) was tunneled subcutaneously to the sciatic nerve. The tip of the catheter was perforated and sutured by two single 9-0 sutures (Ethilon®, Edinburgh, Scotland) to the epineurium (Fig. 3). The contralateral untreated side served as a non-vibrated control. The wounds were sutured and the animals were allowed to recover from anesthesia.
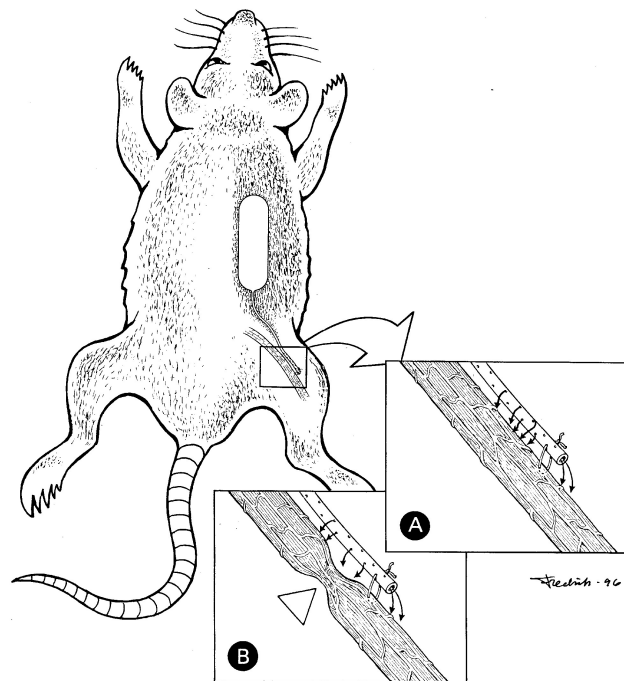

Figure 3. *Experimental procedure paper V. A thin silicone catheter (A) connected to the miniosmotic pump delivered either Ringer's solution only or D600 + Ringer's solution to the sciatic nerve during vibration exposure. Directly after vibration exposure, on the 5th day, test crush lesions (B) were applied bilaterally.*

Evaluation of nerve regeneration

Test crush lesion

In *paper I and V,* test crush lesions were performed (see Fig 3). A test crush lesion is equal to axonotmesis; the axons are disrupted but the basal lamina tubes of the axons are preserved. With specially designed pliers, the sciatic nerve is crushed (2x20s) in a standardized manner.

Directly following release of pressure, or following a recovery period of 3 or 6 days the sciatic nerves were exposed and crushed bilaterally. Vibration exposed nerves were re-exposed and crushed directly after vibration, on the 5th day. The test crush lesions on the non-exposed and the exposed side were applied at similar levels. Before wound closure, the crush sites were labeled with 9-0 epineurial sutures (Ethilon®, Edinburgh, Scotland). The animals were then allowed to recover for 3 *(paper I and V)* or 6 *(paper V)* days before evaluation with the pinch reflex test.

Pinch reflex test

Paper I-II, III-IV: After test crush lesions or nerve grafting the outgrowth of sensory axons was evaluated at various time points. The pinch reflex test [89-91] was performed at day 3 *(I, V)*, 4 *(IV)*, 6 *(IV, V)* and 8 *(II, IV)*. The sciatic nerve was cut far distally and regenerating axons were localized by pinching the nerve, starting from the distal end, in a proximal direction. When the regenerating sensory axons were pinched a withdrawal reflex in the back muscles was elicited. The distance between this site on the nerve and the previously labeled crush lesion, or the proximal suture line of the nerve graft, was measured with a caliper under a microscope and was regarded as the axonal regeneration distance. The animals were killed with an overdose of pentobarbital.

Histology

Paper I: Experimental studies by others have examined structural changes after tourniquet compression in detail [67-69]. Thus, for a morphological overview, routine histology was performed in a separate group of rats (no test crush lesions were applied). The compressed sciatic nerves (300 mmHg, 2 hours, conditioning interval 6 days) were carefully excised. The tibial nerves were excised distal to the knee joints. The nerves were fixed at in-vivo length in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.15) for four hours and then transferred to cacodylate buffer. Three mm lengths of nerve were then obtained from the segment of the sciatic nerve, which was beneath the tourniquet, and from the tibial nerve (distal to the tourniquet). The specimens were post-fixed in 1 % osmium tetroxide in cacodylate buffer for 2 hours followed by a 15 minutes wash in cacodylate buffer. The nerve pieces were dehydrated in ascending concentrations of ethanol and propylene-oxide for 30 minutes infiltrated with a 1:1 mixture of Epon and propylenoxid for 2 hours at room temperature and then with pure Epon overnight. The specimens were embedded in pure Epon for polymerisation at 60 C° for 72 hours. Nerves from the non-compressed side were prepared in the same way. One µm thick longitudinal and cross sections were cut on a microtome and mounted and stained with Richardson's solution (Methylene blue and Azure II). A person who was not aware of the treatment examined the sections in a light microscope.

Immunocytochemistry

BrdU labeling

Paper II: To study cell proliferation in nerves following tourniquet compression (150 or 300 mmHg, 2 hours) 5-bromodeoxyuridine (BrdU) labeling was used. After 6 days, the rats were re-anaesthetized and injected with 5 mg of BrdU in 0.5 ml 0.1 M Tris buffer (pH 7.6) intravenously. After 2 hours a 15 mm nerve segment from the compressed sciatic and tibial nerve was excised using the corresponding contralateral nerve segment as a control. The specimens were fixed in Stefanini's fixative, cryoprotected and embedded in Tissue-Tek® (Sakura Torrence, USA). Longitudinal cryosections, 10 µm thick, were collected on poly-L-lysine coated glass slides. The staining was performed essentially as described by Sondell et al. [92] with the exception that Alexa Fluor antibodies (red A11032 and green A11008, Molecular Probes Inc) were used for visualization of the binding of the primary antibodies. In some sections antibodies to S-100 were used to visualize Schwann cells. The preparations were examined in a fluorescence microscope. The number of BrdU positive cells in the endoneurial space was counted in longitudinal sections of the central portion of the sciatic and tibial nerves and expressed as number of BrdU positive cells/mm^2.

p75 receptor

Paper II: Schwann cells are known to express the low affinity p75 receptor after nerve injury and this was studied by immunocytochemistry in connection to tourniquet compression (150 or 300 mmHg, 2 hours). After a conditioning interval of 6 days, sciatic and tibial nerve segments were excised (as described in the paragraph above) with corresponding contralateral controls. The nerves were embedded in Tissue-Tek® and cryosectioned in 10 μm thick sections. The sections were dried, washed in phosphate buffered saline (PBS), fixed in ice-cold acetone, washed in PBS, treated with 0.3% H_2O_2 in methanol and washed in PBS. The sections were blocked in rabbit serum and then exposed to a mouse monoclonal antibody against p75 receptor (dilution 1:100 Boehringer-Mannheim, Germany) in PBS for 2 hours. After washing in PBS, the sections were exposed to rabbit antimouse horseradish peroxidase-conjugated antibody (dilution 1:50, DAKO) for 45 minutes, washed in PBS and developed in diaminobenzidine tetrahydrochloride (DAB, Sigma, Sweden). After a final wash and dehydration, the preparations were mounted in DPX. Control sections were incubated without the primary antibody. The sections were examined in a light microscope.

CPON

Paper III: In order to investigate if tourniquet compression, as compared to nerve transection, can induce expression of CPON in the nerve cell bodies, the L4-L5 DRG were removed bilaterally by dissection 3, 6, 14 or 28 days after unilateral transection or compression (150 or 300 mmHg, 2 hours). The dissected DRG were immersed in Stefanini's fixative (2% paraformaldehyde and 1.9 % picric acid in 0.1 M phosphate buffer, pH 7.2) for 2 hours, followed by washing and cryoprotection in 20 % sucrose (w/v) in PBS. The DRG were mounted in Tissue-Tek® (Sakura, Torrence, USA). Longitudinal sections, 10 μm thick, were cryosectioned and collected on poly-L-lysin glass slides. Three sections from the mid-axial part of each L4-L5 DRG, at least 50 μm apart, were used. The sections were washed in PBS and then exposed to a primary rabbit antibody against CPON (DAKO, Denmark) diluted 1:1280 in PBS containing 0.25 % Triton-X (Packard, Meriden, USA) and 0.25% bovine serum albumin (Sigma-Aldrich). The sections were incubated at 4° C overnight and the primary antibody was removed. The sections were washed (3 x 5 minutes in PBS) and exposed to fluorescein isothiocyanate (FITC) conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) at a dilution of 1:80 in PBS for one hour, at room temperature and darkness. The sections were finally washed in PBS and mounted in 50% (v/v) glycerol in PBS for fluorescence microscopy. Control sections were incubated without the primary antibody.

Image analysis

Sections subjected to histochemistry or immunocytochemistry were analyzed in an Olympus microscope equipped with fluorescence. They were photographed with a KODAK professional DCS 420 digital camera connected to a Macintosh computer. The software Adobe Photoshop® 5.0 (Adobe Systems Inc.) was used for image analyses.

Statistical analysis

Below follows a brief presentation of the statistical methods that were used in the thesis. The software StatView® 5.0 (SAS Institute Inc. Cary, USA) was used to perform the analysis. Results are presented as mean ± s.e.m in *paper I-IV* and in *paper V,* as median [interquartile range]. A P-value of less than 0.05 was considered as significant.

A one-way *(II)* or two-way *(I-III)* analysis of variance (ANOVA) was used to calculate significant differences when more than two groups of data were compared (except in paper *V*). This was followed by different post-hoc tests: Fisher's protected least significant difference (PLSD) *(I)*, Scheffé's post hoc test *(II-III)* and Bonnferroni-Dunn *(IV)*. Un-paired t-test was used to test for statistical difference when only two groups were compared *(II)*. The Kruskal-Wallis test, including multiple comparisons between different treatments [93], was used to compare the different experimental groups in *paper V*. Wilcoxon signed rank test was used to compare data within the same animal (paired data, *V*).

Results

This section gives a summary of the results and some comments. For further details, the reader is referred to the individual papers.

Paper I

Nerve regeneration enhancement by tourniquet

It was tested if tourniquet compression of a hindlimb can increase the axonal regeneration distances in nerves after a subsequent test crush lesion. Compression with a tourniquet (300 mmHg, 30 minutes) had no significant influence on the regeneration distances at any of the conditioning intervals (0, 3 and 6 days). Tourniquet compression at 300 mmHg for 120 minutes significantly increased axonal regeneration distances. The effect was detectable after a conditioning interval of 3 days but was more pronounced when a conditioning interval of 6 days was employed (up to 36 %). A conditioning interval of 0 days did not influence the regeneration distances after 120 minutes of compression. The fact that a conditioning interval of six days lead to longer regeneration distances may reflect the time needed for Schwann cells to proliferate and produce neurotrophic factors.

The choice of pressure level (300 mmHg) and duration (30 min- 2 hours) is clinically relevant. In connection to upper extremity surgery in humans, the recommended tourniquet pressure is generally the systolic pressure + 50 –70 mmHg [62]. For a rat, the pressure level (300 mmHg) may however be a little higher than what would be recommended, as their systolic pressure is only about 120 mmHg [94].

Histology

Earlier studies have shown that 30 minutes of tourniquet compression (300 mmHg) induces only minor signs of edema and no signs of demyelination or degeneration at one week while changes that are more substantial are evident if the compression is prolonged to 120 minutes [67, 68]. Thus, for a morphological overview, routine histology was performed 6 days after tourniquet compression at 300 mmHg for 120 minutes (without a subsequent

crush lesion). The specimens showed various degrees of pathology. Nerve fiber damage, characterised by vacuolization, myelin indentation, cleavage and variability in myelin sheath thickness, was seen in nerves that had been subjected to compression. Nerve tissue from beneath the cuff (sciatic nerve) was more damaged than the nerve tissue distal to the cuff (tibial nerve). Control nerves showed no pathology.

Paper II

Tourniquet compression: a non-invasive method to enhance nerve regeneration in nerve grafts

In the first set of experiments, the conditioning lesion effect of tourniquet compression, demonstrated in *paper I,* was used in a nerve grafting situation. In the second set of experiments it was examined if compression activates, i.e. induce proliferation of non-neuronal cells which, is seen as increased incorporation of BrdU. The activation can also be observed as and expression of p75 receptor.

Nerve grafts from rat hindlimbs, compressed 6 days earlier, were used to bridge a gap in the contralateral sciatic nerve. Tourniquet compression at 200 or 300 mmHg increased the regeneration distances, measured 6 days after grafting procedure, with up to 30 percent compared to the non-compressed sciatic nerve grafts which corresponds to the results in *paper I*. Tourniquet compression at 150 mmHg had no significant effect on axonal regeneration within the nerve grafts. Compression at that level does probably not induce any signs of degeneration. The lack of effect at that pressure level probably reflects a reversible ischemic conduction block without structural changes. Compression at 200 mmHg has been shown to induce minor structural, although reversible, changes [67], while compression at 300 mmHg causes more substantial changes [67, 68], such as myelin damage and degeneration that may be needed for the conditioning lesion effect.

Using the same experimental setup, regeneration distances in tibial nerve grafts taken from distal to the compression site (300 mmHg, 2 hours), increased with up to 25 percent 8 days post surgery.

Incorporation of BrdU

Proliferation of non-neuronal cells, detected as incorporation of BrdU, was investigated 6 days after tourniquet compression at 150 and 300 mmHg (no grafting procedure was performed). BrdU is only incorporated by cells in the S-phase of the cell cycle. Double labeling of BrdU and S-100 showed that the majority of the proliferating cells were Schwann cells as indicated by the oval shape of the BrdU positive nucleus and the S-100 stained cytoplasm (Fig. 4a). In sections of untreated control nerves, only a few BrdU positive cells (Fig. 4b) were found. Compression at 150 mmHg lead to an increased number of proliferating cells to the same extent in both tibial and sciatic nerves (Fig. 4c) which was unexpected since this level of compression did not increase the axonal regeneration distances. Compression at 300 mmHg markedly increased the number of BrdU positive cells in the tibial nerve (Fig. 4d), but surprisingly not to the same extent as in the sciatic nerve (Fig. 4e). This may be a result of a decrease in blood flow due to a "no reflow phenomenon" in the sciatic nerve during the BrdU labeling period.

p75 receptor expression

The expression of p75 receptor by Schwann cells in sciatic and tibial nerves was studied 6 days after compression at 150 or 300 mmHg (no grafting procedure was performed). In control nerves, the immunoreactivity of p75 receptor was almost undetectable. In contrast, sections from the compressed nerves (both pressure levels) showed a marked expression of p75 receptor, seen as dense immunoreactivity in long strands parallel to the axons. It has been demonstrated that the expression of p75 receptor is partly induced by loss of axonal contact [50] or early axonopathy [51]. Our findings further support that the p75 receptor may be upregulated as a sign of early perturbation in the axon-Schwann cell contact, since the p75 receptor was upregulated already after compression at only 150 mmHg (2 hours). Osmium stained sections of nerves compressed at the same magnitude and duration did not show any structural changes or signs of degeneration (unpublished data).
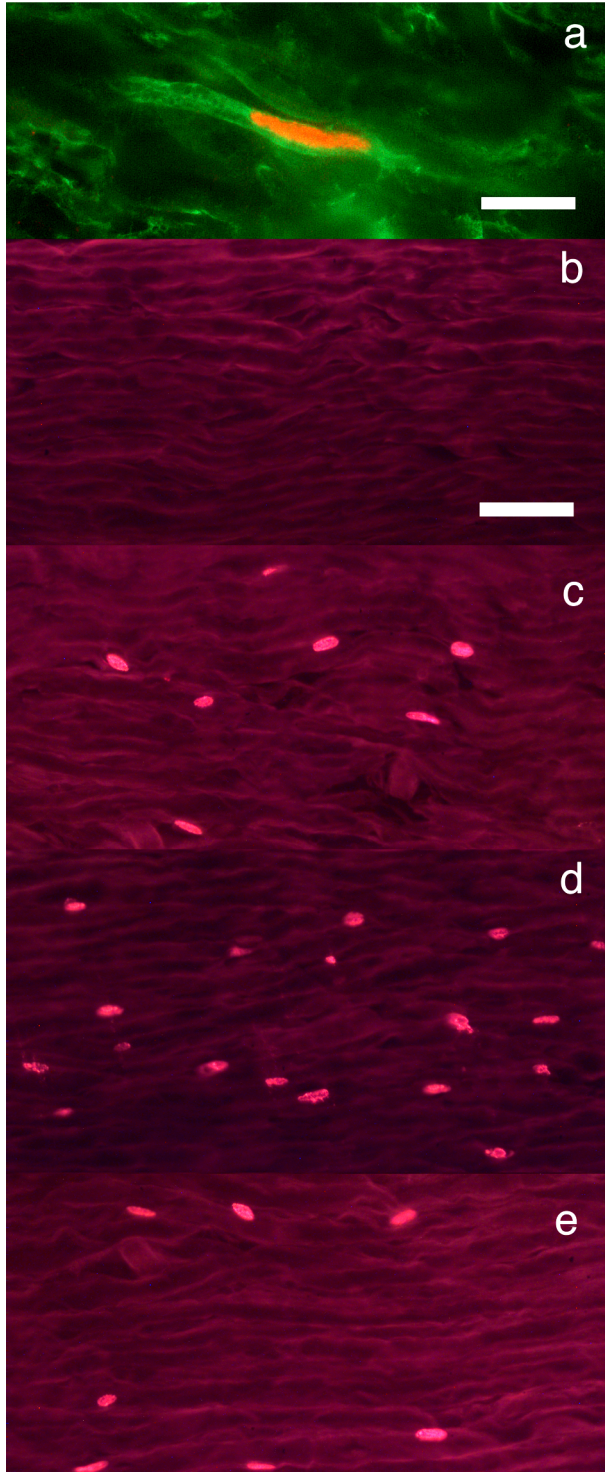
Figure 4. The proliferating cells were mainly Schwann cells, shown by double labeling with S-100 / BrdU (a). Only a few BrdU positive cells were present in control sections (b) whereas tourniquet compression at 150 mmHg induced proliferation of Schwann cells to the same extent in both sciatic and tibial nerves (c). Compression at 300 mmHg markedly increased the number of proliferating cells in tibial nerves (d) but surprisingly not in sciatic nerves (e). Bar = 20 μm (a) and 50 μm (b-e).

