

**PERIPHERAL MECHANISMS OF NEUROPATHIC PAIN
FOLLOWING NERVE LESIONS:
NEUROPHYSIOLOGICAL AND BEHAVIORAL EXPERIMENTS**

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Abbreviations:

BDNF	Brain Derived Neurotrophic Factor
CCI	Chronic Constriction Injury
CCK	Cholecystokinin
CGRP	Calcitonin Gene Related Peptide
CNS	Central Nervous System
CV	Conduction Velocity
DH	Dorsal Horn
DRG	Dorsal Root Ganglia
GAL	Galanin
GDNF	Glial Cell Line Derived Neurotrophic Factor
IB ₄	Isolectin B ₄
IL	Interleukin
iv	intravenous
ip	intraperitoneal
LS	Lesion site
MS	Mechano sensitivity, mechanosensitive
NGF	Nerve Growth Factor
NPY	Neuropeptide Y
P ₂ X ₃	Purino (ATP) receptor
SNI	Spared Nerve Injury
SNL	Spinal Nerve Ligation
SA	Spontaneous Activity
SP	Substance P

Trk A	Tyrosine kinase A receptor
TNF	Tumor Necrosis Factor
TRP	Transient Receptor Potential
TS	Thermosensitivity; thermosensitive
TTX	Tetrodotoxin
TTX-S	Tetrodotoxin-sensitive (Na ⁺ channels)
TTX-R	Tetrodotoxin-resistant (Na ⁺ channels)
VIP	Vasoactive Intestinal Peptide
VR1	Vanilloid Receptor type 1
VRL1	Vanilloid Receptor Like type 1
WDR	Wide Dynamic Range
↑	up-regulation
↓	down-regulation

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1. INTRODUCTION

Almost all tissues are innervated by primary afferent neurones which provide sensory information to the Central Nervous System (CNS) about the environment and the state of the organism. The cell bodies of the afferent neurones are located in the dorsal root ganglia (DRG) or in the cranial nerve ganglia; their receptive terminals transduce and the afferent fibres transmit the information, in form of action potentials, from the periphery to the second order neurones in the dorsal horn. For this purpose sensory neurones have specialised transduction mechanisms which convert different form of stimuli (mechanical, thermal or chemical) into electric signals (generator potentials). According to the tissue they innervate sensory neurones are classified into: cutaneous, deep somatic (skeletal muscle, joint, fascia) and visceral primary afferent neurones.

1.1. CUTANEOUS RECEPTORS IN THE RAT

Physiological classification of the cutaneous receptors

According to the type of stimulus to which they respond three major classes of cutaneous receptors were identified: low threshold mechanoreceptors, thermoreceptors and nociceptors (Table 1.1).

- *Low threshold mechanoreceptors* are activated by mechanical forces applied to the skin. They are classified according to the adaptation rate to a maintained stimulus as rapidly adapting (RA) and slowly adapting (SA) receptors. RA receptors discharge briefly at the onset of a stimulus of constant strength and innervate both hairy skin (G- and D-hairs) and hairless skin. SA receptors continue to discharge during stimuli of constant strength and are subdivided in type I which discharge irregularly and type II which discharge regularly during stimuli of constant strength (Leem et al., 1993; Table 1.1). The myelinated afferent fibres from mechanoreceptors are large diameter ($A\beta$) and small diameter ($A\delta$) fibres and terminate predominately in laminae III-V of the dorsal horn.

Table 1.1
Types of cutaneous receptors in the rat

MECHANORECEPTORS			
Types	Receptive terminals	Stimulus	Fibre
Rapidly Adapting ^{1,3} (RA)	Meissner Corpuscle	Moving displacement	A β
	G-hairs	Hair movement	A β
	D-hairs	Hair movement	A δ
Slowly Adapting ^{1,3} (SA)	Type I (Merkell Cells)	Moving displacement	A β
	Type II (Ruffini Corpuscle)	Stretch	A β
Mechano low threshold ^{1,2,3}	?	Brushing	C
THERMORECEPTORS			
Cold ^{2,3}	Free endings	Innocuous cold (>15°<25°)	A δ ; C
NOCICEPTORS			
Mechano high threshold ^{1,2,3}	Free endings	Noxious mechanical	A δ ; C
Cold ²	Free endings	Noxious cold (>8°<15°)	C
Heat ^{1,2}	Free endings	Noxious heat (>43°)	C
Mechano-cold ^{1,2,3}	Free endings	Noxious mechanical and cold	A δ ; C
* Mechano-heat ^{1,2,3}	Free endings	Noxious mechanical and heat	A δ ; C
Mechano-cold-heat ^{2,3}	Free endings	Noxious mechanical, cold and heat	C
Silent ^{1,2}	Free endings (?)	Inflammation, tissue injury	C

Classification of cutaneous receptors according to their sensory properties.

* Mechano-heat C-nociceptors which respond to noxious mechanical and heat stimuli and to irritant chemicals are called *polymodals*

References: ¹ Handwerker et al., (1991); ² Kress et al., (1992); ³ Leem et al., (1993)

(?) indicates that the identity of receptive terminal is not well established

- *Thermoreceptors* respond to small changes in temperature signalling innocuous cooling stimuli with a temperature range of 15°-25°C (Leem et al., 1993, Clapham, 2002; McKemy et al., 2002). The afferent fibres from cold receptors are unmyelinated (C) fibres and thinly myelinated (A δ) fibres. In studies done on rodents the presence of warm receptors among the

afferent nerve fibres which innervate the skin of the hind paws has not been reported (Handwerker et al., 1991; Kress et al., 1992; Leem et al., 1993; Koltzenburg et al., 1997).

- *Nociceptors* respond to intense forms of mechanical, heat and/or cold stimulation. The afferent fibres connected with nociceptors conduct impulses in the A δ or C-fibre range. The A δ fibres innervate mechano-heat nociceptors which can be classified according to the heat threshold in: type I - with very high activation thresholds (>50°-53°C) and type II - which respond to lower temperatures (activation threshold ~ 43°C ; Raja et al., 1999; Caterina et al., 1999). The C-nociceptors are classified according to the stimulus to which they respond in mechanical, cold, heat, mechano-cold or mechano-heat nociceptors. The mechano-heat C-nociceptors which respond to noxious mechanical and heat stimuli and to chemical stimuli are called "polymodal" (Table 1.1). A certain proportion of cutaneous nociceptors are so called "silent" or "sleeping" nociceptors and do not respond to mechanical or thermal stimulation under normal conditions. They are only activated following tissue inflammation (Handwerker et al., 1991; Kress et al., 1992).

The central endings of myelinated (A δ) cutaneous nociceptive fibres terminate predominantly in laminae I and V of the dorsal horn and the central projections of cutaneous unmyelinated (C) nociceptive fibres terminate primarily in laminae II (Fig. 1.1).

Biochemical classification of C-nociceptors

The cutaneous C-nociceptors can also be classified according to biochemical criteria and trophic factor they depend on into two major populations: peptidergic and non-peptidergic ones.

- *Peptidergic nociceptive neurones* (40- 45% of the DRG neurones) express Trk A receptor (tyrosine kinase A) and require NGF (Nerve Growth Factor) for survival and development during pre- and postnatal life. In these nociceptors a group of neuropeptides is expressed in substantial quantities: CGRP (Calcitonin Gene Related Peptide) and substance P (SP) and other peptides are synthesised at low levels: VIP (Vasoactive Intestinal Peptide), CCK (Cholecystokinin), NPY (Neuropeptide Y) and galanin (Hökfelt et al., 1994). Peptidergic nociceptive afferent neurones project to laminae I and to the outer aspect of laminae II (IIo) (Snider and McMahon, 1998; Fig. 1.1)

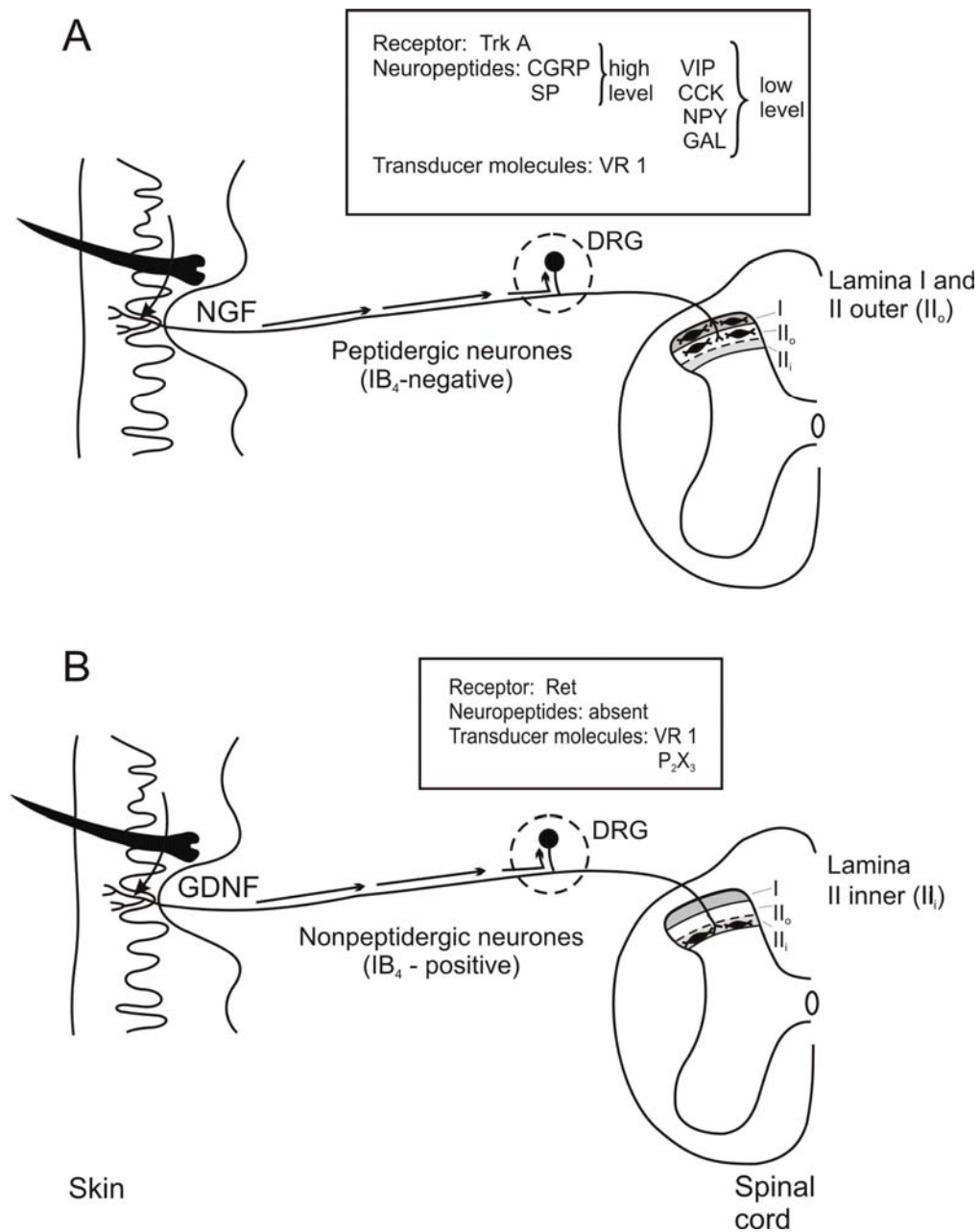


Fig. 1.1. Two distinct classes of C-nociceptive neurones - peptidergic (A) and non-peptidergic (B) - with different trophic factor dependence (NGF and GDNF), neurochemical content (neuropeptides and transducer molecules) and central projections into the dorsal horn. Abbreviations: CCK = Cholecystokinin, CGRP = Calcitonin Gene Related Peptide; DRG = Dorsal Root Ganglion, GAL = galanin, GDNF = Glial Cell Line Derived Neurotrophic Factor; IB₄ = Isolectin B; NGF = Nerve Growth Factor, NPY = Neuropeptide Y; P₂X₃ = (purino) ATP receptor, SP = Substance P, Trk A = Tyrosine kinase A receptor; VIP = Vasoactive Intestinal Peptide, VR1 = Vanilloid Receptor type1 (modified from Snider and McMahon, 1998)

Following noxious stimulation of the skin, neuropeptides are released together with the neurotransmitter glutamate from the central terminals of the nociceptive afferents at the level of dorsal horn where the neuropeptides act as neuromodulators.

- *Non-peptidergic nociceptive neurones* (30% of the DRG neurones) express another type of tyrosine kinase receptor, Ret, and are sensitive to the neurotrophin GDNF (Glial Cell Line Derived Neurotrophic Factor) in the postnatal period. These neurones possess cell surface glycoconjugates which can be identified by binding of lectin IB4 and project to the inner aspect of lamina II (Ili) (Snider and McMahon 1998). (Fig. 1.1)

The nociceptive C-neurones express different types of transducer molecules such as: vanilloid receptors (VR), ATP-receptors on their peripheral and central terminals.

The heat-activated vanilloid receptor type 1 (VR1) is found both in peptidergic and non-peptidergic neurones and acts as thermal transducer activated by noxious heat stimuli ($>43^{\circ}\text{C}$) (Caterina et al., 1997).

The P_2X_3 -ATP-receptor is expressed only on non-peptidergic (IB4-positive) sensory neurones (Fig. 1.1) and is found on both central and peripheral endings of these neurones. The peripheral P_2X_3 receptors are activated by ATP released from lesioned cells sensing local tissue damage (Cook et al., 1997).

Until now it is not known whether these two major classes of nociceptive C-neurones (peptidergic and non-peptidergic) which have different biochemical properties and different central terminations in the dorsal horn have also different functional roles. In the past decade extensive studies of the biochemical and physiological properties of the nociceptors were performed in order to understand the peripheral mechanisms of pain.

1.II. PAIN

Pain is a complex sensory-emotional experience associated with physical insults. Two categories of pain were described: acute (physiological) pain and chronic (pathophysiological) pain. *Physiological* pain is felt when noxious stimuli activate high threshold mechanical and/or thermal nociceptors and the evoked action potentials in thinly myelinated ($\text{A}\delta$) and/or unmyelinated (C) afferent fibres reach a conscious brain. In *pathological* states pain can be

evoked by normally innocuous mechanical and/or thermal stimuli (*allodynia*). The causative factors of pathophysiological chronic pain states might be inflammation or nerve lesion.

1.II.1. Inflammatory pain

During acute and chronic inflammation due to tissue injuries sensory nerve endings are exposed to a variety of cellular breakdown products and inflammatory mediators such as: prostaglandins, bradykinin, purines, cytokines (e.g., TNF α [Tumour necrosis factor]) which are released by inflammatory cells in the tissue. These mediators act on nociceptor endings activating intracellular cascades which result in *post-translational* changes such as phosphorylation of ionic channels or transduction molecules leading to increase in excitability of the nociceptive terminals (for review see Levine and Reichling, 1999). Inflammatory mediators may trigger also increased release of NGF from fibroblasts and other cells. NGF reacts with Trk A receptors in the nociceptor membrane and the complex NGF-Trk A receptor is internalised and transported to the cell bodies of afferent neurones where it induces *transcriptional* changes such as up-regulation or down-regulation of synthesis of neuropeptides, ionic channels and transducer proteins. During inflammatory states VIP, CCK, NPY and galanin are down regulated and CGRP, substance P and BDNF (Brain Derived Neurotrophic Factor) are up-regulated in the DRG (McMahon and Bennett, 1999) (Fig. 1.2A). Co-release of neuropeptides (CGRP, substance P) together with excitatory neurotransmitters (glutamate, aspartate) at the level of dorsal horn can alter the postsynaptic effects of neurotransmitters. Neuropeptides act as central regulators of excitability of the spinal second-order neurones and in this way contribute to abnormal pain states seen during inflammation (Urban et al., 1994). Besides the changes in the neuropeptide content, inflammation leads also to up-regulation of transduction molecules, such as VR1 (Carlton and Coggeshall, 2001; Amaya et al., 2003) and P₂X₃ (Xu and Huang, 2002) in the DRGs of neurones which innervate the affected area. __

The biochemical, morphological and physiological changes occurring in the peripheral and central neurones during inflammation are reversible after the inflammation subsides.