Paper III

C-terminal flanking peptide of neuropeptide Y in DRG following nerve compression

The expression of CPON in DRG, 3, 6, 14 and 28 days after tourniquet compression at 150 or 300 mmHg for 2hours, was examined. The temporal development of the CPON expression was calculated in percent of the total number of counted cells with the nucleus visible.

CPON is not expressed in intact DRG cells but it is distinctly upregulated following peripheral axotomy [21]. It was therefore assumed that CPON could be a suitable marker for minor nerve injury. As expected, no CPON immunoreactivity was detected in any of the cell bodies from control L4 and L5 DRG at any time point. However, tourniquet compression at 150 and 300 mmHg induced a slight and transient upregulation of CPON in DRG (up to 1 and 9%, respectively, at 6 days) while transection of the sciatic nerve induced a marked and persistent increase in CPON positive cells (up to 30 %), in accordance with earlier studies [22, 95]. The transient upregulation of CPON after compression may be explained by a reversible suppression of retrograde axonal transport of target-derived factors. The expression of a variety of neuropeptides, as for example substance P (SP) and CGRP, may be controlled this way [36].

Paper IV

Nerve regeneration in nerve grafts conditioned by vibration exposure

It was tested if vibration exposure can enhance nerve regeneration in nerve grafts. No effect of 2 days of vibration could be detected; therefore the duration of vibration exposure was increased to 5 days. The average regeneration distances for the different experimental groups are depicted in Fig 3 in the original paper. On post operative (p.o.) day 4, both the vibrated recipient side group and the vibrated grafts had significantly longer regeneration distances than control grafts. At p.o. day 6, vibrated grafts had significantly longer regeneration distances than control grafts. At 8 days p.o. the differences between the groups were not statistically significant which may indicate that the stimulation by the vibration exposure had decreased or even that the exposure has a detrimental effect on the regeneration process in a longer perspective. Control grafts had an initial delay of 3.3 days, which is in accordance with other studies [91], whereas both the vibrated recipient side and the vibrated graft had shorter initial delays as compared to controls (1.5 and 1.1 days, respectively). The results indicate that vibration exposure triggers the axons to regenerate earlier and that it can act as a conditioning lesion, similar to that observed after nerve compression. One may speculate how long-term vibration exposure could affect peripheral nerves, since already 5 days of vibration induce functional changes in rat nerves.

Paper V

Nerve injury induced by vibration: prevention of the effect of a conditioning lesion by D600, a Ca^{2+} channel blocker

Paired comparisons showed that the hindlimbs exposed to vibration in both the control group (no miniosmotic pumps) and the group treated with Ringer's solution via miniosmotic pumps had significantly longer regeneration distances at both 3 and 6 days as compared to the non-vibrated hindlimbs. Application of D600 + Ringer's solution via the miniosmotic pump, however, abolished the stimulatory effect of the vibration exposure. One can speculate that local application of D600 may have a neuroprotective effect, but the mechanisms behind the effect are not known. Since D600 blocks L-type Ca^{2+} channels, it is tempting to suggest that vibration induces the influx of Ca^{2+} through such channels. An increased intra-axonal Ca^{2+} level could lead to an activation of Ca^{2+} dependent proteases, which in turn could result in axonal damage, and a concomitant induction of a condition lesion effect. This may be prevented by D600.

A surprising finding was that the non-vibrated nerves in the group of rats treated with D600 + Ringer's solution had significantly longer regeneration distances than the non-vibrated control nerves (at 3 days). The reason for this is not known but previous studies have shown that low systemic concentration of Ca^{2+} channel blocker can accelerate axonal sprouting after surgical repair of transected, but otherwise healthy, nerves [84, 96].

General discussion

Neurobiological considerations

Conditioning lesions

The regeneration process in peripheral nerves can be manipulated by different procedures. Almost 30 years ago, it was shown that the regenerative capacity in a peripheral nerve could be markedly improved if the nerve had been injured previously (i.e. a conditioning lesion) [54]. The initial delay was found to be shortened and regeneration rate was increased. This phenomenon has been observed when the nerve previously had been crushed or transected [54, 61]. However, the conditioning lesion can also be induced by other types of injuries, such as acute and chronic compression [56, 97], vibration exposure [57], and application of electromagnetic fields [58]. The present thesis deals with two types of experimental conditioning lesions - tourniquet compression and vibration exposure. In the short-term perspective, these potentially harmful treatments can increase the regenerative capacity in nerves. The increased regenerative capacity induced by compression and vibration is not only associated with the activation of non-neuronal cells [98], but also of the affected neurons, as discussed below.

Factors influencing the conditioning effect

The pressure level of compression is important for the development of the conditioning effect. Compression at a low magnitude (150 mmHg; *paper II*) did not stimulate axonal regeneration in nerve grafts as higher pressure did (200 and 300 mmHg). In the vibration exposure model only one acceleration level and frequency was employed *(paper IV, V)*. How these parameters influence the response of the nerve is unclear but it has been reported that when a lower frequency and acceleration level (40 Hz/16 m/s^2) was employed in similar experiments the axonal outgrowth was retarded [99].

Another important factor for the development of the conditioning effect was the duration of compression and vibration exposure. Only 30 minutes of compression (300 mmHg) did not affect the axonal regeneration distances after nerve crush, but when the duration of compression was prolonged to 120

minutes the regeneration distances were increased with up to 36 % *(paper I)*. Furthermore, 5 days of vibration were needed to increase the regeneration distances in nerve grafts *(paper IV)*, and 2 days of vibration exposure had no influence on the regeneration distances.

The conditioning lesion effect takes some time to develop (at least 3-6 days), possibly due to the time it takes for both non-neuronal cells (Schwann cells) to initiate proliferation and neurotrophic factor production [91, 100], and to activate the nerve cell bodies (see below). In *paper I*, a conditioning interval of 3 days between the compression and the test crush lesion was needed, but 6 days was more effective for the conditioning lesion effect to develop. This finding is in agreement with previous studies [91]. In *paper V* the test crush lesions were performed on the end of the 5th day of vibration, thus, no conditioning interval was used. Even though no conditioning interval was employed, the regeneration distances increased. This may be explained by the fact that the vibration exposure was extended over a period of 5 days, which may be regarded as the conditioning interval. The Schwann cell activation and proliferation might have started already during the vibration period.

Cell activation - nerve trunk

The nerve injury models used in the thesis – compression and vibration - induced activation and proliferation of cells in the nerve trunk as indicated by the increased incorporation of BrdU and p75 receptor in nerves from the compressed hindlimbs as compared to controls. There was a three-folded increase of proliferating cells in tibial nerves from hindlimbs compressed at 300 mmHg as compared to those compressed at 150 mmHg, implying that also the induction of proliferation of non-neuronal cells is dependent on the severity (i.e. magnitude) of injury. The proliferating cells were mainly Schwann cells in accordance with previous studies [60]. Vibration exposure can also induce a small number of BrdU positive cells in nerves close to the vibration exciter (Dahlin and Kanje, to be published).

The conditioning lesion effect is related to the number of proliferating cells as reported by Sjöberg et al. who used thymidine incorporation to detect proliferation [101]. The BrdU technique has the advantage that the proliferating cells can be morphologically detected in the nerve and identified

by double labeling (S-100). The distribution of proliferating non-neuronal cells can be uneven in chronically locally compressed nerve segments [102, 103]. However, Bergmark et al. and Kanje et al. [102, 103] used silicone tubes to induce chronic nerve compression. One may hypothesize that the increase in the number of proliferating cells observed in such a locally compressed nerve at the edges of the compressed segment may represent an "edge effect", i.e. the pressure gradient was greatest at the edges of the compressed segment causing local structural disarrangement of the myelin sheath, edema and even degeneration [69]. However, such irregular distribution of BrdU was not observed after tourniquet compression.

Activated Schwann cells after compression *(paper II)* were indirectly visualized by the expression of p75 receptors, which are not expressed in mature myelinated Schwann cells. However, p75 can get upregulated when there is a loss of axonal-Schwann cell contact and Wallerian degeneration occurs in the distal stump [44, 49, 104]. Elevation of p75 receptor mRNA is preceding the expression of the receptors [47]. Earlier stages of axonopathy without structural damage or nerve fiber damage can induce such an elevation suggesting it being a sensitive indicator of perturbations in normal axon-Schwann cell interactions [51]. This could explain why p75 receptors were expressed already after compression at 150 mmHg - a pressure level at which it would be unlikely to find nerve fiber degeneration. The role of the p75 receptor is dual; apart from binding NGF, which acts as a trophic agent for the outgrowing axons from TrkA bearing neurons, the p75 receptor can induce cell death by apoptosis [105-107]. The signal transduction pathways used by the receptor is not yet defined but the receptor may have a role in mediating "stress response signals" [53]. Vibration and compression could be considered as such signals. Whether compression and vibration exposure can induce apoptosis remains to be elucidated.

The structural changes observed in *paper I* confirm earlier findings by Nitz et al. [67] who demonstrated that tourniquet compression at 300 mmHg for 2 hours induced degeneration and myelin damage. This level of compression-induced conditioning measured as increased axonal regeneration distances in crushed nerves *(paper I)* and nerve grafts *(paper II)* as well as increased

expression of p75 receptors and BrdU uptake *(paper II)*. Compression at 150 mmHg induced both expression of p75 receptors and BrdU incorporation. Structural signs of injury have not been studied at this level of pressure but, given that tourniquet compression at a magnitude of 200 mmHg induces only minor changes in the nerve [67], it is probable that a pressure of 150 mmHg would cause even less damage. At a pressure of 150 mmHg, conditioning was not observed. This implies that conditioning requires substantial nerve fiber damage. Thus, activation of Schwann cells alone is not sufficient or may be associated with less severe injuries like segmental demyelination.

Cell activation – nerve cell body

The cell body reaction has mainly been investigated after crush/transection injury or application of a chronic constriction injury (CCI) of the sciatic nerve (loose catgut ligature) [108, 109]. It was previously not known if tourniquet compression could induce a cell body reaction (activated cell bodies), but local acute compression can induce "unspecific" morphological changes in the nerve cell bodies [74]. Recently, it was also reported that a chronic compression injury by small diameter silicone tubes could activate nerve cell bodies in DRG observed as an upregulation of CPON [102].

Tourniquet compression at 150 and 300 mmHg for 2 hours *(paper III)* induced a transient upregulation of CPON in DRG in a small subpopulation (9%) of the DRG neurons. CPON, which is not present in adult DRG neurons, therefore proved to be a sensitive marker for activation of one aspect of the cell body reaction, namely the induction of certain neuropeptides. This could be an early sign of nerve injury since more severe injuries as nerve transection robustly induces CPON. While transection results in a persistent induction of CPON upregulation, the upregulation of CPON in response to compression was transient. Possibly, the transient upregulation of CPON may be induced by a reversible inhibition of retrograde transport known to take place during acute local nerve compression [71]. Application of colchicine suppresses the retrograde transport and induces upregulation of NPY in an identical manner to sciatic nerve transection [110] implicating that NPY expression is controlled by target-derived retrogradely transported factors. The finding that administration of neurotrophin-3 (NT3) attenuates injury-

induced NPY expression in the DRG is in line with such a mechanism of regulation [111, 112]. An alternative explanation to the decrease in the number of CPON positive cells with time following compression is that the CPON responsive cells die and thus represent a subpopulation of neurons that are particularly vulnerable to axonal injury. However, since the number of cells that respond with an increase in NPY (and CPON) as a reaction to nerve transection [21] is much larger than the number of neurons that die during the same time interval [113], cell death is not likely to account for the decrease in the number of CPON positive cells in the compressed nerve.

In the present thesis, CPON was used to monitor the effects of compression. This appeared to be a sensitive marker of neuronal activation and possibly nerve injury. It is reasonable to believe that the expression of other neuropeptides, like GAL and CGRP and SP, could also be used to monitor changes in response to tourniquet compression, since the expression of these peptides is also affected by transection and chronic constriction injuries [36, 114]. However, in contrast to NPY and therefore CPON, these peptides are already expressed by many neurons in the intact DRG. A moderate alteration in the number of neurons expressing CGRP, GAL and SP by tourniquet compression might therefore be more difficult to detect. Preliminary observations suggest that also vibration at magnitudes used in this study induces a cell body reaction in a limited population of the DRG neurons (Dahlin and Kanje, unpublished data).

D600 - neuroprotective?

The mechanism by which D600 acts is open to speculation as pointed out previously. One possibility is that D600 interferes with Ca^{2+} influx through voltage sensitive L-type Ca^{2+} channels and thereby limits the extent of nerve injury caused by calcium activated proteases. Such a mechanism of action could explain why D600 prevents conditioning. On the other hand, following systemic administration [84, 96] a L-type Ca^{2+} channel blocker at low concentrations may stimulate regeneration following nerve transection and repair. This could also explain the finding of increased regeneration lengths at the contralateral non-vibrated nerves in D600 treated rats.

Clinical Implications

Stimulation of regeneration

The beneficial effect of conditioning lesions can be utilized to enhance outgrowth of axons following injury. Experimental tourniquet compression at clinically relevant levels and durations, i.e. those used during extremity surgery to generate a bloodless field, acts as a conditioning lesion. In this thesis, conditioning by tourniquet compression increased the lengths of outgrowing axons within nerve grafts with up to 30 % six days after surgery. It is therefore tempting to suggest that pretreatment by tourniquet compression could be used clinically to promote nerve graft repair. In such a situation, the axons should grow faster through the graft with the two suture lines and may reach the distal nerve segment before extensive scarring occurs. In contrast, the use of vibration exposure for conditioning and grafting in the clinical setting is not appropriate, given that one would need 5 hours of vibration during 5 consecutive days to obtain the effect. It should be born in mind, however, that experimental studies of functional recovery are required before any recommendation with respect to use of conditioning by tourniquet compression and nerve grafting can be issued.

Injury - pain

The conditioning lesion effect induced by tourniquet compression and vibration exposure may on the other hand be harmful, i.e. it may be interpreted as a first sign of nerve injury. At relatively moderate levels of compression and vibration, both Schwann cells and neurons were activated. Longer periods of vibration exposure may be detrimental for the nerve regeneration as indicated by the findings of Strömberg et al. [99].

In the clinic, compression and vibration injuries are often encountered. However, surgeons routinely use tourniquet compression during upper and lower extremity surgery. During such procedures, a cuff is inflated around the extremity to create a bloodless operative field. Considering the results presented here, the suitability of this procedure merits discussion. The occurrence of mechanical nerve injury in connection with anesthesia and surgery is probably more common than generally believed [62, 115, 116]. Electromyographic abnormalities up to three weeks after use of tourniquet

(200-290 mmHg, 24-60 min) have been reported in connection to carpal tunnel release. Temporary changes in the muscles of the forearm, probably based on denervation have also been reported [4]. However, in most of the cases in which adverse effects have been reported, the applied tourniquet pressures have been higher. Pressure levels, corresponding to what is used in clinical practice, were used in the thesis and proved to induce both morphological and functional changes in the nerve even at relatively short compression intervals (2 hours). These experimental results imply that it is of great importance to minimize and carefully monitor the pressure levels and duration of tourniquet compression in clinical practice.