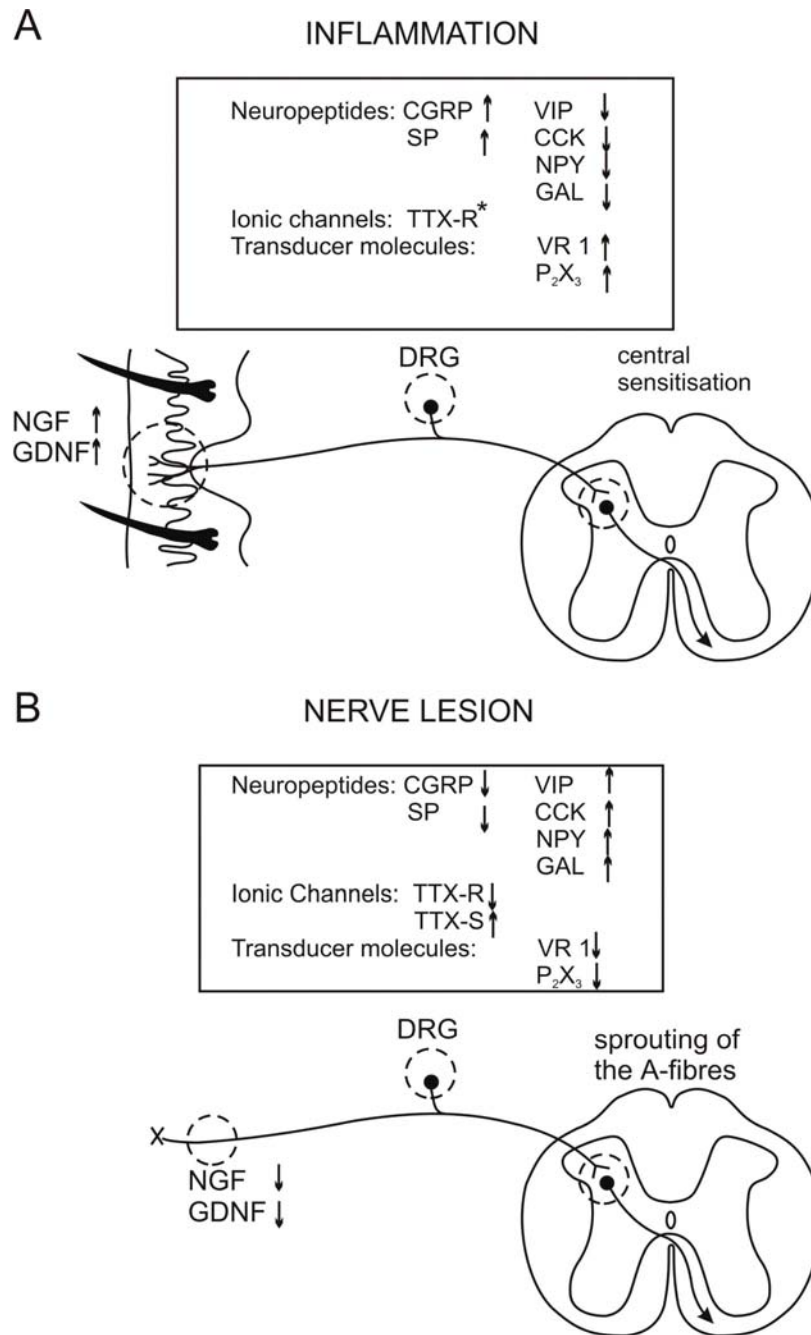


Fig. 1.2. The effect of inflammation (A) and nerve lesion (B) on the neuropeptide content, ionic channels and transducer molecules of the C-nociceptive neurones. Abbreviations: CCK = Cholecystokinin, CGRP = Calcitonin Gene Related Peptide; DRG = Dorsal Root Ganglion, GAL = galanin, GDNF = Glial Cell Line Derived Neurotrophic Factor; NGF = Nerve Growth Factor, NPY = Neuropeptide Y; P₂X₃ = (purino) ATP receptor₂, SP = Substance P, TTX-R = Tetrodotoxin-resistant Na⁺ channel; TTX-S = Tetrodotoxin - sensitive Na⁺ channel; VIP = Vasoactive Intestinal Peptide, VR1 = Vanilloid Receptor type 1. * during inflammation there is no up-regulation of the TTX-R Na⁺ channels but modulation of their activation characteristics. ↑ = up-regulation; ↓ = down-regulation (modified from McMahon and Bennett, 1999)

1.II.2. Neuropathic pain

Neuropathic pain results from injuries or diseases that affect the peripheral and/or central nervous system directly. The causative factors might be metabolic, viral or mechanical nerve lesion. Following nerve injury sensory neurones become disconnected from their targets and the normal retrograde supply of trophic factors (e.g., NGF and GDNF) from the periphery is disrupted. This results in marked phenotypic changes of the sensory neurones which are reflected in changes of their neurochemistry, morphology and functional properties (Fig. 1.2B). In contrast to what happens in the inflammatory state the biochemical, morphological and physiological changes in peripheral and central neurones may become irreversible after lesion of the peripheral neurones especially when the regeneration of their axons to the peripheral target cells is impaired.

1.II.2.1. Possible changes contributing to pathogenesis of neuropathic pain

1.II.2.1.1 Morphological changes

When a nerve is cut and the peripheral axons are prevented to regenerate to the target tissue, neurones undergo considerable retrograde changes. The axons and cell bodies shrink (Jänig and McLachlan, 1984) and this leads to a decrease in the conduction velocity of myelinated and unmyelinated axons (Blumberg and Jänig, 1982). Many neurones may finally degenerate - especially the postganglionic neurones and afferent unmyelinated neurones innervating the skin (Jänig and McLachlan, 1984; Hu and McLachlan, 2003) - and this results in a partial central deafferentation. Woolf et al., (1992) suggested that partial denervation of the second order neurones in lamina II due to death of afferent neurones with unmyelinated fibres trigger collateral sprouting of large diameter (A β) fibres from their terminal area in laminae III-V into lamina II that normally has synaptic input exclusively from unmyelinated primary afferents. Sprouting of the A β fibres into nociceptive spinal regions following peripheral nerve injury was considered to provide a possible substrate for pain symptoms such as mechanical allodynia but recent studies (Hughes et al., 2003) do not support this hypothesis.

Studies done *ex vivo* on a skin-nerve-spinal cord preparation (Köerber et al., 2001) show that partial denervation of the second order neurones in the laminae II due to loss of primary

afferent neurones with unmyelinated (C) fibres leads to sprouting of thinly myelinated (A δ) nociceptive fibres from lamina I to lamina IIo which normally is the exclusive site for the termination of small unmyelinated C fibres. This suggests a prominent role of A δ nociceptors in some chronic pain states following peripheral nerve injury (Köeber et al., 2001).

1.II.2.1.2 Biochemical changes

After different types of nerve lesion significant changes in the content of neuropeptides, channel proteins and transducer molecules occur in primary sensory neurones. Neuropeptides which are expressed in substantial quantities in intact neurones such as CGRP and substance P are down-regulated and peptides which are synthesised at low levels in normal conditions (VIP, CCK, NPY and galanin) are up-regulated following axotomy (Hökfelt et al., 1994). (Fig. 1.2B). Up-regulation of these neuropeptides in DRG neurones and their central termination areas following partial nerve injuries (Ma and Bisby, 1998) may have an important impact on spinal processing of the sensory information in the dorsal horn. For example, White and Mansfield (1996) reported that NPY and VIP increase neurite outgrowth of dissociated DRG neurones. The up-regulation of these neuropeptides following peripheral nerve injury might also be involved in induction of central sprouting of intact primary afferents into the denervated area of the dorsal horn, contributing to the mechanism of neuropathic pain (Ma and Bisby, 1998). Nerve lesion leads to down-regulation of transducer molecules, such as VR1 (Michael and Priestley, 1999) and P₂X₃ receptors (Brandbury et al., 1998; Tsuzuki et al., 2001) in the DRGs of axotomized neurones.

1.II.2.1.3 Physiological changes

Following nerve lesion, axotomized afferent neurones with myelinated and unmyelinated axons develop many abnormal functional changes. They generate ectopic spontaneous activity which may originate at the lesion site or in the DRG (Wall and Devor, 1983; for review see Devor and Seltzer, 1999). Ectopic spontaneous activity is the result of changes in membrane excitability due to modulation or changes in expression of voltage dependent Na⁺, K⁺, Ca²⁺ channels in the neuronal membrane (McCleskey and Gold, 1999). Based on their sensitivity to tetrodotoxin (TTX) two types of Na⁺ channels were identified: TTX-sensitive (TTX-S)

channels with fast inactivation and slow recovery from inactivation and TTX-resistant (TTX-R) channels with slow activation and inactivation and rapid recovery from inactivation. The expression of TTX-R Na^+ channels is regulated by NGF. When the access to the peripheral source of NGF is interrupted by axotomy the TTX-R Na^+ channels are down-regulated and a TTX-S Na^+ channel with fast recovery from inactivation is up-regulated (Cummins and Waxman, 1997). The rapid recovery from inactivation of the TTX-S Na^+ channels following axotomy shortens the refractory period of injured neurones making them able for abnormal firing.

K^+ currents, by their hyperpolarizing effect on the neuronal membrane, are also important for regulation of firing properties of the afferent fibres. Nerve injury diminishes the overall K^+ current and the reduction in membrane hyperpolarisation can contribute to ectopic impulse firing (Everill and Kocsis, 1999).

The abnormal spontaneous and evoked activity developed by lesioned afferent neurons might be important for initiation of long term alterations in excitability of spinal nociceptive neurones which are responsible for the distortion of sensory perception. The mechanisms by which peripheral and central changes following peripheral nerve lesions lead to different symptoms of pain and sensory disorders are incompletely understood. Multiple mechanisms at multiple sites may operate either alone or together and with different time courses to produce the complex clinical pictures.

1.II.2.2. Experimental approaches to explore the mechanisms of neuropathic pain

Many potential neurobiological mechanisms have been studied which may contribute to the pathogenesis of neuropathic pain. Human and animal models were developed in the past decade to investigate the mechanisms involved in initiation and maintenance of sensory abnormalities which appear following nerve lesion.

1.II.2.2.1. Human models

Human models provide access to the conscious experience of individual subjects so that spontaneous and evoked pain can be measured quantitatively. In this way psychophysical data and experimental interventions can be correlated. The human models are limited by the

restriction of the invasive surgical and pharmacological interventions making the investigation of specific pathophysiological mechanisms difficult or even impossible. The limitations of human experimental models to study the underlying mechanisms of neuropathic pain are overcome by using animal models.

1.II.2.2.2 Animal models

Understanding of the mechanisms of clinical pain has been accelerated by the development of animal models that reflect as closely as possible the behavioural components of clinical pain. The most frequently used species is the rat.

2.2.1 Behavioural animal models

Animals undergo nerve lesions which are clinically relevant and behavioural experiments are performed before and after the surgical interventions. The signs of pain are measured by quantifiable behavioural components such as paw withdrawal latency to thermal stimulation and paw withdrawal threshold to mechanical stimulation with von Frey hairs. These reactions are interpreted as being equivalent to thermal and mechanical allodynia in humans. The quantitative data obtained in behavioural experiments guide the design of further reduced animal models which can offer an insight into the neuronal mechanisms of sensory perception.

2.2.2 Reduced animal models in vivo

In order to understand the mechanisms behind different types of neuropathic pain, reduced animal models *in vivo* are used. In reduced animal models neurones and neurone populations are investigated in anaesthetised animals *in vivo* under controlled conditions. Recordings from single afferent neurones are performed in order to quantify the abnormal ectopic activity which develops after nerve trauma and which might be responsible for the initiation and maintenance of abnormal pain behaviour. Pharmacological interventions on the lesioned neurones allow to study the underlying mechanisms of the abnormal spontaneous activity.

2.2.3 Reduced animal models *in vitro*

The underlying mechanisms of ectopic activity generation at the cellular and sub-cellular level are studied in *in vitro* experiments. Reduced models *in vitro* use isolated parts of tissues and organs (including skin-nerve preparation, dorsal root ganglia with attached nerves, isolated nerves with a nerve end neuroma or isolated DRG cells) from animals which have undergone a controlled nerve lesion. Using these preparations it is possible to investigate, at cellular and subcellular level, the biochemical and physiological changes in the afferent neurones following nerve lesion. Discovery of nociceptor-specific ion channels such as VR1, TTX-R Na⁺ channels encourages the use of ion channels as targets for treating a variety of persistent pain conditions. The limitations of this approach include an altered physiology of isolated tissues and neurones compared to their behaviour *in situ*. On this basis, the data obtained in these investigations must be interpreted together with results obtained from reduced animal models *in vivo* and data from behavioural experiments on animals and humans.

1.II.2.3. Rat models to study neuropathic pain

Development of animal models that reflect elements of clinical pain syndromes has led to advances in understanding the pathophysiological mechanisms of initiation and maintenance of neuropathic pain. The following animal models have been introduced to investigate pathophysiological mechanisms of neuropathic pain: the chronic constriction injury model (CCI; Bennet and Xie, 1988), the partial sciatic nerve ligation model (Seltzer et al., 1990), the segmental spinal nerve ligation model (SNL; Kim and Chung, 1992) and the spared nerve injury model (SNI; Decosterd and Woolf, 2000). The SNL model is the most extensively used animal model to study the mechanisms of neuropathic pain.

1.II.2.3.1 Spinal Nerve Ligation Model

The spinal nerve ligation model (SNL) consists of a tight ligature of one (L5) or two (L5 and L6) spinal nerves close to the dorsal root ganglion, leaving the L4 component of the sciatic nerve intact. The L5 spinal nerve lesion leads in rats to an early onset and long-lasting mechanical and thermal allodynic behaviour. The mechanical allodynic behaviour is supposed to be triggered and maintained by ectopic activity generated in lesioned afferent neurones

(Sheen and Chung, 1993). Three to 8 days and 20 to 53 days after L5 spinal nerve lesion spontaneous discharges were present in 56% and respectively 29% of the L5 dorsal root filaments, spontaneous activity being observed in afferent neurones with myelinated fibres but not in those with unmyelinated nerve fibres (Liu et al., 2000). A temporal but weak correlation between ectopic activity and allodynic behaviour was found (Liu et al., 2000) suggesting that the ectopic activity in lesioned afferent neurones might be important for the maintenance of the neuropathic pain behaviour. Studies conducted in the same laboratory by Eschenfelder et al., (2000) demonstrated that mechanical allodynic behaviour which develops in rats after L5 spinal nerve lesion did not disappear after transection of the dorsal root L5, suggesting that besides the spontaneous activity in the lesioned afferent nerve fibres there is another mechanism which account for the abnormal reactions to mechanical and thermal stimuli in rats with SNL.

Wu et al., (2001) proposed that the abnormal pain behaviour which develops in rats after lesion of the L5 spinal nerve might be dependent on ectopic activity in intact (non-lesioned) afferent neurones. Recordings made from C-fibres in the intact L4 spinal nerve after ligation and transection of the L5 spinal nerve showed that one day after lesion, spontaneous activity developed in approximately half of the afferent C fibres. The alterations in the properties of intact L4 afferent neurones might be due to their interaction, distal to the lesion site, with the products associated with degeneration of the lesioned L5 neurones.

1.II.2.3.2 Spared Nerve Injury Model

Decosterd and Woolf (2000) developed a new animal model which consists of ligation and section of two branches of the sciatic nerve (namely tibial and common peroneal nerves) sparing the sural nerve (Spared Nerve Injury [SNI] model). The SNI model results in early (less than 24 hours), prolonged and substantial changes in mechanical and cold sensitivity that closely mimic the features of clinical neuropathic pain. The SNI model differs from the SNL model in that the intermingling of intact and injured neurones in peripheral target tissues is restricted to the border territory between the lesioned tibial nerve and intact sural nerve. This permits behavioural testing of the non-injured sural nerve territory (adjacent to the denervated areas) and also enables to investigate the neurophysiological changes which take place in the

peripheral afferent terminals of the intact sural afferent neurones following injury of neighbouring nerves.