Entrapment neuropathy and vibration syndromes may be associated with pain of which there is a lack of efficient treatments. The understanding of pain mechanisms and further development of effective treatments of pain is essential. One frequently used experimental pain model is to study the expression of different neuropeptides, such as NPY, in relation to CCI and transection [109, 110]. The exact function of NPY upregulation, in the nerve cell bodies in DRG is not known, but it may modify pain impulses [17]. NPY is considered to have a nociceptive modulating effect due to its inhibitory function. Rats that lack NPY show increased pain behavior following nerve lesions [117]. Through studies of NPY receptors, it has been suggested that NPY may be released from injured large sensory neurons and that transfer of nociceptive information from small and medium sized neurons is blocked following peripheral nerve injury [23]. The dramatic upregulation of NPY and CPON following axotomy may be explained by its suggested antinociceptive function. Following tourniquet compression and chronic compression with silicone tubes [102] only a small subpopulation of the DRG neurons expressed CPON. This may reflect the severity of trauma and perhaps the grade of pain modulation needed.

Future

Nerve trauma, particularly transection of major nerve trunks, generally results in significant disability in adults. Although microsurgical techniques have been refined, the functional recovery is unsatisfactory. It is conceivable that the approach to the repair of peripheral nerve injuries must be altered in order to improve functional recovery. An increased understanding of how the neuron and the supportive cells respond to trauma is needed as well as better knowledge regarding mechanisms regulating axonal outgrowth. One way to improve the results after nerve injury could be to use pre-conditioned nerves in nerve grafting situations. The present results showed that this could be achieved by a non-invasive technique. However, based on our present knowledge of graft repair it seems likely that the surgical approaches must be supplemented with pharmacological treatment in order to optimize nerve regeneration. On the other hand, it may even be more important to develop such treatment aimed at rescuing injured neurons or limit the extent of nerve injury. Whether or not Ca^{2+} channel inhibitors like D600 could be used in such a context remains to be investigated.

Conclusions

- Tourniquet compression enhances axonal regeneration - measured as an increased axonal regeneration distance after a crush injury - and acts thereby as a conditioning lesion.

- The local conditioning effect by tourniquet compression can be used to improve the early regenerative capacity of axons in a nerve graft and is associated with an increased proliferation of Schwann cells and a concomitant expression of the p75 receptor.

- Tourniquet compression induces a transient upregulation of CPON in a subpopulation of nerve cell bodies of the dorsal root ganglia.

- Vibration exposure also acts as a conditioning lesion, primarily by shortening the initial delay. The conditioning lesion effect may also be considered as an injury.

- The conditioning lesion induced by vibration exposure can be prevented by local treatment with the Ca^{2+} channel blocker D600.

Summary in Swedish

Populärvetenskaplig sammanfattning

Bakgrund

Varje nervskada, oavsett orsak, utgör ett stort problem för den drabbade individen och slutresultatet är i regel dåligt. Personerna som drabbas av nervskador är ofta i arbetsför ålder. Rehabilitering efter en nervskada medför vanligtvis lång sjukskrivning och ibland måste patienten skolas om till ett nytt yrke. Detta innebär stora kostnader för både individen och för samhället. Man kan därför vinna mycket på att förbättra slutresultatet hos dessa patienter.

Nervskada - nervreparation

Nervcellskropparna är belägna i ansamlingar (ganglier) i anslutning till ryggmärgen. Nervtrådarna (axonerna) löper som långa utskott från cellkropparna till olika muskler och hudområden. Efter en skada på en nervstam förloras den del av nervtråden som finns bortom skadan (mot periferin) medan dess bindvävshölje (nervskidan) finns kvar. Efter en avskärning av nervstammen med t ex en kniv kan nervskidan sys ihop, varefter de avskurna nervtrådarna i nervstammen försöker växa ut igen till muskler och hud. Nervtrådarna växer emellertid långsamt och de har svårt att hitta rätt.

I allvarligare fall av nervskador där nervstammen slitits av och det uppstått ett "gap" mellan den skadade nervstammens båda ändar kan det bli nödvändigt att utföra en nervtransplantation. Genom att skarva in delar från en annan mindre betydelsefull känselnerv, som "offras", kan man överbrygga gapet i nervstammen. Trots välutvecklad mikrokirurgisk teknik är utväxten av nervtrådar efter en nervtransplantation sämre än efter en skärskada där nervändarna sytts direkt mot varandra. Det skulle därför vara en fördel om man kunde utveckla icke-invasiva metoder att "förbehandla" nervtransplantat så att utväxten av nervtrådar genom dessa stimulerades.

Stimulering av utväxt

Avhandlingen är inriktad på experimentella studier kring två icke-invasiva behandlingar som kan påverka perifera nervsystemet. Särskilt undersöks om dessa behandlingar kan påverka kapaciteten hos nervceller och deras nervtrådar att återutväxa efter en nervskada. Det är känt att nervtrådars utväxt kan stimuleras om nervstammen tidigare utsatts för en skada eller annan manipulering. Detta fenomen kallas för konditioneringseffekt. Effekten kan dels bero på att nervstammens stödjeceller stimuleras, dels att nervcellerna ställer om sin ämnesomsättning och är "beredda" på reparation när nervstammen skadas på nytt.

Vid den ena typen av behandling anlades ett tryck med en blodtrycksmanschett (tourniquet) runt ena bakbenet på en råtta. Blodtrycksmanschetter används rutinmässigt vid operationer i "blodtomt fält" för att undvika störande blödning under operationen. Trycket från manschetten stoppar inte bara blodflödet i blodkärlen utan påverkar även muskelvävnad och nervstammar i extremiteten. Vid den andra typen av behandling exponerades nerverna i råttans bakben för vibration (som vid användning av handhållna vibrerande verktyg).

Genom behandling med ett högt tryck (200-300 mmHg), eller vibrationsexposition av bakbenet, kunde utväxten av nervtrådar påskyndas i råttans ischiasnerv efter en tillfogad nervskada (lokal klämskada). Effekten var särskilt tydlig när en del av en nerv som på detta sätt behandlats med tryck användes som ett nervtransplantat för att överbrygga en skada, ett gap, i en för övrigt obehandlad nerv.

Vad händer i nervstammarna efter stimulering?

Tryck med en blodtrycksmanschett, även vid lägre trycknivåer än de som stimulerade nervtrådarnas utväxt (150-300 mmHg), aktiverade nervtrådarnas stödjeceller (Schwannceller) så att dessa delade sig (cellproliferation). Stödjecellerna började också uttrycka så kallade p75 receptorer för nervtillväxtfaktorer som ett tecken på aktivering efter kompression.

Vad händer i nervcellskropparna?

Nervcellskropparna aktiverades av tryckbehandling genom att dessa började producera en neuropeptid, CPON, vilket är en substans som produceras av nervcellkroppar efter t ex nervavskärning. Produktionen av CPON var dock övergående och inte lika riklig som efter en avskärningsskada men tydde ändå på en aktivering.

Stimulering eller skada?

De använda metoderna att stimulera en nerv kan, sett ur en annan synvinkel, också betraktas som potentiellt skadliga. Att stimulering leder till att nervtrådarna börjar växa tidigare efter en skada eller nervtransplantation kan tolkas som ett tecken på tidig nervskada. Exponeringstid, tryck- och vibrationsnivåer är sannolikt avgörande för graden av skada. Den potentiellt skadliga konditioneringseffekten av vibration kunde blockeras om en nervstam i en vibrerad extremitet samtidigt behandlades med ett läkemedel, en kalciumkanalblockerare (D600).

Sammanfattning

Exponering för vibration eller lokalt tryck med en blodtrycksmanschett runt en extremitet ökade nervtrådarnas kapacitet att återutväxa (regenerationsförmåga) efter en nervskada. Behandlingarna ledde till att nervcellskropparna och nervens stödjeceller aktiverades. Troligtvis är en sådan aktivering nödvändig för att uppnå en ökad regenerationsförmåga. I vissa situationer skulle man kunna utnyttja denna effekt för att förbättra operationsresultatet efter en nervskada.

Om man å andra sidan betraktar aktiveringen som en skada, framstår det som viktigt att begränsa exponeringen av tryck och vibration. I synnerhet i samband med operationer i blodtomt fält bör man noggrant kontrollera och minimera både trycknivå och behandlingstid för att inte skada nerver och muskler under blodtrycksmanschetten. Om behandling med D600 kan förebygga nervskador som orsakas av t ex vibrationsexposition, eller kompression kvarstår att utreda.

Mången målar en kalv som en sol
Och anses för rena genit
Men den som vill göra en hederlig stol
Har inga genvägar dit

Mången diktar med grumliga ord
Och folk säger: Underbart
Men den som vill snickra ett användbart bord
Är tvungen att tänka klart

Hjärnans drömskhet är utan gräns
Och ögat ser vad det vill
Men handens medfödda intelligens
Kan ingen konstra till

Alf Henriksson

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"Not everything that can be counted counts, and not everything that counts can be counted"

Albert Einstein

Appendix I-V

Nerve regeneration enhancement by tourniquet

I

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NERVE REGENERATION ENHANCEMENT BY TOURNIQUET

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The use of tourniquet compression as a non-invasive method to enhance axonal regeneration was assessed in the rat sciatic nerve. One hind limb of the rat was subjected to compression by a tourniquet set at 300 mmHg for 30 or 120 min followed by bilateral test crush lesions performed either directly or after a conditioning interval of 3 or 6 days, with the non-compressed side serving as a control. Axonal regeneration distances were evaluated after 3 days by the pinch reflex test. We found that compression caused an increased outgrowth length of sensory axons compared to the controls. The effect was most obvious after 120 min of compression with a conditioning interval of 6 days. Tourniquet compression has a conditioning lesion effect on peripheral nerve and may enhance nerve regeneration.

INTRODUCTION

Despite the use of advanced microsurgical techniques during the two last decades, the functional and sensory recovery after repair of peripheral nerve injuries is still poor (Birch and Raji, 1991; Lundborg, 2000; Lundborg et al., 1997). A successful outcome may be limited by several factors, including slow and incomplete outgrowth of axons which allows atrophy of the end organ. One way to overcome this obstacle is to develop a method of stimulating axonal outgrowth. Clinically, such a method has to be harmless to the patient and it should also be non-invasive. It has been previously demonstrated that a conditioning lesion such as a transection, a crush lesion or a local compression injury to a peripheral nerve enhances the axonal outgrowth after a subsequent test crush lesion (conditioning lesion effect) (Dahlin and Kanje, 1992; Dahlin and Thambert, 1993; Forman et al., 1980; Kerns et al., 1993; McQuarrie and Grafstein, 1973). The effect is not fully understood but may be explained by the activation of the neuron itself, as well as stimulation and proliferation of non-neuronal cells in the nerve trunk (Jacob and McQuarrie, 1993; Sjöberg and Kanje, 1990). It seems that a relatively mild injury is sufficient to cause activation of the non-neuronal cells (Kanje et al., 1995). The purpose of this experimental study was to evaluate whether nerve regeneration can be stimulated by acute compression of a nerve with an inflatable tourniquet.

MATERIALS AND METHODS

The study was carried out at a laboratory with controlled room temperature and air humidity and with the same operator throughout the study. Thirty-six female Wistar rats weighing 180–200 g (Möllegard, Denmark) were randomly divided into six groups, each of six animals. The rats were anaesthetized with an intraperitoneal injection of 10.8 mg pentobarbital. The hindlimbs of each rat were shaved and one was compressed with a 1.6 cm wide inflatable tourniquet (Hokanson, Blidö, Sweden), which was applied at thigh level, at a pressure of

300 mmHg for 30 minutes in three groups and 120 minutes for the other three groups (Fig 1). The pressure was controlled by a calibrated inflator (Stille-Werner, Sweden). The distal and proximal edges of the compression site were marked with a pen on the skin for identification. Directly following release of pressure, or following a conditioning interval of 3 or 6 days (Table 1), both sciatic nerves were exposed and crushed for 20 seconds with specially designed pliers approximately 2 mm proximal to the previously compressed nerve segment. The test crush lesions on the non-compressed (control) and the compressed side were applied at a similar level and the crush sites were labelled with 9/0 epineurial sutures (Ethilon®, Edinburgh, Scotland). The wounds were closed and the animals were allowed to recover.

Three days after the crush lesions were performed, the animals were lightly anaesthetized again using the same anaesthetic technique (9.0 mg pentobarbital). The person who performed the observations was blinded to which conditioning interval was studied and which tourniquet duration had been used. The outgrowth of sensory axons
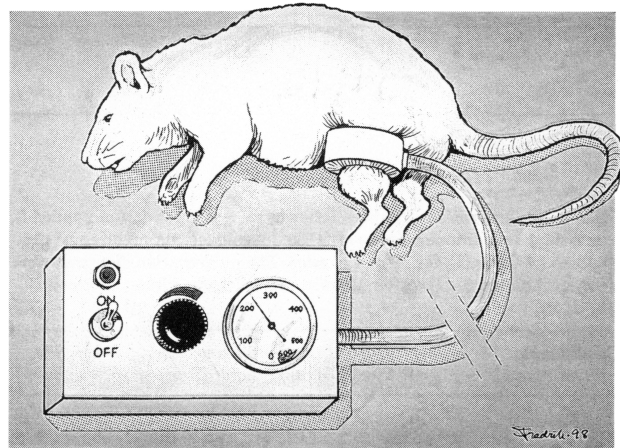

Fig 1   The tourniquet model. The mini-tourniquet was applied to the hind limb of the anaesthetized rat for 30 or 120 min at 300 mmHg. It was controlled by a calibrated inflator. The dotted line indicates that the tube is longer than illustrated.

was evaluated by the pinch reflex test (Bisby and Pollock, 1983; Forman et al., 1980; Kanje et al., 1988; Kerns et al., 1993). This was done by cutting the sciatic nerve distally. The distal extent of regenerating axons was then localised by pinching the distally cut nerve in disto-proximal direction until a withdrawal reflex in the back muscles was elicited. The distance between this labelled point and the previously marked crush level was measured with a calliper under a microscope and was regarded as the regeneration distance. The animals were then killed with an overdose of pentobarbital (120 mg).

For a morphological overview, routine histology was performed in a separate group of three rats. One of their hindlimbs was subjected to compression at 300 mmHg for 120 min with a conditioning interval of 6 days (no test crush lesion was applied). The sciatic nerves were carefully excised from 2 mm proximal to the labelled crush site to 13 mm distal to the crush site. 15 mm lengths of the tibial nerves were also excised distal to the knee joints. The specimens were loosely tied to wooden sticks at in-vivo length. The nerves were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.15) for 4 hours and then transferred to cacodylate buffer. Three mm lengths of nerve were then obtained from the segment of the sciatic nerve which had been beneath the tourniquet and from the tibial nerve (distal to the tourniquet). The specimens were post-fixed in 1% osmium tetroxide in cacodylate buffer for 2 hours followed by a 15 minutes wash in cacodylate buffer. The nerve pieces were dehydrated in ascending concen-

Table 1—Design of the study. A tourniquet with a pressure of 300 mmHg was applied around the rat hind limb for 30 or 120 minutes. After various time periods (conditioning intervals) test crush lesions were applied to the sciatic nerves bilaterally (n = number of rats)

Conditioning interval (days)	Duration of compression (min)	
	30	120
0	n = 6	n = 6
3	n = 6	n = 6
6	n = 6	n = 6

Table 2—Regeneration distances following a test crush lesion applied 0, 3 or 6 days (conditioning interval) after tourniquet compression of the hindlimb (300 mmHg for 30 or 120 min). The regeneration distances were always evaluated 3 days after the test crush lesion. Values are mean [SEM; n]

Conditioning interval (days)	Duration of tourniquet compression (min)			
	30	control	120	control
0	5.9 [0.3; 6]	5.5 [0.2; 6]	5.6 [0.5; 6]	5.3 [0.3; 6]
3	6.0 [0.2; 6]	5.5 [0.2; 6]	7.0 [0.2; 6][a]	5.5 [0.1; 6]
6	6.0 [0.2; 6]	5.7 [0.2; 6]	7.7 [0.5; 6][b]	5.7 [0.3; 6]

[a] and [b] indicate statistical significant difference between control group (no tourniquet) and tourniquet group: a $P = 0.03$ and b $P = 0.005$.

trations of ethanol and propylene-oxide for 30 minutes, infiltrated with a 1:1 mixture of Epon and propylenoxid for 2 hours at room temperature and then with pure Epon overnight. The specimens were embedded in pure Epon for polymerisation at 60°C for 72 hours. Nerves from the non-compressed side were prepared in the same way. One μm thick longitudinal and cross sections were cut on a microtome and mounted and stained with Richardson's solution; the coded sections were examined by light microscopy.

Statistical methods

After the inter-individual differences in regeneration distances between the control side and the tourniquet side had been eliminated by pairwise subtraction, a two way Anova test (factors; conditioning interval and duration) was used to determine if some of the effects in the model were significant. A post-hoc study (Fisher's PLSD) was carried out to examine if there were any significant differences between the groups in regeneration distances for either the conditioning interval or the duration of tourniquet compression. A P-value less than 0.05 was considered statistically significant. The values are presented as mean and standard error of the mean (SEM).

RESULTS

The results of the pinch reflex test performed 3 days after the application of the test crush lesions are presented in Table 2. Pooled data from the control side (no tourniquet, $n = 36$) resulted in a regeneration distance of 5.5 (SEM, 0.1) mm. Compression with a tourniquet set at 300 mmHg for 30 minutes had no significant influence on the regeneration distance at any of the conditioning intervals (Fischer's PLSD analysis, $P > 0.05$). However, tourniquet compression at 300 mmHg for 120 minutes significantly increased the axonal regeneration distance (Fig 2). This effect was detectable after a conditioning interval of 3 days ($P = 0.03$), but was more pronounced (up to 36%) after a conditioning interval of 6 days ($P = 0.005$). A conditioning interval of 0 days did not influence the axonal regeneration distance in this group. The two-way Anova analysis identified the positive interaction between duration and conditioning interval as the predominant factor responsible for the improved regeneration ($P = 0.01$), but both duration and conditioning interval alone had significant effects ($P = 0.03$ and $P = 0.02$ respectively).

Six days after tourniquet compression at 300 mmHg for 120 minutes (without a subsequent crush), longitudinal and cross sections of the sciatic and tibial nerves, directly under and distal to the cuff, were evaluated by light microscopy. The specimens showed various degrees of pathology. Nerve fibre damage, characterized by vacuolization, myelin indentation, cleavage and variability in myelin sheath thickness, was seen in nerves that had been subjected to compression. Based on these

features, nerve tissue from beneath the cuff zone (sciatic nerve, Fig 3a) was more damaged than the nerve tissue distal to the cuff (tibial nerve, Fig 3b), with more irregularities in myelin thickness and even rupture and disintegration of the myelin sheath. The presence of myelin ovoids can represent axonal degeneration but this was not clearly detected in our sections. Indentations in the myelin sheath in the longitudinal sections of the compressed sciatic nerve indicates, however, nerve fibre injury. These findings contrasted with the findings in the control groups (Fig 4a and b), in which the nerve tissue was organized with a constant myelin sheath thickness and there were easily detectable nodes of Ranvier in longitudinal control sections.
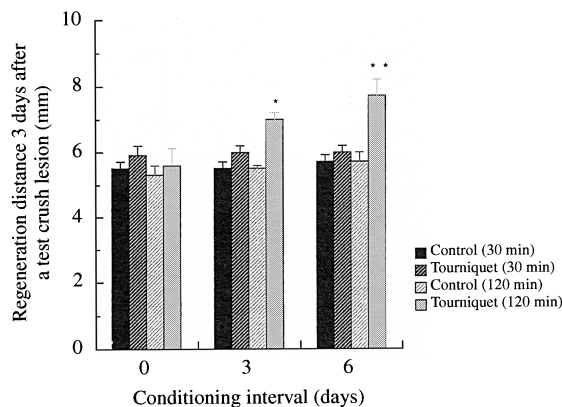
Fig 2   Summary of the findings from Table 2. Hind limbs of rats were compressed at 300 mmHg for 30 or 120 minutes and the contralateral side served as a control. Various conditioning intervals (0, 3 and 6 days) were used prior to application of a test crush lesion. Regeneration distances, obtained with the pinch reflex test 3 days after the test crush lesion, are indicated on the y-axis. *indicates $P < 0.05$ and **indicates $P < 0.01$. error bars indicate SEM

DISCUSSION

The present study shows that tourniquet compression of the rat hind limb can increase the regenerative capacity of sensory nerve fibres in the sciatic nerve following a test crush lesion (Dahlin and Thambert, 1993). The effect seems to depend on the duration of the compression, since 120 minutes, but not 30 minutes, was needed. The effect is also dependent on the magnitude of the pressure (Dahlin and Thambert, 1993). The choice of 30 and 120 minutes may represent two common durations of tourniquet application in clinical practice. Our study showed that the changes induced by tourniquet compression are more pronounced following a conditioning interval of 6 days, a time when morphological signs of nerve injury are also evident. This is in accordance with studies of transection-induced predegeneration which indicate that such a procedure improves the outgrowth of axons (Dahlin et al., 1988; Danielsen et al., 1995; Kerns et al., 1993). A conditioning interval of 3 and 6 days is sufficient to cause such a stimulation of nerve regeneration (Danielsen et al., 1995).

It has already been shown that a nerve injury before a test crush lesion increases the regeneration potential of the peripheral nerve (McQuarrie and Grafstein, 1973). When conditioning lesions of various severity are applied to a peripheral nerve before a test crush lesion, the regeneration distance of the axons from the site of the test crush lesion increases. Different types of conditioning lesions include exposure to pulsed electromagnetic fields of various duration (Sisken et al., 1989), chronic and acute nerve compression (Dahlin and Kanje, 1992; Dahlin and Thambert, 1993) and even a minor injury such as vibration exposure (Dahlin et al., 1992; Widerberg et al., 1997). The effects of these conditioning lesions are not fully understood but the most appealing explanation is that they cause a

a   b

Fig 3   Morphology of a longitudinal section (a) of a rat sciatic nerve and cross-section of a tibial nerve (b) 6 days after compression for 120 min at 300 mmHg. The specimen in (a) was from directly beneath the tourniquet and in (b) was distal to the tourniquet. Note the irregularities and indentations of the myelin sheath and the signs of scattered myelin degradation in (a) (arrows). In (b) the myelin sheaths are creased and there are signs of intraneural edema but the damage is not as severe as in the compressed nerve segment (a). Length of bar 20 μm.
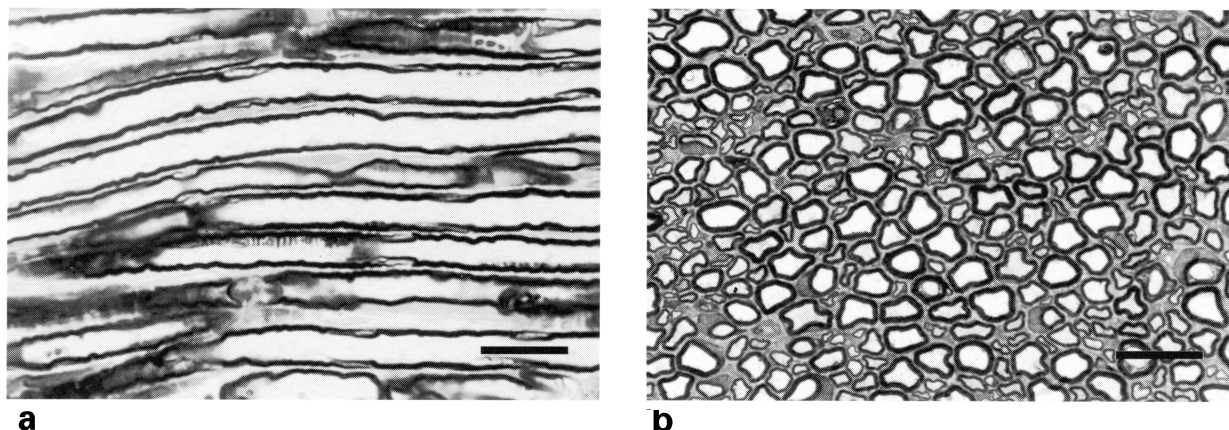
a **b**

Fig 4 Morphology of a longitudinal section of a rat sciatic nerve (a) and of a cross-section of a tibial nerve (b) in controls. There are no signs of edema and organized structures with little variation in myelin sheath thickness are seen. Length of bar 20 µm,

combination of peripheral and central changes within the nerve trunk (proliferation of Schwann cells), the neuron (nerve cell body) and the immediate surroundings of the nerve (inflammatory response with macrophage invasion) (Jacob and McQuarrie, 1993; Kanje et al., 1995; Sjöberg and Kanje, 1990).

In this study, tourniquet compression of a rat hind limb at 300 mmHg, a pressure which is probably a little higher than what is relevant in human extremity surgery, was investigated and was found to act as a conditioning lesion on peripheral nerve. The observed regenerative response is probably explained by the microscopic findings of local damage in the nerve, such as demyelination and axonal degeneration, which was seen in nerves that had been subjected to compression at 300 mmHg for 120 minutes together with subsequent changes in the nerve aiming to put the components in the nerve into a regenerative state. One should also consider the effects on the microcirculation with local microvascular permeability changes (Nitz et al., 1989; Rydevik and Lundborg, 1977). The fact that the conditioning lesion effect seemed to be more pronounced after a conditioning interval of 6 days may reflect the time needed for Schwann cell proliferation and for the nerve cell body to switch to a growth mode (Danielsen et al., 1995; Fu and Gordon, 1997). Furthermore, the tourniquet had to be applied for 120 minutes to get a regenerative effect and 30 minutes of compression was insufficient to condition the nerve. It has previously been shown that, in accordance with the present results, compression at 300 mmHg for 30 minutes only induces minor signs of edema and no signs of demyelination or degeneration, while substantial morphological changes are evident if the compression is prolonged to 120 minutes (Nitz et al., 1989; Nitz and Matulionis, 1982; Pedowitz et al., 1991).

The severity of the nerve injury caused by tourniquet compression seems to depend on several factors. The mode of compression, its magnitude and its duration are important factors (Crenshaw et al., 1988; Dyck et al., 1990; Nitz et al., 1989; Nitz and Matulionis, 1982; Pedowitz, 1991). Compression of a peripheral nerve trunk, e.g. with a tourniquet, induces a so called 'edge effect' which is an injury to nerve fibers and blood vessels at both edges of a tourniquet where the pressure gradient and shear forces are maximal (Ochoa et al., 1972; Rydevik and Lundborg, 1977; Rydevik et al., 1989). Ochoa and co-workers described a specific lesion of the node of Ranvier in teased fiber preparations, (nodal invagination), which, was only evident in nerves compressed at high pressure (1000 mm Hg) (Fowler et al., 1972; Ochoa et al., 1972). A substantially lower pressure was used in the present study (300 mm Hg). Teased fiber preparations were not used in the present study since the aim of its morphological part was simply to give an overview of the changes. Mechanical factors seem to be relatively more important at higher, rather than lower pressures and time is a significant factor at both high and low pressures.

Another important factor that influences the severity of the nerve injury brought about by tourniquet compression is ischaemia. Two hours of ischaemia is shorter than the time needed to induce changes in the *distal* part of a nerve subjected to only ischaemia (Lundborg, 1970). Tourniquet compression at the pressure level used in this study will jeopardise the microcirculation of the nerve. Endoneurial oedema and increased endoneurial pressure after cuff release could cause prolonged ischaemia beneath the tourniquet (Lundborg et al., 1983; Rydevik and Lundborg, 1977). The combination of mechanical and ischaemic factors (Dyck et al., 1990; Powell and Myers, 1986) might be responsible for the morphological changes seen in the sciatic nerve 6 days after compression at 300 mmHg for 120 minutes. The degenerative changes seen in the tibial nerve, which was distal to the tourniquet and thus not subjected to direct mechanical forces, is probably a result of Wallerian degeneration.

A further aspect of our study and previous studies (Nitz et al., 1989; Pedowitz et al., 1991) is that tourniquet compression at clinically relevant pressure levels and durations may cause neuronal injury which may interfere with functional recovery after extremity surgery. However, the level of 300 mmHg is probably above the 'recommended level' for a rat as their systolic blood pressure is about 120 mmHg (Friberg, 1985).

The tourniquet pressure in upper extremity surgery should be approximately 50–70 mmHg above the systolic blood pressure and the corresponding pressure level for lower extremity surgery is 100–150 mmHg (Räf and Netz, 1999).

The important conclusion of the present study is that a non-invasive procedure such as tourniquet compression could be used as a method to stimulate nerve regeneration in selected situations. Its potential application in nerve grafting procedures is currently under further investigation. Application of a tourniquet to the limb from which a nerve graft is to be harvested might condition the nerve graft and potentiate nerve regeneration within the graft. However, compression of the proximal part of the divided nerve trunk which requires a nerve graft will probably not give any additive effect (Kerns et al., 1993).

Acknowledgements

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Tourniquet compression: a non-invasive method to enhance nerve regeneration in nerve grafts

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Abstract

One hind limb of a rat was subjected to tourniquet compression (150, 200 and 300 mmHg; 2h). After 6 days a 10 mm sciatic or tibial nerve graft from the compressed limb was sutured to bridge a 3-4 mm gap in the sciatic nerve of the non-compressed limb. The distances of regenerating sensory axons were measured 6 days post surgery (tibial grafts; 8 days). Compression at 200 and 300 mmHg lead to significantly longer regeneration distances compared to controls. Incorporation of BrdU and expression of p75 receptor by non-neuronal cells (Schwann cells) in sciatic nerves 6 days after compression (150 and 300 mmHg; 2h) was also increased as a sign of Schwann cell activation. Tourniquet compression may be used as a non-invasive method to enhance nerve regeneration in nerve grafts.

Introduction

In the clinical situation, a peripheral nerve injury with substantial loss of nerve tissue demands nerve grafting. A frequently used method is to bridge a defect by an autologous nerve graft from the sural nerve. Unfortunately, the outgrowth of axons in the graft is slow and incomplete with poor functional recovery as a result. Various methods, including conditioning, have therefore been tested experimentally to enhance nerve regeneration through nerve grafts [1,2] but so far none of them have come to a clinical use. We have recently shown that tourniquet compression of a rat hind limb can increase the regeneration distance of sensory axons after a test crush lesion on one and the same nerve [3]. Tourniquet compression also activates the nerve cell bodies in the dorsal root ganglia (DRG) observed as a reversible induction of the C-terminal flanking peptide of neuropeptide Y (CPON) [4]. The effects of tourniquet compression are, however, complex and include direct local damage to the nerve during the compression but probably also indirect damage during reperfusion [5]. Compression induces signs of degeneration in the nerve trunk depending on the magnitude and duration of the pressure [3,6,7]. It is therefore reasonable to assume that both local changes and distant changes at the level of the nerve cell body are responsible for the

conditioning lesion effect of tourniquet compression. In the present study we tested the hypothesis that tourniquet compression of a limb can be used to condition a nerve segment intended for nerve grafting and thereby improve nerve regeneration. We also investigated if compression as such, induced two regeneration-related changes in the compressed nerve; proliferation of non-neuronal cells, mainly Schwann cells [8], measured as incorporation of 5-bromodeoxyuridine (BrdU), and expression of the low affinity neurotrophin p75 receptor.

Materials and methods

Animals and tourniquet compression

Experiments were performed on female Wistar rats weighing 180-200 g (Möllegaard, Denmark) after permission from the local ethical committee of Lund University. Prior to tourniquet compression the animals were divided into two main experimental groups in order to study: 1) axonal regeneration distances into sciatic and tibial nerve grafts taken from a compressed hind limb; 2) regeneration related changes: incorporation of BrdU by non-neuronal cells (Schwann cells) and expression of p75 receptors in sciatic and tibial nerves after compression. The rats were anaesthetised with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and the hind limbs were shaved. One of the hind limbs was compressed (conditioned) with a 1.6 cm wide inflatable tourniquet (Hokanson, Blidö, Sweden) at a pressure of 150, 200 and 300 mmHg for 2 h, at midthigh level [3,4]. A calibrated inflator controlled the pressure (Stille-Werner, Sweden). The distal and proximal edges of the compression-site were marked with a pen on the skin for identification. Sciatic nerve grafts were taken from the compression-site, and tibial nerve grafts were taken from distal to the compression-site. In a control group the tourniquet was applied to the hind limb without any pressure. After removal of the tourniquets the animals were allowed to recover from anaesthesia.

Nerve grafting and evaluation of regeneration distance

Six days after tourniquet compression (conditioning interval) the rats were re-anaesthetised. A 10 mm nerve graft from the compressed sciatic nerve (n=5 at each pressure level) was transplanted to bridge a freshly made 3-4 mm long gap in the sciatic nerve of the non-compressed side. In another group of rats (n=5) one hind limb was compressed at 300 mmHg for 2 h and after 6 days, a 10 mm tibial nerve graft was used to bridge a gap in the contralateral sciatic nerve as described above. The sciatic and tibial nerve grafts were sutured using three single 9/0 epineurial sutures at each end of the graft. The wounds were closed and the animals were allowed to recover for 6 days (sciatic nerve grafts) or 8 days (tibial nerve grafts). The length of regenerating sensory axons into the two different nerve grafts was evaluated by the pinch reflex test which has previously been described [3,9]. The distance between the proximal suture line and the positive pinch reflex site, measured by a caliper, was regarded as the axonal regeneration distance.