1.II.2.3.3 Nerve lesion followed by regeneration

A characteristic of the nerve lesions in the animal models presented above is a permanent prevention of nerve regeneration following injury. When a nerve is lesioned and the regeneration to the periphery is prevented (e.g., neuroma lesion) the injured myelinated and unmyelinated fibers develop ectopic spontaneous and evoked activity. Studies done on rats, showed that 6-30 hours after sural nerve ligation and section about 18% of the axotomized unmyelinated and about 30% of the myelinated nerve fibres developed ectopic activity (Blenk et al., 1996; Michaelis et al., 1995, 1999). The unmyelinated units became responsive to mechanical, cold or heat stimuli and could develop spontaneous activity. The myelinated afferents developed sensitivity to mechanical stimuli applied to their novel axon endings and only few of them had spontaneous activity (Blumberg and Jänig, 1984; Michaelis et al., 1995).

In some clinical situations after peripheral nerve injury the lesioned afferent nerve fibres are free to regenerate to the peripheral target tissues. In this case, the cut distal end of the lesioned afferent nerve fibres send sprouts which tend to reach the target tissues. Growth factors synthesised by the non-neuronal cells post nerve injury are believed to be responsible for the sprouting of the lesioned fibres towards the target tissues (Heumann et al., 1987; Derby et al., 1993; Ahmed et al., 1999). Following nerve lesion, cytokines (e.g. interleukines, IL; tumor necrosis factor, TNF α) are produced and released by Schwann cells during Wallerian degeneration in the distal stump of the nerve (Shamash et al., 2002). The intimate contact of the lesioned and regenerating nerve fibres with the rich cytokine environment distal to the lesion site may influence the excitability of the injured myelinated and unmyelinated fibres during the regeneration process. The pattern and distribution of the abnormal discharge properties of the lesioned fibres can be different depending on the type of lesioned afferent fibre (A- or C-fibre), on the type of nerve lesion and can vary in time during the regeneration process and after the axons have reinnervated the target tissues. The change in the characteristics of spontaneous and evoked activity influences the status of excitability in

spinal nociceptive neurones and consequently the sensory abnormalities associated with neuropathic pain.

1.III. AIMS OF THE STUDY

Both injured afferent neurones and their uninjured neighbouring nerves develop abnormal spontaneous and evoked activity following nerve injury and both populations of afferents are hypothetically involved in initiation and maintenance of sensory abnormalities observed after nerve lesion. Analysis of the pattern of this ectopic activity and its underlying mechanisms is important for understanding the peripheral mechanism of neuropathic pain.

In the present study two models of nerve injury were used:

1. Sural nerve *crush* or sural nerve *section and resuturing (Regeneration model)*. The abnormal spontaneous and evoked discharge properties developed by *lesioned* afferent A- and C-fibres during the process of regeneration and after the axons have reinnervated the peripheral target tissues were investigated using neurophysiological methods.
2. In spared nerve injury model (*SNI model*) the common peroneal and tibial nerves were lesioned sparing the sural nerve. In this model the electrophysiological changes occurring in *intact* sural nerve fibres after lesioning of the neighbouring nerves were studied. Furthermore, components of the pain-like behaviour elicited by sensory stimulation of the skin in the rats were additionally investigated.

2. MATERIALS AND METHODS

Behavioural and neurophysiological experiments were performed on 98 male Wistar rats weighting 200-250 grams at the beginning of the experiments. The animals were housed singularly in plastic cages with sawdust as bedding material and kept on a 12 h light /dark cycle (6:00 – 18:00 – light). Water and food were provided ad libidum. The experimental protocols were approved by the local Animal Care Committee.

2.1. Nerve lesion procedure

Nerve injury was performed under pentobarbital sodium anaesthesia (Narcoren, 60mg/kg, i.p) and aseptic precautions. Animals were assigned to the following experimental groups.

Group A - Sural nerve lesion

Crush: In 32 rats the left sural nerve was exposed about 10 to 25 mm proximal to the ankle and injured by crush with fine watch maker forceps 15 - 20 mm proximal to the ankle. The sural nerve was squeezed three times (10s duration) at the same location over 1 to 1.5 mm, leading to transparency of the nerve at the lesion site.

Section and resuturing: In 26 rats the sural nerve was exposed and sectioned 15 - 20 mm proximal to the ankle. The proximal nerve end was reanastomosed to the peripheral stump using two epineural sutures (Prolene, 10/1, BV-4, Ethicon, Norderstedt, Germany).

Group B - Spared Nerve Injury

In 23 rats the sciatic nerve and its three terminal branches (the sural, common peroneal and tibial nerves) were exposed at the trifurcation site. The common peroneal and the tibial nerves were tightly ligated and sectioned distal to the ligation, and 2-4 mm of the distal nerve stump were removed. The sural nerve was spared and great care was taken to avoid any contact with or stretching of the intact sural nerve.

Muscle and skin were closed in two layers. The recovery of the rats was uneventful.

Group C - Control

In 17 rats no nerve lesion was performed.

2.II. Behavioural Testing

Rats which underwent Spared Nerve Injury (SNI) were tested for signs of mechanical and thermal allodynia post nerve injury. In order to assess mechanical and cold sensitivity of the hind paws the rats were placed in transparent plastic boxes above a wire mesh floor which allowed full access to the paws. Animals were tested in groups of four. Behavioural accommodation was allowed for at least 10 min before testing started. All the behavioural tests were done on the hind paws both ipsi- and contra-lateral to the nerve lesion. The area tested was the medial plantar surface of the hind paws for the *tibial nerve territory* and lateral plantar surface of the hind paws for the *sural nerve territory* (Fig. 2.1).

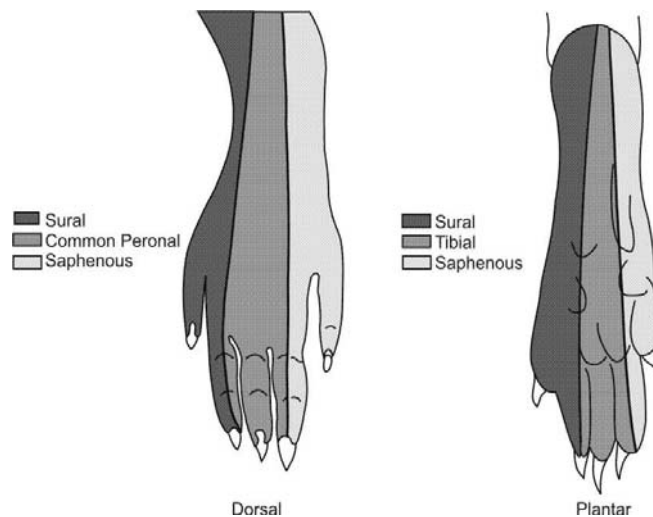


Fig. 2.1. Different zones of the dorsal and plantar surfaces of the rat hind paw innervated by the three terminal branches of the sciatic nerve (sural, tibial and common peroneal nerves) and by the saphenous nerve (from Decosterd and Woolf, 2000).

Following SNI, the animals showed an abnormal paw exposure due to denervation produced by lesion of the tibial and common peroneal nerves. Therefore, cold and heat sensitivity of the medial plantar surface of the hind paw (tibial nerve territory) ipsilateral to the lesion side could not be assessed. Standard testing procedures were used to quantify signs of mechanical and thermal allodynia. The baseline measurements were taken 3 times before nerve lesion. The rats were then tested on day 1 after lesion, twice a week until day 13 post operation and further on once a week until day 55 post nerve lesion.

2.II.1 Testing of mechanical sensitivity

Mechanical sensitivity was quantified using the up-down method (Fig. 2.2). An ascending series of von Frey filaments that delivered approximately logarithmic incremental forces (7.5 to 220 mN) were applied to the test area for about 8s. The 45 mN stimulus was applied first. Whenever a positive response to the stimulus (flexion reaction) occurred the next weaker von Frey hair was applied; whenever a negative response (no reaction) occurred the next stronger force was applied. The test was continued until: a) the response to 5 more stimuli after the first change in response had been obtained or b) the upper/lower end of the von Frey set was reached before a positive or negative response had been obtained. The pattern of positive and negative responses, respectively, was converted to a threshold value using the formula given by Dixon (1980): $\text{threshold} = 10^{(X+Kd)}$ where X = value of the final von Frey hair used (in log units); K = the tabular value for the pattern of positive/negative responses (obtained from Dixon, 1980; Chaplan et al., 1994) and d = mean difference in strength (in log units) between stimuli (here 0.183). In cases when continuous positive or negative responses to stimuli at either end of the stimuli range used were observed, values of either 5.8 mN (for continuous positive responses) or 271.9 mN (for continuous negative responses) were assigned. These numbers correspond to a von Frey hair 1 log increment below the smallest or 1 log increment above the highest von Frey hair used.