Proliferation of non-neuronal cells (BrdU-incorporation)

Six days after compression at 150 or 300 mmHg, the rats (n=6 at each pressure level) were re-anaesthetised and intravenously injected with 5 mg of BrdU in 0.5 ml 0.1 M Tris buffer (pH 7.6) that was allowed to circulate for 2 h. A 15 mm nerve graft from the compression site (sciatic nerve) and from distal to the tourniquet (tibial nerve) was excised using the corresponding contralateral nerve segment as a controls. The specimens were fixed in Stefanini's fixative, cryoprotected and embedded in Tissue-Tek® (Sakura Torrence, USA). Longitudinal cryosections, 10 μm thick, were collected on poly-L-lysine coated glass slides. Immunocytochemistry of BrdU, and in some experiments of S-100 (added as a general marker for Schwann cells), was performed essentially as described by Sondell et al. [10]with the exception that Alexa Fluor antibodies (red A11032 and green A11008) were used for visualisation of the binding of the primary antibodies. The preparations were examined in a fluorescence microscope. The number of BrdU positive cells in the endoneurial space was counted in longitudinal sections of the central portion of the sciatic and tibial nerves and expressed as number of BrdU positive cells/mm².

p75 receptor-immunocytochemistry

The expression of the low affinity p75 receptor was studied by immunocytochemical staining of the nerves compressed at 150 (n=6) or 300 (n=3) mmHg. After a conditioning interval of 6 days sciatic and tibial nerve segments were excised as above with their corresponding contralateral controls. Longitudinal cryosections,10 µm thick, were embedded in Tissue-Tek®. The sections were dried, washed in PBS, fixed in ice-cold acetone, washed in PBS, treated with 0.3% H_2O_2 in methanol and washed in PBS. The sections were blocked in rabbit serum and then exposed to a mouse monoclonal antibody against p75 receptor (dilution 1:100 Boeringher-Mannheim, Germany) in PBS for 2 h. After washing in PBS the sections were exposed to rabbit antimouse HRP antibody (dilution 1:50, DAKO) for 45 minutes and washed in PBS and developed in diaminobenzidine tetrahydrochloride (DAB, Sigma, Sweden). After a final wash and dehydration the preparations were mounted in DPX. Control sections were incubated without the primary antibody. The sections were examined in a light microscope.

Statistics

Axonal regeneration distances (mm) in sciatic and tibial nerve grafts and the number of BrdU positive cells (cells/mm²) in sciatic and tibial nerves following compression are expressed as mean ± s.e.m. Statistical analysis of variance (ANOVA) was performed on data of regeneration distances in sciatic grafts followed by Sheffés post hoc test. A two way ANOVA (factors: pressure level, nerve) was used to determine statistical differences in the number of BrdU positive cells, followed by Sheffés post hoc test. The un-paired t-test was used to analyse statistical differences in axonal regeneration distances in the tibial grafts. A p-value less than 0.05 was considered statistically significant.

Results

Regeneration distance

In the first set of experiments compressed nerve grafts conditioned for 6 days were used to bridge a gap in the contralateral sciatic nerve. After an additional six days axonal regeneration distances were measured. The axonal regeneration distances (mm) into sciatic nerve grafts are summarised in Table 1. Tourniquet compression at 200 or 300 mmHg of the sciatic donor nerve, before nerve grafting procedure, increased the regeneration distance with up to 30 percent compared to the non-compressed sciatic nerve grafts, whereas tourniquet compression at 150 mmHg had no significant effect.

Using the same paradigm, tibial nerve grafts taken from distal to the compression site (300 mmHg) increased regeneration distances with up to 25 percent 8 days post surgery (12.3 mm ± 0.29) as compared to the non-compressed tibial nerve grafts (9.8 mm ± 0.62; p=0.007).

Magnitude of tourniquet	Axonal regeneration distance (mm)	Number of rats
0 (control)	6.1 ± 0.4	5
150	6.4 ± 0.2	5
200	8.0 ± 0.2*	5
300	7.6 ± 0.3*	5

Table 1. Regeneration distances of axons in sciatic nerve grafts. The sciatic nerve intended for nerve grafting was subjected to tourniquet compression 6 days before nerve graft surgery (conditioning interval) and grafted to a fresh defect in the sciatic nerve at the contralateral side. The regeneration distances (values are mean ± s.e.m) were evaluated after an additional 6 days.
***Significantly different from control, p < 0.05, ANOVA / Sheffé's post hoc test.**

BrdU-incorporation

In the second set of experiments, incorporation of BrdU and expression of p75 was investigated 6 days after tourniquet compression at 150 and 300 mmHg (no grafting procedure was performed). The number of BrdU positive cells in segments of compressed sciatic and tibial nerves is presented in Table 2. BrdU is only incorporated by proliferating cells in the s-phase of the cell cycle. Proliferating cells are mainly Schwann cells [8] as indicated by the oval shape of the BrdU positive nucleus and the S-100 stained cytoplasm (Fig. 1a). In segments from untreated control nerve there were only a few (0-1 cells/mm^2) BrdU positive cells (Fig. 1b). Compression at 150 mmHg lead to an increased number of proliferating cells to the same extent in both tibial and sciatic nerves (Fig. 1c). Compression at 300 mmHg markedly increased the number of BrdU positive cells in the tibial nerve (Fig. 1d) as compared to compression at 150 mmHg, but surprisingly not in the sciatic nerve (Fig. 1e). The distribution of BrdU positive cells was similar in all sections.

Nerve type	Magnitude of tourniquet compression			
	150 mmHg		300 mmHg	
Sciatic nerve	16.9 ± 1.8	n=6	16.8 ± 2.4	n=6
Tibial nerve	17.5 ± 0.6	n=6	52.4 ± 2.5*	n=6

Table 2. Number of BrdU positive cells (cells/mm2) presented as mean ± s.e.m. in segments from sciatic and tibial (distal to the tourniquet) nerves 6 days after tourniquet compression at 150 or 300 mmHg for 2 hours (n = number of nerves). * *Significantly different from tibial nerve at 150 mmHg, p < 0.05, ANOVA/Sheffé's post hoc test.*
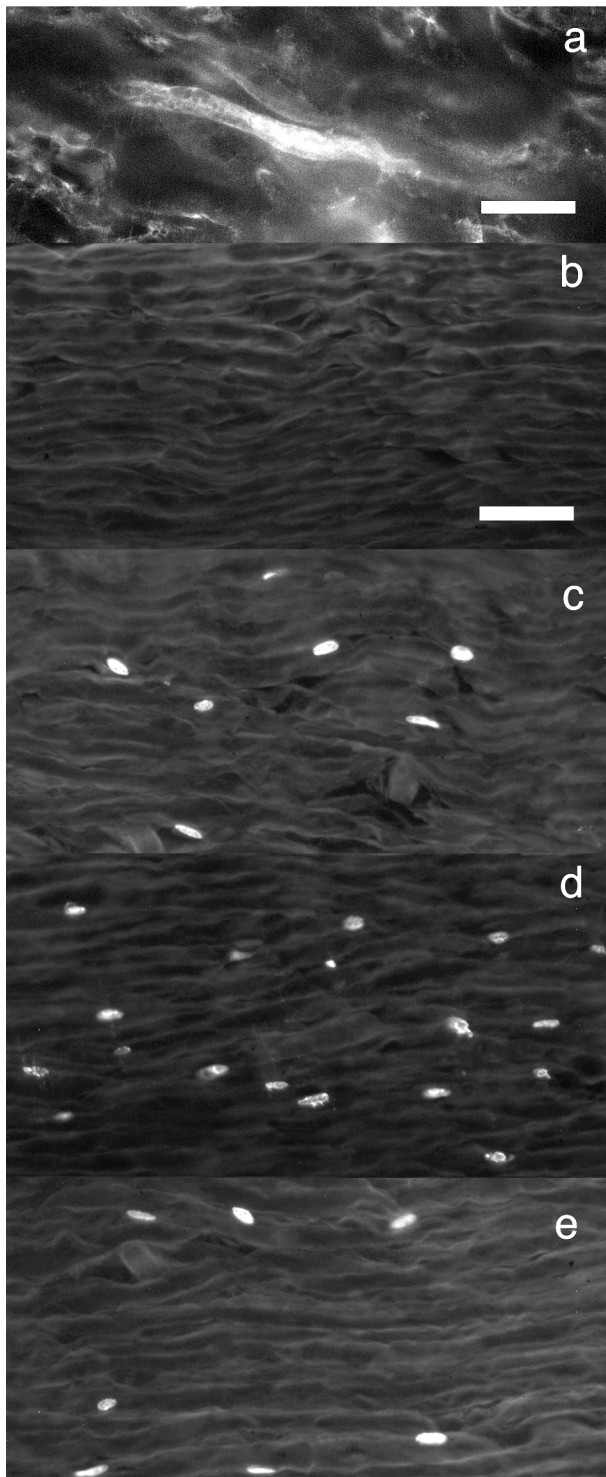
Figure 1. Immunocytochemistry of proliferating cells in nerve segments 6 days after compression. The proliferating non-neuronal cells were mainly Schwann cells, shown by double labelling with S-100/BrdU (a). Only a few BrdU positive cells were present in control sections (b) whereas tourniquet compression at 150 mmHg induced proliferation of non-neuronal cells at the same extent in both sciatic and tibial nerves (c). Compression at 300 mmHg markedly increased the number of proliferating cells in tibial nerves (d) but surprisingly not in sciatic nerves (e). Bar = 20 (a) and 50 (b-e) µm.

p75 receptor-expression

The expression of p75 receptor by Schwann cells in sciatic and tibial nerves 6 days after compression at 150 or 300 mmHg, was studied (no grafting procedure was performed). In sections of sciatic and tibial nerves from the non-compressed side the immunoreactivity of p75 receptor was almost undetectable. The weak staining of these sections may be associated with Schwann cells containing thin unmyelinated nerve fibres (Fig. 2a). In contrast, all nerve sections from the compressed nerves showed a marked expression of p75 receptor. Dense immunoreactivity was seen in long strands parallel to the axons in sections from both sciatic (Fig 2b) and tibial (Fig. 2c) nerves and there was no obvious difference in the distribution of the expression of p75 receptor.
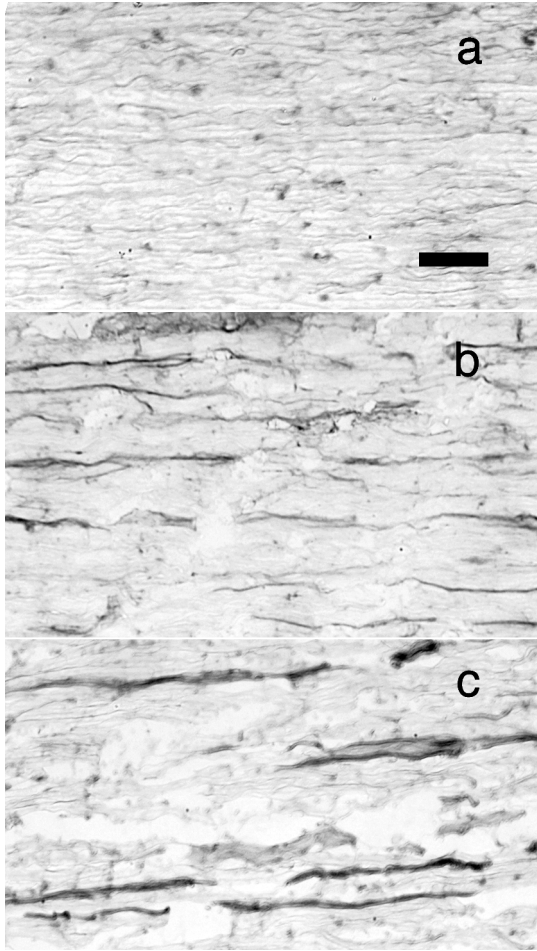
Figure 2. A faint expression of p75 receptor was seen in sections of nerve segments from controls (a), but 6 days after tourniquet compression at 300 mmHg the expression of p75 receptor was markedly induced in both sciatic (b) and tibial nerves (c). Bar=50μm.

Discussion

Our initial assumption, that tourniquet compression can condition a nerve intended for grafting and that regeneration into such grafts can be enhanced, proved correct. Tourniquet compression at 150 and 300 mmHg for 2 hours also induced an increase of cell proliferation and in expression of the low affinity neurotrophin p75 receptor in the sciatic and tibial nerves observed 6 days after the single compression episode. Double labelling of BrdU and S-100 suggests that the majority of the proliferating cells are Schwann cells [8]. Such a proliferative and p75 receptor response also occur following other types of nerve injuries, like nerve transection or

demonstrated in one and the same nerve by two consecutive, but temporally separated lesions (conditioning interval), has been ascribed to changes both at the level of the nerve cell body and the site of the conditioning lesion, i.e. locally in the nerve trunk [12,13].We have previously demonstrated using one and the same nerve (sciatic) that compression can induce conditioning and that the duration and magnitude of the tourniquet pressure as well as the time of the conditioning interval are important factors for the conditioning effect [3,14]. In the present study a "two"nerve approach was used. First, conditioning was induced by compression on one nerve (sciatic or tibial). This nerve was subsequently used as a graft in another (contralateral) unconditioned nerve (sciatic) in the same animal. We used a conditioning interval of 6 days, which is optimal for conditioning [1,3], and a compression period of 2 hours since short periods, like 30 minutes, are ineffective [3]. Furthermore, we varied the magnitude of compression. Tourniquet compression at 150 mmHg had no significant effect on nerve regeneration into the grafts. In contrast, compression at a magnitude of 200 or 300 mmHg increased axonal regeneration distance significantly.

The reason for the enhanced regeneration potential of a nerve subjected to compression is not fully known. Tourniquet compression at the higher levels used in this study (200-300 mmHg) have been shown to induce local structural changes at the compression-site. Thus, Nitz et al. [6] demonstrated demyelination, axonal degeneration and edema in rat sciatic nerves compressed at 300 mmHg for 2 hours, while compression at 200 mmHg induced minor, but reversible, structural changes. Degenerative alterations in peripheral nerves, like those occurring after nerve crush and axotomy, are associated with Schwann cell proliferation and upregulation of p75 receptor in these cells [15,16]. We found that tourniquet compression induced proliferation and an increase in the expression of p75 receptor both at 150 and 300 mmHg. However, only the latter magnitude gave rise to a nerve graft in which stimulated outgrowth of nerve fibres were observed. It is thus tempting to speculate that conditioning requires both activation locally of cells in the nerve (proliferation of the Schwann cells and increased expression of p75 receptors) and degeneration of nerve fibres. The role of Schwann cells seems to be production of factors which support regeneration of axons,

including cell adhesion molecules, basal lamina components and various growth factors [11,17].

Our result shows that tourniquet compression induces a marked increase of proliferation of cells in the nerve, mainly Schwann cells, as visualised by double labelling of BrdU and S-100 protein in both the sciatic and tibial nerves. In the tibial nerve, a significantly higher number of BrdU positive cells were observed at 300 mmHg than at 150 mmHg. Surprisingly, the number of BrdU positive cells was dramatically lower in the sciatic than in the tibial nerve at (300 mmHg). We assume that this is a result of a decrease in blood flow due to a "no-reflow" phenomenon and therefore the delivery of BrdU during the labelling period. A "no reflow phenomenon" following compression caused by injury to the intraneural vessels of directly compressed nerves has been demonstrated [18,19]. Indeed ischemia as such may contribute to the lower number of BrdU positive cells. However, we favour the hypothesis that BrdU delivery, rather than energy metabolic impairment, is responsible for the lower number of BrdU positive cells in the sciatic nerve, since we could not observe any difference in expression of the p75 receptor between tibial and sciatic nerve segments at 300 mmHg. This increase requires energy and occurs in the same cells that incorporate BrdU. In fact, the expression of the p75 receptor has been shown to be a sensitive indicator of Schwann cell responsiveness to nerve injury and can be used as a marker of degeneration-related changes in Schwann cells [20]. One should also consider the possibility that blockage of anterograde and retrograde transport can increase p75 receptor levels locally which occurs following a cold block of axonal transport [21].

Clinical implications

From the clinical perspective two aspects can be discussed. First, our results points to a possible way by which axonal outgrowth in nerve grafts can be enhanced. In elective reconstructive surgery, involving e.g. sural nerve grafting, the graft could be conditioned by tourniquet compression a week before the grafting procedure. Although conditioning of the nerve graft could also be induced by a nerve crush [1], conditioning by tourniquet compression may be an advantage since it is a non-invasive technique. Secondly, our results raise questions as to whether or not even moderate compression levels may be harmful. For extremity surgery tourniquet compression for extended periods (up to 2 hours) are used routinely to create blood less field. The highest level of compression that was used in our study (300 mmHg) is probably higher than what would be a "recommended" level for the rat as their systolic pressure is only about 120 mmHg. A pressure of 300 mmHg for 2 hours is associated with degenerative changes in rat sciatic nerves [6]. However, at a tourniquet pressure of 200 mmHg, which may be comparable to what can be relevant in clinical situations in upper and lower extremity surgery of humans [22], a conditioning lesion effect was found, which could imply that even such moderate pressures result in degenerative events. Temporary neurophysiological abnormalities after upper extremity tourniquet in carpal tunnel release in humans have been reported [23]. Furthermore, even lower pressures (150 mmHg for 2 hours), i.e. such where no signs of nerve degeneration in experimental animals has been observed, resulted in activation of Schwann cells and expression of p75 receptor. Whether or not such activation could be detrimental or not merits attention.

Conclusion

We report that tourniquet compression at 200-300 mmHg for 2 hours can condition a nerve that is intended to be used as a nerve graft. Compression at 150 mmHg had no effect on axonal regeneration distance into such a graft even though proliferation of Schwann cells and the expression of p75 receptor increased. Compression at 200 and 300 mmHg increased axonal regeneration distance with up to 30% in the nerve grafts as compared to sham-operated rats. The expression of the p75 receptor was similar after compression at 300 and 150 mmHg, but a higher number of proliferating Schwann cells was seen after compression at 300 mmHg. These findings suggest that the magnitude of compression is of importance for the conditioning effect and, the conditioning of a nerve may require both activation locally of cells in the nerve and degeneration of nerve fibres. Tourniquet compression as a non-invasive method to enhance axonal regeneration into nerve grafts may have a potential clinical value.

## Acknowledgements

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C-terminal flanking peptide of neuropeptide Y in DRG following nerve compression

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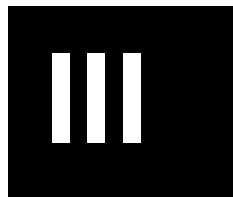
C-terminal flanking peptide of neuropeptide Y in DRG following nerve compression

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The C-terminal flanking peptide of neuropeptide Y (CPON) was studied in dorsal root ganglia (DRG) by immunocytochemistry after different recovery periods (3, 6,14 and 28 days) following tourniquet compression of the rat hindlimb (sciatic nerve; 150 or 300 mmHg; 2 h). Compression induced a transient increase in the number of CPON-positive DRG-neurons (the contralateral uninjured side was devoid of CPON-positive cells). The compression-induced increase in CPON was less than that observed in separate rats subjected to sciatic nerve transection. The results show that compression induces regenerative changes in peripheral neurons and that such an injury of the nerve trunk is not limited to the site of the compression but results in the activation of the entire neuron. *NeuroReport* 12:3193–3196 © 2001 Lippincott Williams & Wilkins.

Key words: C-terminal flanking peptide of neuropeptide Y (CPON); Nerve compression; Nerve injury; Neuron; Neuropeptide Y; NPY; Tourniquet

INTRODUCTION

Transection of a rat sciatic nerve induces regenerative changes in the nerve cell body, including modification of the expression of different neurotransmitters and neuropeptides like neuropeptide Y (NPY) in the neurons of the dorsal root ganglion [1–4]. Peripheral nerve compression results not only in local changes at the site of injury of the nerve trunk but also alterations in the nerve cell body reminiscent of those observed after more severe lesions such as nerve crush and transection [5,6]. For instance, we have demonstrated that tourniquet compression of the rat hind limb acts as a conditioning lesion and thereby stimulates axonal outgrowth after a subsequent crush injury [7], an effect ascribed to the activation of the nerve cell body. Whether a compression injury also results in alteration in neuropeptide expression is unknown.

The C-terminal flanking peptide of neuropeptide Y (CPON) is a marker for neuropeptide Y, and it has the same distribution as NPY in the nervous system [8]. NPY, and consequently CPON, is not expressed in uninjured neurons [9], but is distinctly up-regulated in neurons after peripheral nerve transection [2,10]. Since NPY is not expressed in uninjured neurons we assumed that CPON could be used as a sensitive marker for less traumatic nerve injuries such as nerve compression. The purpose of the present study was to investigate whether tourniquet compression induces expression of CPON in the nerve cell bodies of the DRG of the rat sciatic nerve

MATERIALS AND METHODS

Animals and tourniquet compression: Wistar rats weighing 180–200 g (Möllegard, Denmark) were randomly divided into three experimental groups. The rats were anaesthetised with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and the hindlimbs were shaved. One of the hindlimbs was compressed with a 1.6 cm wide inflatable tourniquet (Hokanson, Blidö, Sweden) at a pressure of 150 or 300 mmHg. A calibrated inflator (Stille-Werner, Sweden) controlled the pressure [7]. After 2 h the tourniquets were removed and the animals were allowed to recover. In a third group of rats, the sciatic nerve in one of the shaved hind limbs was carefully exposed and transected at midthigh level. A 5 mm long nerve segment was resected to prevent regeneration. The wounds were closed and the animals were allowed to recover. The L4–L5 DRG were removed bilaterally by dissection following 3, 6, 14 or 28 days of recovery ($n = 3$ at each time period and injury). Before DRG harvesting the rats were killed by i.p injection of pentobarbital sodium (600 mg/kg) followed by heart puncture. A total of 36 animals was used. The experiments were approved by the local ethical committee at Lund University and carried out according to the European Communities Council Directive regarding care and use of animals for experimental procedures.

Immunocytochemistry: The dissected DRG were immersed in Stefaninis fixative (2% paraformaldehyde and 1.9% picric acid in 0.1M phosphate buffer, pH 7.2) for 2 h, followed by washing and cryoprotection in phosphate buffered saline (PBS) containing 20% sucrose solution. The DRG were mounted in Tissue-Tek (Sakura, Torrence, USA) and 10 μm longitudinal sections were cut on a cryostat and collected on poly-L-lysine objective slides. Three sections from the mid-axial part of each L4–L5 DRG, ≥ 50 μm apart

were used. The sections were washed in PBS and then exposed to a primary rabbit antibody against CPON (DAKO, Denmark) diluted 1:1280 in PBS containing 0.25% Triton-X (Packard, Meriden, USA) and 0.25% bovine serum albumin. The sections were incubated at 4°C overnight. The primary antibody was removed and the sections were washed (3×5 min in PBS) and exposed to fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) at a dilution of 1:80 in PBS for 1 h, at room temperature in darkness. The sections were finally washed in PBS and mounted in 50% glycerol in PBS for fluorescence microscopy. Control sections were incubated without the primary antibody.

Image analysis: The preparations were examined in an Olympus fluorescence microscope (BX 60) and pictures were taken by a KODAK Professional DCS 420 digital camera (around $\times 200$ magnification) linked to the microscope and a Power Mac computer. The digital images (one/section) were stored on compact discs. The images were processed using Adobe Photoshop 5.0 software, and used for determination of the number of CPON-positive nerve cell bodies and the total number of nerve cell bodies in the images. Only cells with a clearly visible nucleus were counted.

Statistics: A two-way ANOVA was used to test for differences in the material (factors: time and injury) and the calculations were made using the software StatView 5.0. Six sections from each side of each animal were analysed. The value obtained from each section was initially transformed by the formula: arcsinus ($^2\sqrt{}$ value) in
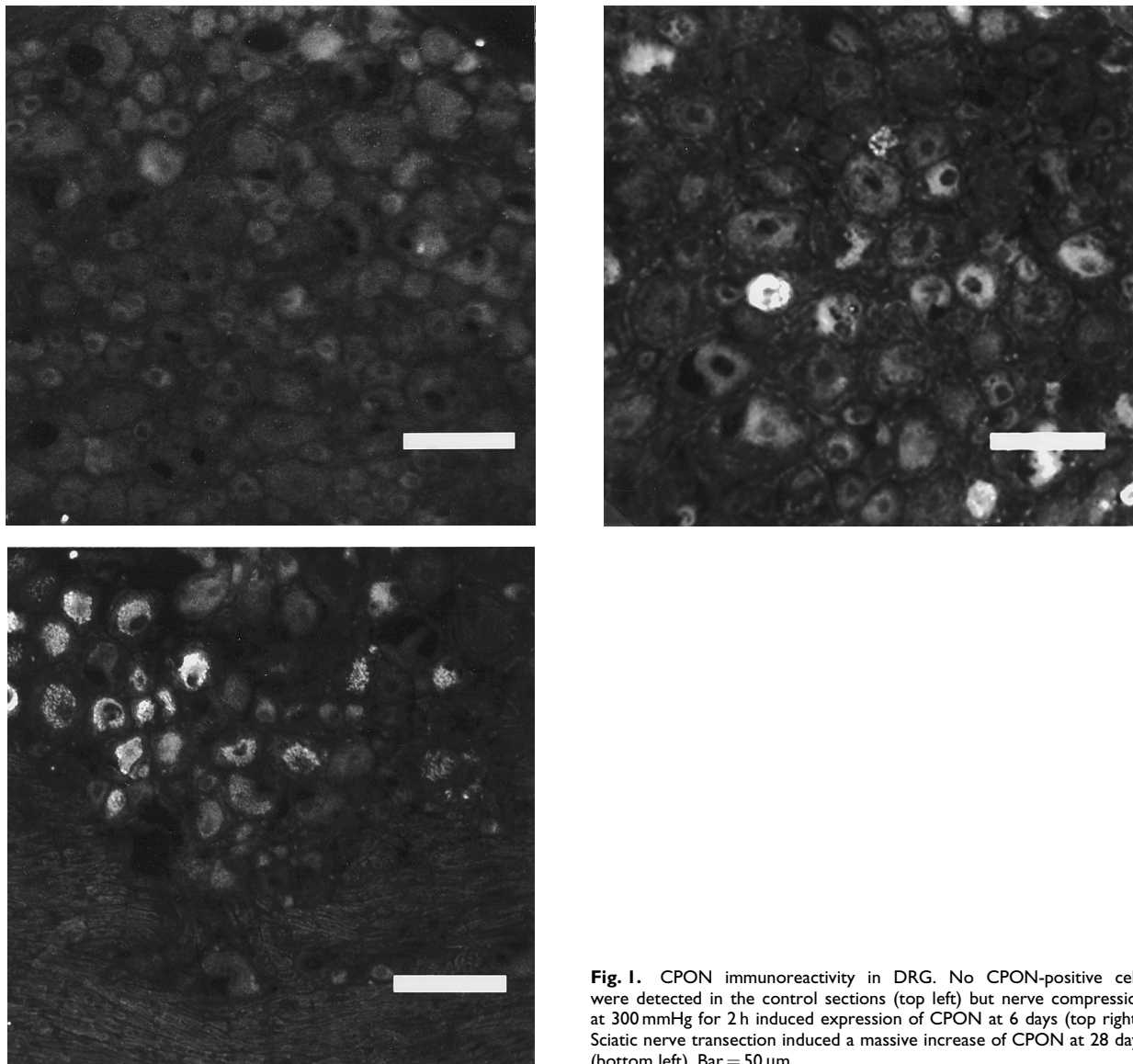

Fig. I. CPON immunoreactivity in DRG. No CPON-positive cells were detected in the control sections (top left) but nerve compression at 300 mmHg for 2 h induced expression of CPON at 6 days (top right). Sciatic nerve transection induced a massive increase of CPON at 28 days (bottom left). Bar = 50 μm.

order to stabilise the variance of the relative frequencies (the number of CPON-positive cells) and thereby be able to correctly compare the values. A *post-hoc* study was carried out to examine if there were any significant differences between the groups for either the time or the injury. A value of $p < 0.05$ was considered statistically significant. Values in figures are presented as CPON-positive cells in percent of the total number of counted cells.

RESULTS

No CPON immunoreactivity was detected in any of the cell bodies from the uninjured (control) L4 and L5 DRG at any timepoint (Fig. 1 top left). However, some CPON-immunoreactive axons, mainly associated with blood vessels, were seen in most sections. Compression at 150 and 300 mmHg induced up-regulation of CPON in DRG (Fig. 1 top right), as did transection of the sciatic nerve (Fig. 1 bottom left). The two-way ANOVA identified a positive interaction between time and injury, i.e. compression, but the latter had alone significant effect. A *post-hoc* test (Fisher's PLSD) demonstrated significant differences between the various injuries. The temporal development of the CPON expression is shown as a percentage of the total number of counted cells in Fig. 2. At 3 days, 3 and 6% of the neurons that were compressed at 150 and 300 mmHg, respectively, expressed CPON, but there was no significant difference between the groups. At 6 days, 1 and 9%, respectively, of the cells were positive ($p < 0.0001$) and after that the number of positive cells declined. Only around 1% of the cells expressed CPON at 28 days in both the compression groups. In contrast, sciatic nerve transection caused a more dramatic increase of CPON immunoreactivity. At 3 days > 13% ($p = 0.02$) of the cells expressed

CPON and at 14 and 28 days, 28 and 30%, respectively, of the cells were CPON positive.

DISCUSSION

Our study shows that tourniquet compression of a peripheral nerve induces an increase of the expression of CPON in the sensory nerve cell bodies in DRG, i.e. one aspect of regenerative response of the cell bodies [4,11]. We have previously shown that tourniquet compression can condition the nerve. Taken together, these findings suggest that local compression of the nerve trunk activates the entire neuron and puts it into a regenerative mode, similar to that of more severe injuries such as crush lesions [7]. Furthermore, our assumption that CPON could be used as a marker for minor nerve injury was justified since the number of positive cells seems to be correlated to the severity of the injury. The results show that compression at 150 and 300 mmHg caused CPON up-regulation in up to 3 and 6%, respectively, of the counted neurons at 3 days. After 6 days, 1 and 9%, respectively, of the cells were CPON positive. The up-regulation of CPON after compression was transient compared to that seen after transection, where the number of positive cells was still around 30% at 28 days, which also is in accordance with earlier studies [3,12,13].

The expression of a variety of neuropeptides, including NPY and substance P, is regulated by target-derived factors that are retrogradely transported to the cell body [14,15]. One such retrograde signal which has been shown to affect NPY expression is neurotrophin 3 (NT3) [16,17]. Transection disrupts target contact while compression is known to inhibit axonal transport [6,18,19]. It is noteworthy that application of colchicine on rat sciatic nerve causes suppression of retrograde transport and up-regulation of NPY in DRG in an identical manner to sciatic nerve transection [20]. One explanation of the differences in the CPON response could therefore be that compression transiently suppresses retrograde signalling and that CPON is downregulated when the retrograde axonal transport is re-established. The functional consequences of the up-regulation of CPON are unknown but may be related to modification of pain impulses [4]. Mice that are deficient in NPY show increased pain behaviour following nerve lesions [12].

A period of compression as short as 2 h was detectable as a CPON response at least 1 week after the injury. In a clinical setting, compression of the duration and magnitude of those in the present study is frequently used during surgical procedures. The possibility that such a treatment aimed at the creation of a bloodless field results in long-term changes in neuropeptide synthesis in the inflicted neuron and thereby pain modulation must be considered.

CONCLUSION

In this study we report that CPON, a marker for NPY, is transiently up-regulated in DRG as a response to peripheral nerve compression. We also report that compression causes far less up-regulation of CPON in nerve cell bodies than nerve transection. We suggest that the transient CPON response to the compression injury may be ex-
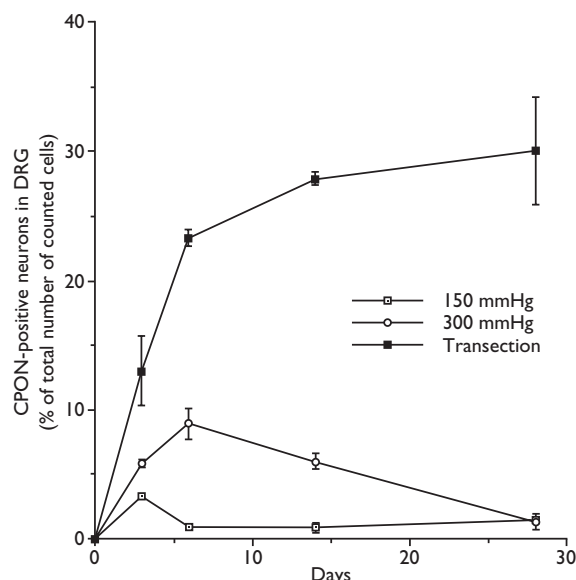

Fig. 2. Temporal development of the CPON induction in DRG following sciatic nerve compression and transection injuries. Values are means ± s.e.m., $n = 3$.

plained by the reversible inhibition of retrograde axonal transport of target derived factors.

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Nerve regeneration in nerve grafts conditioned by vibration exposure.