Fig. 2.2. Testing procedure for mechanical sensitivity of the hind paws. Animals were placed in transparent plastic boxes above a wire mesh floor which allows full access to the paws. Von Frey filaments were applied perpendicularly to the tested area with sufficient force to cause buckling against the paw and held for about 8s.

2.II.2 Testing of cold sensitivity

Cold sensitivity was quantified by measuring the duration of paw withdrawal in response to acetone application. An acetone drop was formed at the end of a piece of a small polyethylene tube which was connected with a syringe. The drop was gently applied to the medial or lateral plantar surface of the hind paws. The acetone was applied 5 times (once every 3-5 min) to each paw and the duration of paw withdrawal (in seconds) was measured.

2.II.3 Testing of heat sensitivity

Heat sensitivity was quantified using the Hargreaves test (Hargreaves et al., 1988). Rats were placed in a transparent plastic chamber with a glass floor (Fig. 2.3). Measurements of withdrawal latency to heat stimuli began after a period of accommodation of 10-20 minutes. A radiant heat source consisting of a high intensity projector lamp bulb (Osram 58-8007, 8V, 500W) was focused on the test territories of the hind paws. Onset of the radiant stimulus triggered a timer which was stopped by subsequent paw movement. Five heat stimuli were delivered to the lateral plantar surface of the ipsi- or contra-lateral hind paws at 3-5 min intervals. Following SNI the rats did not place the entire foot on the glass floor. Every attempt was made to focus the thermal stimulus on the sural innervation territory that was in full contact with the glass floor since it was reported that the withdrawal latency (which is related to the heat transfer) may vary with the degree of paw contact.



Fig. 2.3. Testing procedure for heat sensitivity of the hind paws. Rats were placed in a transparent plastic chamber with a glass floor. Thermal stimuli were applied to the tested territories using a radiant heat source.

For a given test day the sequence of the above mentioned behavioural tests was: 1) mechanical stimulation 2) cold stimulation 3) heat stimulation. The order of the tests was kept the same throughout the whole investigation period for all animals. The rationale for the choice of such order is that the least stressful test was performed first to minimise the influence of one test on the result of the next one.

2.III. Neurophysiological Experiments

2.III.1 Experimental groups of animals

Group A

Rats with *sural nerve lesion* were assigned to two experimental subgroups according to the time period post lesion when they were taken for neurophysiological experiments.

Period I: 4 days to 3 weeks post nerve lesion when the lesioned fibres were in the process of regeneration to the peripheral target tissues. **Group AI:** N = 18 rats with sural nerve crush and N = 16 rats with sural nerve section and resuturing.

Period II: 1 to 4 months post nerve lesion when the regeneration process is supposed to be completed (assuming a regeneration rate of about 1 to 3 mm/day; Forman et al., 1979; for review see Lisney, 1989). **Group A II:** N = 14 rats with sural nerve crush and N = 10 rats with sural nerve section and resuturing.

Group B

Rats with *Spared Nerve Injury* were assigned to three experimental subgroups, according to the time period post nerve lesion when they were taken for neurophysiological experiments:

Period I: 1 day - 1 week post lesion; **Group BI:** N= 7

Period II: 12 –26 days (2 – 4 weeks) post lesion; **Group BII:** N=6

Period III: 50 – 70 days (7 – 10 weeks) post lesion; **Group BIII:** N=7

Group C

Rats without nerve lesion were used as *Control* group

2.III.2. Anaesthesia and maintenance of the rats during the experiments

On the day of the final experiments the rats were re-anaesthetised with pentobarbital sodium (Narcofen, 60 mg/Kg, i.p) and catheters were inserted into the tail artery for arterial blood pressure measurement (transducer LM-22, List, Darmstadt, Germany) and into the jugular vein for administration of fluid and drugs (Fig. 2.4).

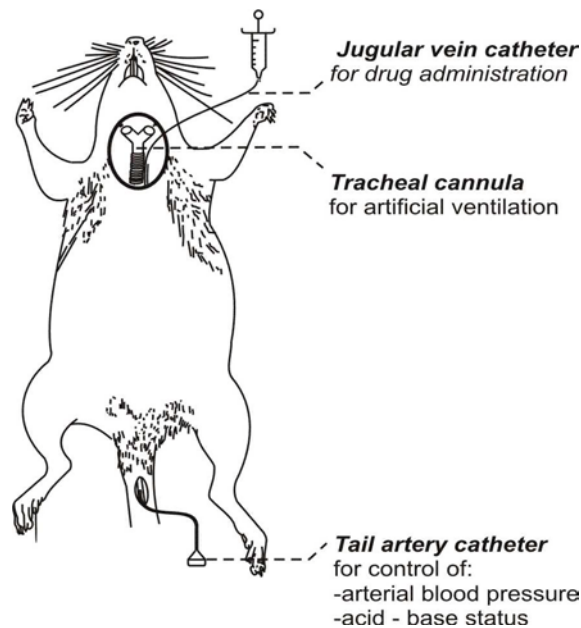


Fig. 2.4. Preparation of the animal. Jugular vein catheter was used for drugs and fluid administration, tail artery catheter for blood pressure recording and acid-base status control and tracheal cannula for artificial ventilation.

Additional doses of pentobarbital were given regularly (10-20 mg/kg/h, i.v) in order to maintain a sufficient level of anaesthesia judged from the absence of spontaneous blood pressure fluctuations. Mean arterial blood pressure was always higher than 70 mmHg. The trachea was cannulated, the rats were paralysed with Pancuronium (Organon, initially 1mg/kg, i.v; maintenance with 0.4 mg/kg/h) and artificially ventilated at 70 strokes per minute (ventilation pump RUS 1300, FMI, Egelsbach, Germany) using O₂-enriched room air. Blood gases were measured regularly throughout the experiment (ABL30, Radiometer Copenhagen); arterial pO₂ always exceeded 70 mmHg and pH was close to 7.4. Rectal temperature was monitored and kept close to 37.0°C by means of a servo-controlled heating blanket.

At the end of experiments the anaesthetised animals were killed by an intravenous injection of a saturated potassium chloride solution. All experiments were approved by

local animal care committee of the state administration and were conducted in accordance with German Federal Law.

2.III.3. Surgical procedure for the neurophysiological experiments

The left sural nerve was exposed from the ankle to its junction with the sciatic nerve where it was sectioned, isolated from connective tissue and placed on a rigidly fixed black Perspex platform for isolation of single filaments (Fig. 2.5A). In rats with sural nerve lesion (crush respectively section and resuturing), the sural nerve was isolated from the surrounding tissue 5 to 7 mm proximal and 5 to 7 mm distal to the lesion site and mounted on two pairs of platinum wire electrodes for electrical stimulation (Fig. 2.5B). In rats with spared nerve injury and in the control group one pair of stimulation electrodes was placed ~10 mm proximal to the ankle and ~25 mm distal to the recording site. The exposure was covered with warm (30°C) paraffin oil in a pool made from the skin flaps.

2.III.4. Recording and electrical stimulation

Under a stereo microscope fine filaments were dissected with sharpened watch maker forceps from the proximal cut end of the sural nerve and put on the recording electrode. Action potentials were recorded unipolarly using a pair of platinum wire electrodes with the indifferent electrode connected to the nearby tissue. The signals were differentially amplified (input resistance of the amplifier 10M Ω), filtered by a bandwidth from 120 Hz to 1.0 -1.2 kHz (unmyelinated fibers) or a bandwidth from 120 Hz to 40 kHz (myelinated fibers) and fed through voltage window discriminators (Fig. 2.6). Spike discrimination was controlled by a delay circuit; the action potentials were delayed by 5 ms and displayed on a storage oscilloscope.