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IV

Nerve regeneration in nerve grafts conditioned by vibration exposure

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Abstract

Regeneration distances were studied in nerves from vibration-exposed limbs. One hind limb of anaesthetized rats was attached to a vibration exciter and exposed to vibration (80 Hz/32 m/s^2) for 5 h/day for 2 or 5 days. Seven days after the latest vibration period a 10-mm long nerve graft was taken from the vibrated sciatic nerve and sutured into a corresponding defect in the contralateral sciatic nerve and vice versa, thereby creating two different models within the same animal: (i) regeneration from a freshly transected unvibrated nerve into a vibrated graft and (ii) regeneration from a vibrated nerve into a fresh nerve graft (vibrated recipient side). Four, 6 or 8 days postoperatively (p.o.) the distances achieved by the regenerating axons were determined using the pinch reflex test. Two days of vibration did not influence the regeneration, but 5 days of vibration reduced the initial delay period and a slight reduction of regeneration rate was observed. After 5 days of vibration an increased regeneration distance was observed in both models at day 4 p.o. and at day 6 p.o. in vibrated grafts. This study demonstrates that vibration can condition peripheral nerves and this may be caused by local changes in the peripheral nerve trunk and in the neuron itself.

Keywords: Vibration; Nerve regeneration; Nerve injury; Conditioning lesion; Schwann cell; Nerve grafts

1. Introduction

Vibration exposed operators may show up with a complex of peripheral neurological, circulatory and musculoskeletal dysfunctions. The symptoms occurring after vibration exposure have been extensively studied [2,3,7,22]. From the experimental point of view the situation is the opposite. There are only a few experimental studies describing the changes in various tissues after vibration exposure and trying to explain the pathophysiologic mechanisms behind vibration injury. More knowledge is required to understand and to treat this syndrome [8]. We have previously shown that experimental vibration to a rat hind limb induces changes in both muscle and nerve tissue [6,16,17,21]. Studies by us and others show that changes in nerve tissue span from edema formation [16], temporary structural changes in non-myelinated nerve fibers [17] to ultrastructural changes in myelinated nerve fibers [4,10]. We have also shown that there is an increased regenerative capacity after vibration exposure [6]. Thus, the vibration expo-

sure can act as a conditioning lesion [6,19,20]. It has recently been shown that nerve regeneration into experimental nerve grafts can be improved if the graft is conditioned, e.g. by predegeneration [15] The purpose of the present study was to investigate if nerve regeneration could be altered in nerve grafts conditioned by vibration exposure and to see which mechanism(s) are responsible for the increased regenerative capacity after vibration exposure.

2. Materials and methods

Sixty-four female Wistar rats, weighing 180–200 g, were used for the study and they were anaesthetized by an i.p. injection of a mixture of pentobarbital (60 mg/ml), diazepam (5 mg/ml) and saline in 1/4/1 volume proportions prior to vibration. The animals were placed in a soft network support and one of the hind paws was attached to a plate horizontally which was fixated on the top of a vibration exciter. In this way the paw could be exposed to a sinuswaved vibration for 5 h/day during 2 or 5 consecutive days with a defined frequency and acceleration level (80 Hz; 32 m/s^2). The details of the

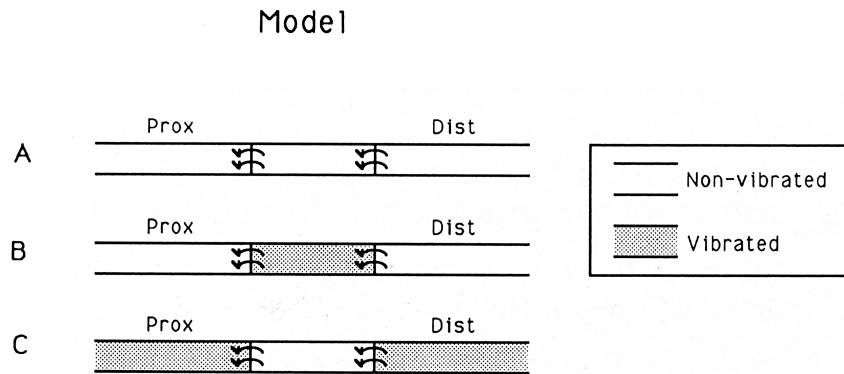---

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Model

Fig. 1. Schematic drawing of the experimental set up. A 10-mm long nerve segment was sutured as a nerve graft between transected nerve ends at the contralateral side in control rats (A). In model B a nerve segment from a vibrated nerve was sutured as a graft (vibrated graft) into an uninjured transected nerve. Finally, in model C an uninjured nerve segment was sutured as a graft into a vibration-exposed transected nerve (vibrated recipient side).

vibration exciter for rats are described elsewhere [6,21].

Seven days after the end of vibration exposure the rats were reanaesthetized and the sciatic nerves exposed and mobilized bilaterally. A 10-mm long nerve graft was taken from the vibrated side and transposed to the contralateral side and vice versa, thereby creating two different models (Fig. 1) within the same animal: (i) regeneration from a freshly transected unvibrated nerve into a vibrated graft, and (ii) regeneration from a vibrated nerve into a fresh nerve graft (vibrated recipient side). The nerve grafts were sutured using three single 9-0 perineurial stitches (Ethilon®). The wounds were closed and the animals allowed to recover. The control experiments consisted of normal rats not exposed to vibration. In these control experiments a 10-mm long nerve segment of the sciatic nerve was removed and placed as a nerve graft in the contralateral nerve defect and sutured as described above. This procedure was done on both sides in the control rats.

After 4, 6 or 8 days the rats were reanaesthetized and the sciatic nerves were re-exposed. The regeneration distances in the nerve grafts were evaluated by the pinch reflex test [6,13,15,29]. Briefly, the peroneal and tibial branches of the sciatic nerve were cut as distally as possible and then consecutively pinched by forceps. When the tips of the fastest growing sensory nerve fibers were pinched, a slight reflex movement of the back limb was elicited and the distance between that point and the proximal suture line of the nerve graft was referred as the regeneration distance. The pinch reflex test is a valuable and accurate method to determine the outgrowth length of sensory axons from a crush lesion or from a nerve suture as shown by concomitant staining of neuroflaments [15,25,26] or by evaluation with axonal transport using radioactive substances [1].

All individual regeneration distances (including 0 mm of regeneration) were plotted on a diagram and the corresponding regression line was calculated according to Holmquist et al. [11] in order to determine the regeneration rate (slope of the regression line) and the initial delay period (i.e. the period before regenerating nerve fibers enter the graft; the interception of the regression line and the x-axis). The model accounts for the 'regeneration failures' (i.e. those nerves showing 0 mm regeneration distances within the evaluation period; nerves which have not yet started to regenerate). If no 0 values are recorded the method gives the same result as ordinary regression analysis. The statistical differences between regeneration distances for the various experimental groups at p.o. days 4, 6, and 8, respectively, were calculated using ANOVA (Bonferroni–Dunn; $P < 0.05$ was considered significant). The statistical calculations were done on a Macintosh® computer equipped with StatView® 4.02, FPU version.

3. Results

Initially we tested the effects of 2 days of vibration on nerve regeneration. Two days of vibration did not increase the regeneration distances during the examined time period (data not shown) as compared with control grafts. Therefore, we increased the vibration exposure to 5 days and the results from these experiments are summarized in Fig. 2. In the control series three out of 36 examined nerve grafts had 0-mm regeneration distance, one at each examined time point. No 0-mm regeneration was detected in either vibrated grafts or on the vibrated recipient side. The calculated regeneration rate was 1.6 mm/day for control grafts, 1.4 mm/day for the vibrated recipient side and 1.2 mm/day for vibrated grafts, respectively. Control grafts had an initial delay of 3.3 days, whereas both the vibrated recipient side and the vibrated graft had a decreased initial delay as compared
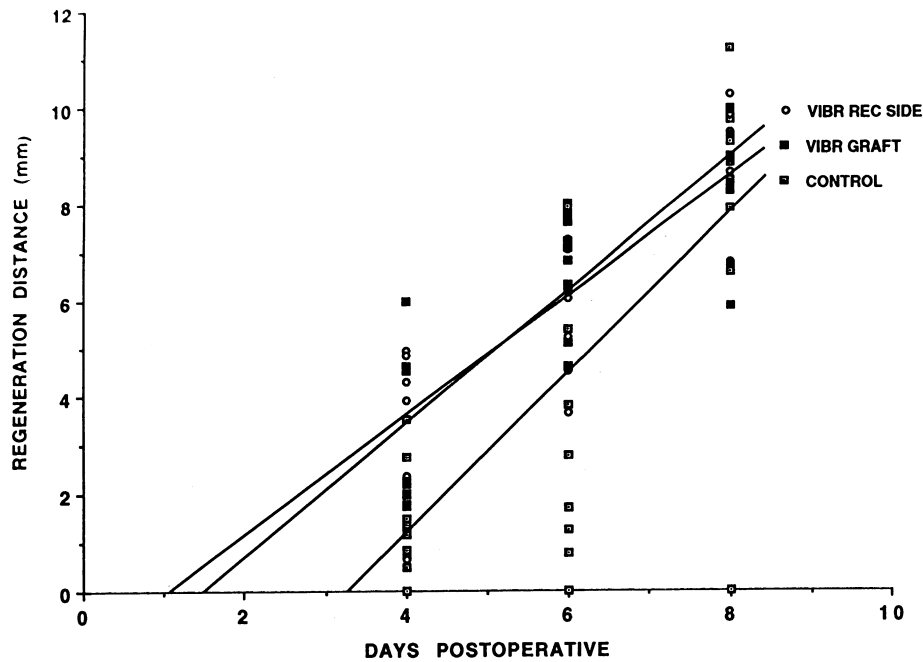
Fig. 2. Regeneration distances from the sensory pinch test at 4, 6 or 8 days p.o. for control grafts, vibrated recipient side (vibr rec side) and vibrated grafts (vibr graft). The rat hind limb was vibrated for 5 h/day for 5 days (80 Hz/32 m/s^2) 7 days prior to surgery. The regression lines (based on all individual data points including 0-mm regeneration distances) are calculated according to Holmquist et al. [11].

with controls (1.5 days for vibrated recipient side and 1.1 days for vibrated grafts, respectively).

The average regeneration distances for the different experimental situations at various postoperative days

are depicted in Fig. 3. On p.o. day 4 both the vibrated recipient side group and the vibrated grafts had a significantly longer regeneration distance than the control grafts. Two days later (p.o. day 6) both experimen-
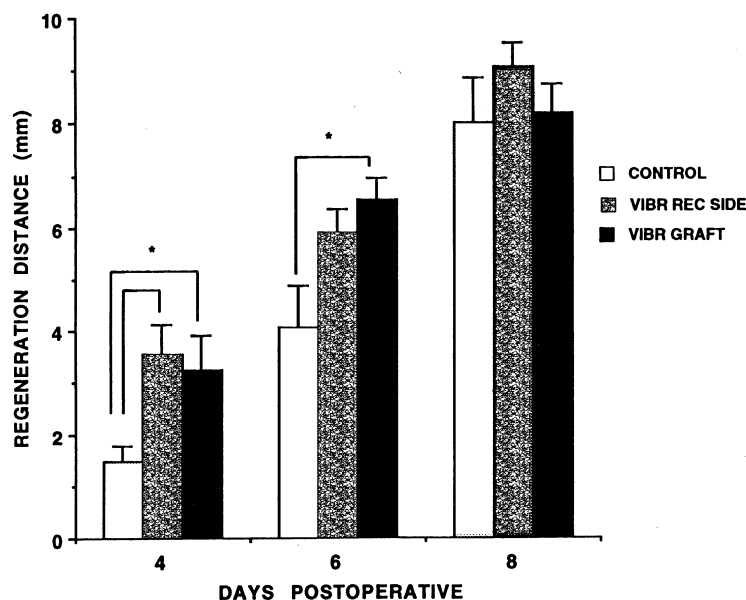

Fig. 3. Histogram showing the regeneration distances (mean + S.E.M.) for control grafts (n = 12 at each time point), vibrated recipient side (vibr rec side; n = 7 for p.o. day 4 or 8 and n = 8 for postoperative day 6) and vibrated grafts (vibr graft; n = 7 for postoperative day 4 or 8 and n = 8 for postoperative day 6). The rat hind limb was vibrated for 5 h/day for 5 days (80 Hz/32 m/s^2) 7 days prior to surgery. *Statistically significant difference, $P < 0.05$.

tal groups had increased regeneration distances as compared with controls, although a statistical difference could only be detected between vibrated grafts and control grafts. At 8 days p.o. the differences between the various groups were not statistically significant.

4. Discussion

This study demonstrated that 5 days of vibration, but not 2 days, of a rat limb at a certain frequency and amplitude increased the regenerative capacity of the affected nerve during the first days of regeneration. The rate and initial delay periods for control grafts were similar to another control series published previously [15]. Our previous study on the effects of vibration on nerve regeneration showed that 2 days of vibration was enough to stimulate regeneration. In that study [6] we used a nerve crush as the test lesion and not transection and repair as in the present one. A crush injury represents a slighter injury with preserved endoneurial tubes and preservation of circulation in the segment where the axons grow. A crush injury is also easier to standardize since the quality of the nerve suture may be variable even by a trained surgeon. The results of our previous study [6] showed that there was no effect on the contralateral, unexposed side, indicating that there was no whole body effect by the vibration. Furthermore, in the present model we could also investigate the effects on the cells in the nerve trunk and on the neuron itself. In that respect the present model represents a better model to study the mechanism(s) behind the observed effect.

The regeneration distances achieved by axons regenerating from a vibrated recipient nerve (vibrated recipient side) or into a vibrated graft were longer at the earliest evaluation period as compared with control grafts. Furthermore, vibrated grafts had an increased regeneration distance at 6 days p.o. as compared with controls. It should also be noted that no 0-mm regeneration distances were detected in both experimental groups, which was the case for control grafts. It seems that vibration exposure triggers the axons to regenerate earlier, as indicated by the reduced initial delay period, but since the two vibrated groups have a slightly slower regeneration rate the effect is lost with longer evaluation periods. One might speculate that the effect might have persisted longer if the nerves had been transected and repaired immediately after vibration exposure. However, our previous data (Ref. [6] and unpublished data) indicate that the effect take some time to develop; i.e. an optimal effect is achieved with a recovery of 7 days as in the present study.

The precise mechanism(s) for the enhancement effect of vibration on nerve regeneration is not known, but the vibration can act as a conditioning lesion [19,20], similar to that observed after nerve compression [5]. This condi-

tioning lesion phenomenon can be regarded as an indicator or 'alarm-reaction' that the neuron on the vibrated recipient side has been affected/injured by the vibration of its axon in the limb [5,6], and/or that the non-neuronal cells, like the Schwann cells, in the nerve trunk is affected/stimulated. Schwann cells are known to produce a variety of growth factors [9,14,23,28] and one might speculate that vibration exposure triggers the synthesis of such factors thus improving regeneration in vibrated grafts. In this respect the interaction between axons and Schwann cells is important [24,27]. The importance of the local environment that the regenerating axons are facing has been demonstrated in experiments where predegenerating nerve grafts (triggered Schwann cell proliferation) has been transposed to freshly created nerve defects [15]. Thus, the present study indicates that both the above mentioned mechanisms contribute to the observed effect since the regeneration was improved in the two experimental groups. Furthermore, it was observed that there was no difference between the two experimental groups after 5 days of vibration exposure and the control group at day 8 p.o. This indicates that the stimulation by the vibration exposure has decreased or that even the exposure has a detrimental effect on the regeneration process.

The frequency used in the present study, i.e. 80 Hz, is a very common frequency among vibratory handheld tools used in industry, especially the bulk of rotatory tools (e.g. grinding machines have rotational speeds — 4000–6000 rev./min — causing vibration with this frequency [18]). The acceleration level (32 m/s^2) and the used frequency was set to a level which is considered to generate vascular disorders, such as white fingers, in a population exposed to vibration of 10% within 5 years if exposed for 4 h/day over an extensive period of time [12]. The present study indicates that this frequency and acceleration level already after 5 days, but not at 2 days, of vibration induces functional changes in rat nerves. We can only speculate how longer exposition periods could affect peripheral nerves, including the nerve fibers regulating vascular resistence.

Acknowledgements

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Nerve injury by vibration: prevention of the effect of a conditioning lesion by D600, a Ca^{2+} channel blocker

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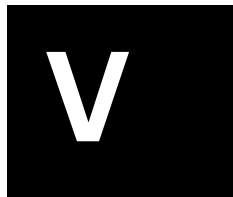
Nerve injury induced by vibration: prevention of the effect of a conditioning lesion by D600, a Ca²⁺ channel blocker

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Abstract

Objectives—Exposing a hind leg of a rat to vibration induces an injury to the sciatic nerve—a so called conditioning lesion. After such injury induced by vibration the regenerative capacity of the nerve is improved and can be detected as an increased axonal outgrowth from a test crush lesion to the same nerve. The purpose was to study whether the effect of a conditioning lesion induced by vibration can be prevented by local treatment with a Ca²⁺ channel blocker D600.

Methods—D600 (methoxyverapamil) or Ringer's solution was locally applied to the sciatic nerve on one side through a silicone tube connected to a miniosmotic pump, which was implanted subcutaneously. During the same period the hind leg was exposed to vibration (80 Hz; 32 m/s² root mean squared) for five hours daily for five consecutive days. The other hind leg was not vibrated. After the end of exposure to vibration the sciatic nerves were crushed bilaterally (test crush lesions) and three or six days later the regeneration distances of sensory axons were measured by the pinch reflex test.

Results—Nerves in the control animals (without implanted miniosmotic pumps and nerves on to which Ringer's solution was locally applied) that were exposed to vibration showed a significantly increased outgrowth length of sensory axons from the test crush lesion compared with the non-vibrated side. Such an effect of a conditioning lesion from the exposure to vibration was suppressed by local application of D600.

Conclusions—Local administration of a Ca²⁺ channel blocker D600 can prevent the effect of a conditioning lesion—that is, the nerve injury induced by vibration can be inhibited by D600. This may have implications for the treatment of patients with neuropathy of the hand induced by vibration.

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We have previously shown that axonal outgrowth in the rat sciatic nerve after a test crush lesion is increased when the same hind leg had previously been exposed to vibration of a defined acceleration, frequency, and duration that produced a conditioning lesion.[1 2] It has been suggested that such an improved outgrowth of axons can be explained by local changes occurring in the nerve trunk or even changes in the neuron itself.[2] Such an effect of a conditioning lesion is probably an expression of a harmful nerve injury and might explain the clinical symptoms occurring in patients exposed to vibration. It has been suggested that these symptoms, like vibration induced white finger (VWF), may have a neurological pathophysiological component.[3 4] Neurological symptoms can also occur without other symptoms in patients exposed to vibration.[4–9] The therapeutic possibilities for these patients are limited, but various pharmacological treatments such as the use of Ca²⁺ channel blockers have been suggested.[10–12] Theoretically, a Ca²⁺ channel blocker might attenuate the cellular damage after a neuronal injury.[13 14] Experimentally, local treatment with such a Ca²⁺ channel blocker (D600; methoxyverapamil) can block the effect of the conditioning lesion resulting from exposure to magnetic fields.[15] In the present study, we investigated if this substance, which interferes with the entry of calcium through the channels in the cell membrane, can also alter the effect of the conditioning lesion induced by exposure to vibration expressed as stimulation of nerve regeneration.

Materials and methods

ANIMALS AND Ca²⁺ CHANNEL BLOCKER

Female Wistar rats (n = 70) weighing 180–200 g were used. The study was approved by the local ethics committee of Lund University. The rats were anaesthetised by an intraperitoneal injection of a mixture of pentobarbitone (60 mg/ml) and saline in the proportion 1/10 by volume. One of the sciatic nerves was exposed in the thigh region under sterile conditions. D600 (methoxyverapamil; Sigma) was given locally to the nerve through a miniosmotic pump (2002, 0·5 µl/h, Alzet, USA) which was implanted subcutaneously in the back of the rat. A 6 cm long silicone catheter with an inner diameter of 0·7 mm (4 FR Mentor Corporation, Goleta, CA) was pulled subcutaneously from the pump to the sciatic nerve in the midthigh region. The distal part of the catheter was perforated and sutured by two single 9–0 Ethilon stitches to the epineurium (figure). The pump and the catheter were filled with Ringer's solution (in mM: NaCl 139; KCl 2·4; CaCl₂ 1·4; MgSO₄

2·0; NaH$_2$PO$_4$ 0·6; Na$_2$HPO$_4$ 3·25; pH 7·4) only or with Ringer's solution containing D600 at a concentration of 0·2 mg/ml. The wounds were sutured and the animals allowed to recover. The miniosmotic pumps were filled under sterile conditions and pre-activated before implantation with connected silicone tubing in sterile saline (37°C) according to the recommendations of the manufacturer. The method has previously been described by Rusovan and Kanje.[15] In their original work the concentration of D600 was 0·1 mg/ml and the pump delivery rate was 1·0 μl/h. As our experimental protocol demanded an administration time > 7 days we used miniosmotic pumps with a longer delivery time but a lower pumping rate (0·5 μl/h) and a doubled concentration (0·2 mg/ml), thus giving the same dose to the nerve as described previously.

EXPOSURE TO VIBRATION
Three days later the rats were reanaesthetised before vibration by an intraperitoneal injection of 0·2 ml of a mixture of pentobarbitone (60 mg/ml), diazepam (5 mg/ml), and saline in the proportions 1:2:1 by volume. Animals were placed in a soft network support and the hind paws, to which Ringer's solution with or without D600 was applied to the sciatic nerve, were attached horizontally to a plate which was fixated on the top of a vibration exciter. By this method the hind leg of the rat could be

exposed to sinusoidal vibration for five hours daily for five consecutive days (80 Hz; 32 m/s² root mean squared; displacement 127 μm root mean squared) according to previous reports,[1] and the other hind leg served as a non-vibrated control.

EVALUATION OF REGENERATION DISTANCES
Directly after vibration on the fifth day, the sciatic nerves on both sides were exposed, crushed with special pliers, and a 9 0 Ethilon suture was attached to the epineurium at the site of the crush. After three or six days the rats were reanaesthetised and the sciatic nerves were again exposed. The regeneration distance was measured by the pinch reflex test.[1 16-18] Briefly, the peroneal and tibial branches of the sciatic nerve were cut as distally as possible and consecutively pinched by forceps. When the tips of the fastest growing sensory nerve fibres were pinched the back leg showed a slight reflex movement and the distance between that point and the suture marked crush site was referred to as the regeneration distance. Several previous studies have shown that the pinch reflex test is an accurate method to evaluate regeneration distances as it corresponds to the distance measured by immunostaining of neurofilaments.[17-19] After the pinch reflex test the animals were killed and the miniosmotic pumps were taken out and opened to confirm that they had been working throughout the experiment.

In control experiments, rats without implanted miniosmotic pumps were vibrated in the same way for five hours daily for five consecutive days and the sciatic nerve was crushed bilaterally on the fifth day of vibration. Evaluation of the regeneration distance by the pinch reflex test was done after three or six days.

STATISTICS
The results are presented as median (interquartile range (IQR)). The Wilcoxon signed rank test was used to compare the data within the same rat (paired data). The Kruskal-Wallis test including multiple comparisons between different treatments[20] was used to compare the different experimental groups at three or six days. A P value ≤ 0·05 was accepted as significant. The calculations were done on a Macintosh computer equipped with StatView 4·02; FPU version.

Results
The table shows the results. Paired comparisons showed that in the control animals the leg exposed to vibration (no miniosmotic pumps) had significantly longer regeneration distances after both three and six days of regeneration (P = 0·045 and P = 0·049, respectively). In animals treated only with Ringer's solution the regeneration distances were also significantly increased on the leg exposed to vibration after three and six days of regeneration (paired comparison; P = 0·046 and 0·019, respectively). Application of D600 + Ringer's solution, however, abolished the
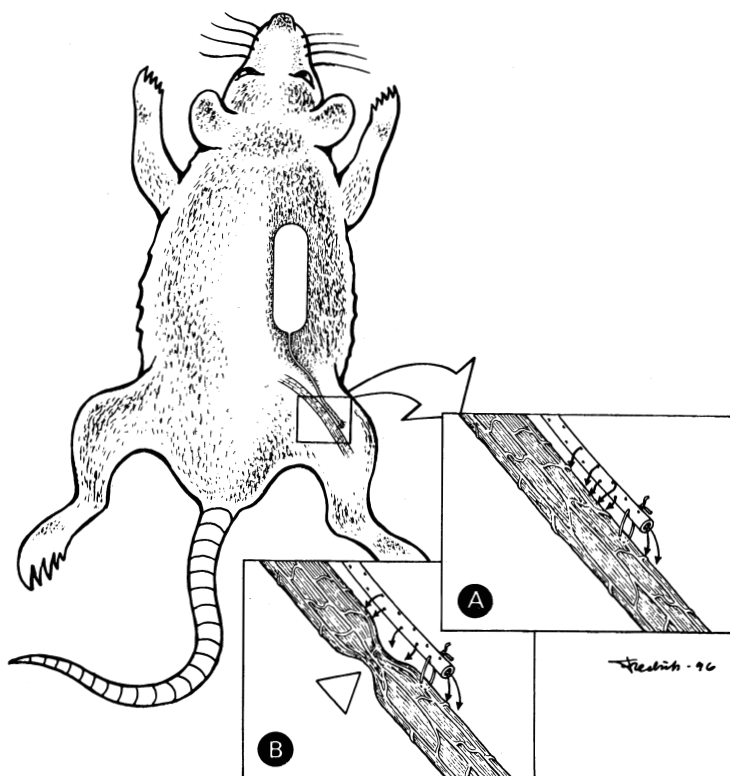
Schematic drawing of the experimental procedure. A miniosmotic pump was implanted subcutaneously in the back and Ringer's solution or D600 + Ringer's solution was locally applied to the sciatic nerve through a silicone catheter (A). The corresponding hind leg was exposed to vibration of defined acceleration, frequency, and duration for five days. Directly after exposure to vibration on the fifth day a test crush lesion was applied to the sciatic nerves bilaterally (B) and the regeneration distances of the sensory nerve fibres were evaluated three or six days later.

Effects of exposure to vibration (80 Hz, 32 m/s² rms; 5 hours daily for 5 days) and local administration of the Ca²⁺ channel blocker D600 on nerve regeneration after a nerve crush of the rat sciatic nerve

	Regeneration distances (median mm (IQR))					
	Three days			Six days		
	Non-vibrated leg	Vibrated leg	P value*	Non-vibrated leg	Vibrated leg	P value*
Control n Ringer's	5·0 (0·8)† 12	6·1 (1·0) 12	0·045	15·6 (1·6) 8	16·5 (1·3) 8	0·049
solution n D-600	5·4 (1·4) 14	6·3 (2·1) 14	0·046	16·1 (3·8) 13	17·5 (3·3)‡ 13	0·019
+ Ringer's solution n	6·4 (2·2) 11	5·5 (2·0) 11	0·13	16·1 (2·3) 12	14·8 (2·0) 12	0·071

*Wilcoxon signed rank test for paired data.
†P = 0·040 v D600 + Ringer solution, Kruskal-Wallis test.
‡P = 0·027 v D600 + Ringer solution, Kruskal-Wallis test.

stimulatory effect of the exposure to vibration. In these experiments, in which D600 + Ringer's solution was applied to one leg, the regeneration distances after three and six days had a tendency (paired comparisons), although not significant (P = 0·13 and 0·07 respectively), to be even shorter in the leg exposed to vibration compared with the hind leg not exposed to vibration.

The Kruskal-Wallis test showed that there were significantly shorter regeneration distances in nerves exposed to vibration treated with D600 + Ringer's solution than in nerves treated only with Ringer's solution at six days (P = 0·027). In the D600 + Ringer's solution series non-vibrated nerves had significantly longer regeneration distances at three days than non-vibrated control nerves (P = 0·040). The Kruskal-Wallis test did not show any significant difference between non-exposed nerves at six days (P = 0·64) or between nerves exposed to vibration at three days (P = 0·58).

Discussion

The present study showed that a Ca²⁺ channel blocker (D600), which is known to block the influx of Ca²⁺ through voltage sensitive calcium channels,[21][22] can abolish the effect of the conditioning lesion in peripheral nerves, which is induced by exposing the hind leg of a rat to vibration. A similar phenomenon has been found in rats exposed to 50 Hz sinusoidal magnetic fields.[15] The present study also further confirmed the fact that exposure to vibration can induce the effect of a conditioning lesion, which is an increased regenerative capacity of the nerve from an initial trauma—for example, exposure to vibration—and which is detected as an increased axonal outgrowth from a second test crush lesion. The phenomenon of a conditioning lesion is considered to be a sign of nerve injury.[1][2][23] To study whether the application of the miniosmotic pump and the small silicone tube used to locally apply the drug to the nerve influenced the regenerating axons, experiments were performed in which the miniosmotic pumps were loaded only with Ringer's solution. In these experiments an effect of the conditioning lesion was still found on the vibrated

leg three and six days after exposure to vibration as in previous experiments without pumps. Thus we conclude that the application of Ringer's solution to the nerve in itself did not affect the regenerating axons.

A surprising finding was that the regeneration distances were longer in the nerves in the leg not exposed to vibration (table, P = 0·040), when treated with D600 + Ringer's solution, than in the corresponding nerves in controls. Such an effect has not previously been found in studies in which regeneration distances were investigated in rats exposed to exogenous magnetic fields and treated with D600.[15] It may be explained by the fact that a low concentration of Ca²⁺ channel blocker can accelerate axonal sprouting in healthy nerves not injured by exposure to vibration. This has been found in previous studies.[24][25] This phenomenon also implies that there may be a systemic effect of D600 at the level of the nerve cell body, which may even work on the vibrated leg, partly explaining the neuroprotective effect.

We have suggested that exposure to vibration causes injury to the peripheral nerve as vibration can act by causing a conditioning lesion, which is probably a sign of nerve injury.[1] Local application of the calcium channel blocker D600 to the nerve abolished the effect of the conditioning lesion when paired comparison was made and there was even a significantly (P = 0·027) shorter regeneration distance at the vibrated side at six days compared with nerves treated with only Ringer's solution. This indicates that, due to the action of the Ca²⁺ channel blocker, the initial trauma to the nerve (exposure to vibration) does not induce any injury which can be detected by the second test crush lesion. The abolishing effect of D600 indicates that the drug has a neuroprotective effect on neuronal injury induced by vibration. Such an effect is not only related to exposure to vibration, but also to another technique for inducing conditioning lesions—namely, magnetic fields.[15][19] The mechanisms behind this neuroprotective effect in our study are not known but the Ca²⁺ channel blocker D600 stops the influx of Ca²⁺ through voltage sensitive calcium channels.[21][22] Therefore, it can prevent the activation of proteases dependent on Ca²⁺ and generation of free radicals which have a toxic action on axons and thereby the drug can have a neuroprotective effect on the neurons exposed to vibration.[13][14][26]

The hand-arm vibration syndrome represents a considerable clinical problem usually dominated by neurological and vasospastic symptoms.[4] There are limited possibilities for medical treatment of patients with hand-arm vibration syndrome. Blocking of Ca²⁺ channels has been used for the treatment of primary Raynaud's phenomenon as well as Raynaud's phenomenon induced by VWF. Such treatment has been reported to be mostly effective,[10][11] and is recommended.[12] However, many patients have neurological symptoms alone or in combination with vasospastic symptoms. The present study indicates that

Ca^{2+} channel blockers have a potential clinical value for treatment not only of vasospastic symptoms, but also of neuropathy of the hand induced by vibration. Good relief of such symptoms has also been found in symptomatic stone workers treated with Ca^{2++} channel blockers in Sweden (G Wemmenborn; personal communication).

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Abstract

The effect of short time vibration exposure and tourniquet compression on nerve regeneration in rats was studied with special reference to cell activation.

One of the hindlimbs was conditioned by either vibration exposure (5 hours per day, during 5 consecutive days) or compression (150-300 mmHg for 30-120 minutes), which was followed by a recovery period of 0-7 days. Test crush lesions or a transplantation of a conditioned nerve segment into a freshly made gap in the contralateral nerve (and vice versa after vibration), were performed. Axonal regeneration distances were measured after an additional 3-8 days. Furthermore the reaction of neuronal cellbodies, in the dorsal root ganglia, and non-neuronal cells was studied following compression.

Vibration exposure, and tourniquet compression in particular, increased axonal regeneration lengths after both test crush lesions and transplantation (up to 36%). This so called conditioning effect, which may be regarded as a sign of injury, was in one experiment prevented by treatment with D600, a calcium channel blocker.

The non-neuronal cells, which were mainly Schwann cells, increased their proliferation following compression along with an upregulation of the low affinity nerve growth factor receptor (p75). The neuronal cellbodies responded to compression by increased expression of the C-terminal flanking peptide of Neuropeptide Y (CPON).

Thus, tourniquet compression and vibration exposure, which are non-invasive methods, can increase the regenerative capacity of neurons. This requires activation of both Schwann cells and neuronal cellbodies. Conversely, the observed effect of such treatments may be regarded as a potentially harmful alarm reaction in the peripheral nervous system. Whether D600 prevents nerve injury caused by vibration exposure or compression remains to be investigated.

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