The sural nerve was electrically stimulated with square wave pulses of 0.1 ms (A-fibres) or 0.5 ms (C-fibres) in duration at a frequency of 0.3 - 3 Hz and with variable intensities up to 40V. Conduction velocity (CV) of the myelinated and unmyelinated fibres was calculated from the latency of the response to single pulse electrical stimulation and the distance between recording and stimulation electrodes (Fig. 2.5C). Recorded fibres were categorised as A- or C-fibres based on their conduction velocity and their spike wave form (for A-fibres: spike width 0.3 - 0.6 ms, CV > 2m/s; for C-fibres spike width: 0.7 - 1.8 ms; CV \leq 2 m/s) (Matzner and Devor, 1987; Handwerker et al., 1991; Michaelis et al., 1994).

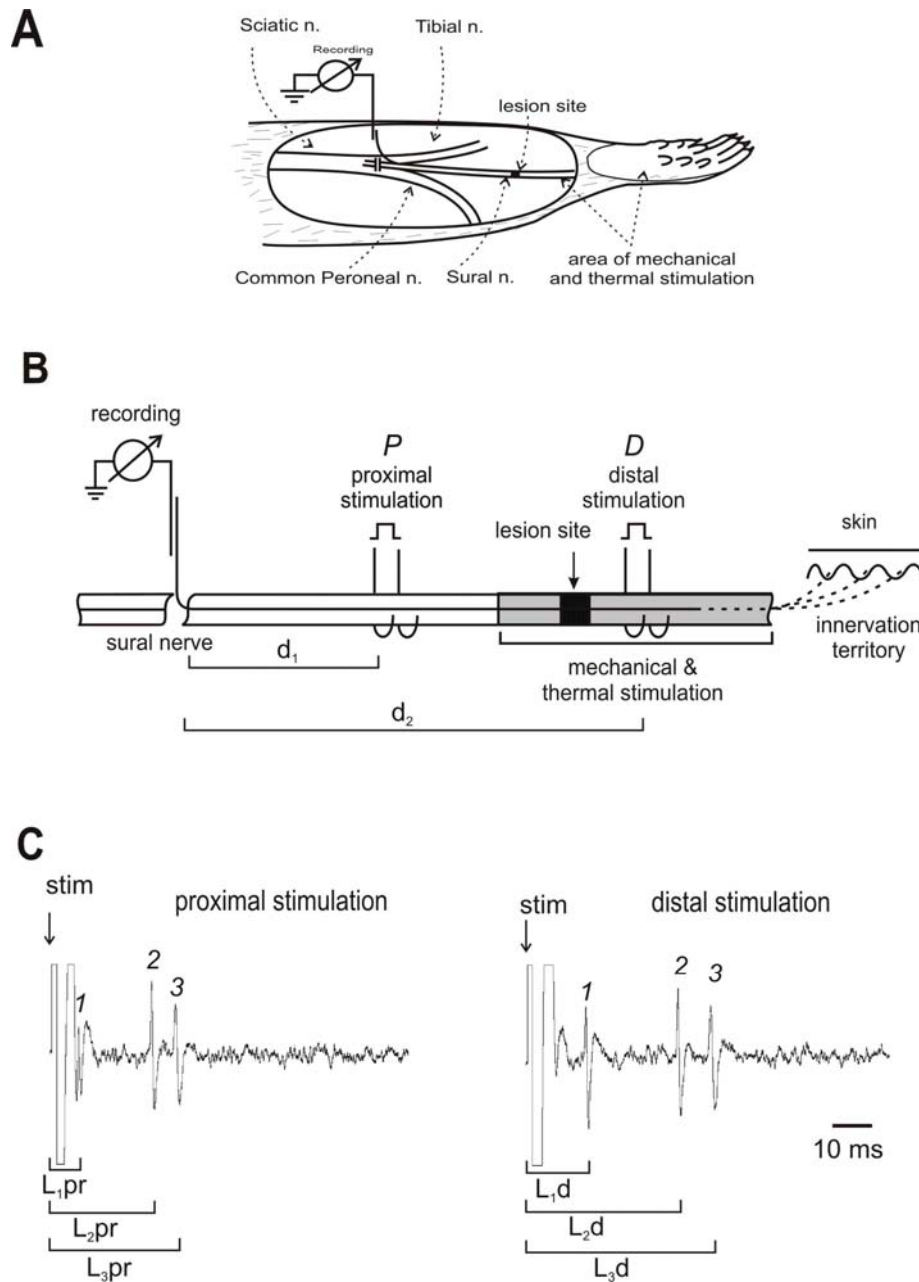


Fig. 2.5 A. Diagram indicating the position of the pool and the sciatic nerve with its 3 terminal branches (tibial, common peroneal and sural nerve). B. Schematic presentation of the sural nerve preparation. Fibre activity was recorded in fine filaments teased from the sural nerve rostral to the lesion site and put on a platinum recording electrode; the reference electrode was positioned on the nearby tissue. The nerve was placed on two pairs of stimulation electrodes proximal (*p*) and distal (*d*) to the lesion site for electrical stimulation of the nerve fibres. Natural (mechanical and thermal) stimuli were applied along the lesioned sural nerve at the area of sprouting of the nerve fibres, both proximal and distal to the lesion site and to the sural nerve innervation territory of the hind paw. C. Recordings for identification and calculation of conduction velocities (CV) of the fibres activated from the proximal respectively distal pair of stimulation electrodes. In this filament 3 C-fibres (marked 1,2,3) could be activated electrically both from proximal and distal stimulation electrodes. CV of the fibres excited from proximal electrode was calculated dividing the distance (in mm) between the recording electrode and the proximal stimulation electrode (d_1) by the time interval (in ms) between the onset of stimulus (arrow) and the onset of the responses ($L_{1,pr}$, $L_{2,pr}$, $L_{3,pr}$). Stimulation of the nerve from the distal electrodes yields systematically longer latencies ($L_{1,d}$, $L_{2,d}$, $L_{3,d}$) due to longer distances (d_2) between stimulation electrodes and recording electrode.

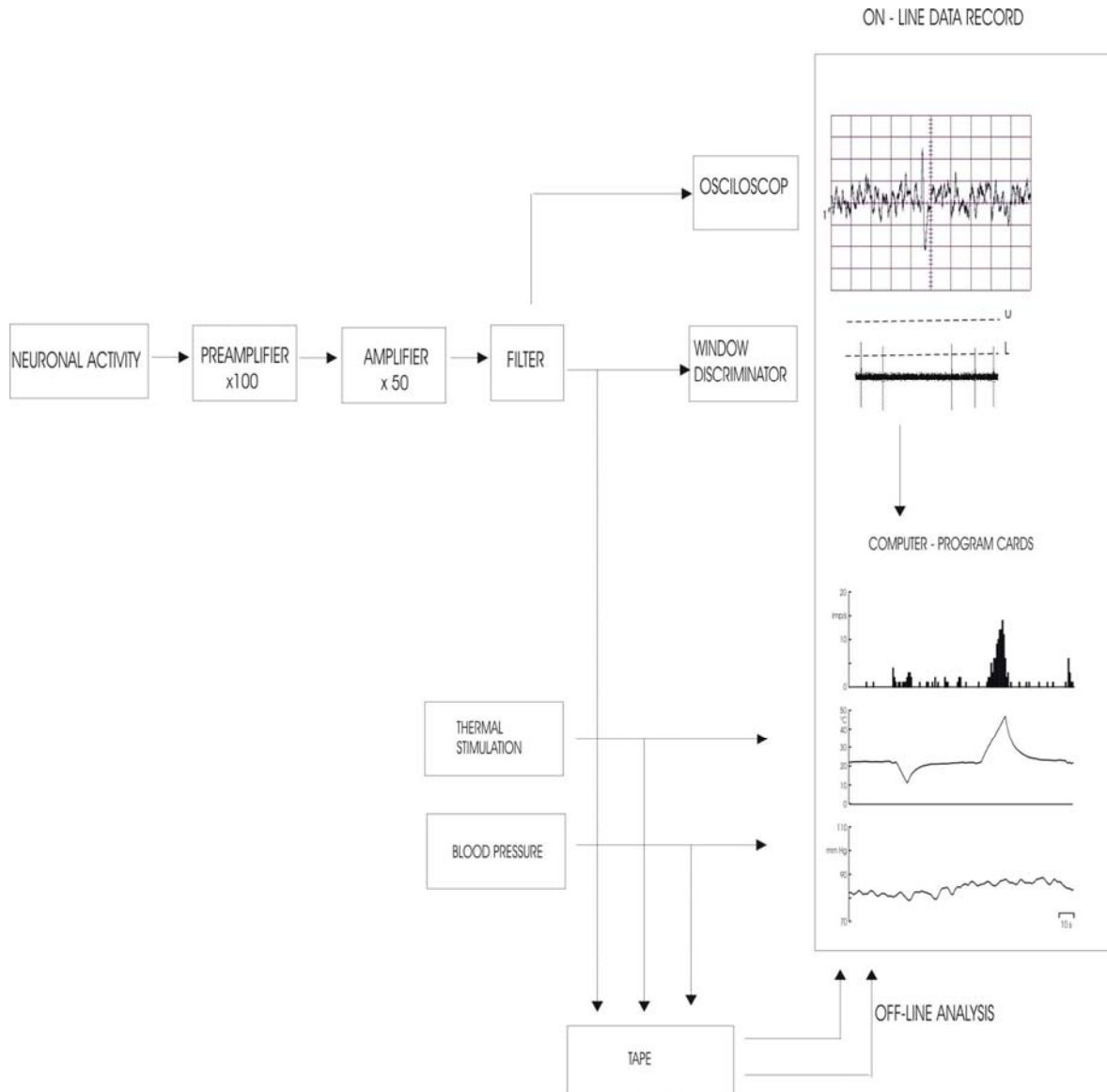


Fig. 2.6 - Recording and analysis of the data. Neuronal activity (action potentials) recorded from the afferent nerve fibres was amplified, filtered and fed into an amplitude window discriminator. The upper (u) and lower (l) discrimination lines were positioned so that the spike of the action potentials was placed between the two lines. The discriminated action potentials together with the blood pressure and temperature of the thermal stimulus were fed into a computer (software CARDS by Tiedemann). Discriminated action potentials were also visualised on an oscilloscope. All the data were recorded simultaneously on a digital tape recorder for further off-line analysis.

2.III.5. Natural stimulation

In the animals with sural nerve injury mechanical and thermal stimuli were applied along the lesioned sural nerve and to the target tissues of sural nerve territory. In the control animals and in rats with SNI where the sural nerve was not lesioned natural stimuli were applied only to the hind paw area innervated by the sural nerve.

Mechanical stimulation.

Mechanosensitivity of the afferent units was investigated by careful probing of the lesioned sural nerve and the area of the sural nerve innervation with a small fine-tipped blunt glass rod under visual control using a stereomicroscope. In rats with sural nerve lesion, mechanical stimuli were applied along the sural nerve in order to identify the size and location of receptive field(s) for individual afferent fibres. The mechanical sensitivity was tested in 1 mm steps starting 10 mm proximal to the lesion site up to the most distal point available on the exposed sural nerve (13-18 mm distal from the lesion site). After identification of the receptive field, mechanical stimuli were applied either repetitively at 1Hz (phasic stimulation) or continuously over about 10s (tonic stimulation). The mechanical threshold of the afferent fibers was determined using calibrated von Frey glass-fiber filaments with circular tips of 0.5 mm diameter and forces of 0.45 to 100 mN (*MARSTOCK^{nervertest}*, Fruhstorfer, Marburg, Germany). First stimuli of 7.5 mN were applied. In case of activation (at least 3 impulses/10s) the next weaker filament was used, in case of no response after three 10s stimuli the next stronger filament was used. The strength of the finest filament that either evoked at least three action potentials in silent fibres and fibres with rates of spontaneous activity below 0.5 imp/s or increased the spontaneous activity by $\geq 100\%$ in fibres with rates of spontaneous activity of >0.5 imp/s was defined as threshold stimulus strength.

In the control group, SNI group and sural nerve lesion group A II in addition to von Frey mechanostimulation, pinch stimuli with forceps were applied to the skin of sural nerve territory in order to identify the high threshold mechanonociceptors.

Thermal stimulation.

Thermosensitivity of the injured myelinated and unmyelinated fibres which had their receptive fields along the lesioned sural nerve was tested using a U-shaped water-perfused thermode. (Fig. 2.7, see legend for characteristics of the thermode).

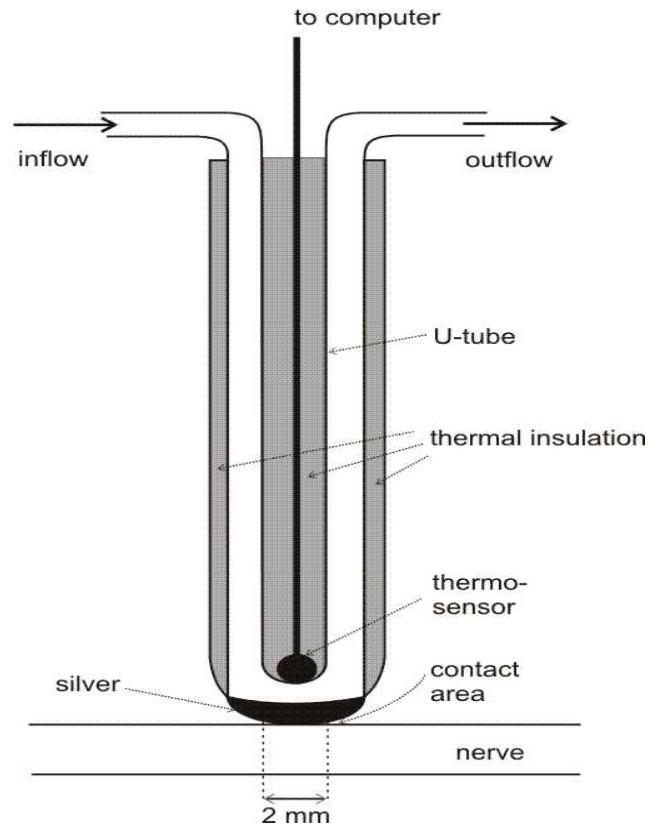


Fig. 2.7. Device used for thermal stimulation of the nerve. The thermode consisted of a U-shaped metal tube in which water at different temperatures was perfused. At its convexity (the contact area with the nerve) the thermal insulation of the U-tube was interrupted at 1mm x 2mm to allow optimal thermal conduction to the nerve. A small thermoelement of about 1 mm diameter (KTY 21-7, Siemens, Germany) was placed in the concavity of the U-tube, opposite to the nerve, in order to measure continuously the temperature on the surface of the U-tube.

The surface of the thermode tube contacting the nerve was 2 mm long. Due to thermal conduction the stimulus covered a distance of 4 mm along the nerve. For testing the thermosensitivity the thermode was therefore placed at five sites of the nerve: the lesion site, 4-6 mm proximal to the lesion site as well as 4-6 mm, 9-11 mm and 14-16 mm distal to the lesion site. In this way the area from 7 mm proximal to 17 mm distal to the lesion site was tested. For identification of cold sensitive fibres the thermode was initially perfused with ice water, the lowest temperature reached was in the range 3.5-5 °C. If a fibre was activated before the minimal temperature was reached, a series of testing stimuli at higher temperatures (10-19°C) was applied. For initial testing of heat sensitive fibres stimuli of 47-50°C were applied (duration 3-5s: longer stimuli were avoided to prevent nerve damage). If a fibre was activated before peak temperature was reached, a series of test stimuli at lower temperatures (30-42°C) was applied. These temperature stimuli were

maintained for approximately 15s and started from a baseline temperature of about 22 to 24°C.

Thermosensitivity of the lesioned sural nerve fibres which have regenerated and reached the skin and of the intact sural nerve fibres (in control and SNI group) was tested using a Peltier element which delivered ramp stimuli at rates of 0.5°C/s, 1°C/s and 1.5°C/s starting from an adaptation temperature of 23°C. Every A- and C-fibre tested was at least once exposed to a ramp cold stimulus (which decreased from 23 to 4°C) and to a ramp heat stimulus (which increased from 23 to 49°-52°C). When the receptive field was not accessible for placing the Peltier thermode the thermosensitivity was tested by applying a hot or cold metal rod on the receptive field. In this case the activation of the fibres by thermal stimuli could not be determined quantitatively but the fibres could be classified as being cold or heat sensitive.

A fibre was regarded as thermosensitive if more than three action potentials were generated during increase or decrease of the temperature in silent fibres and fibres with rates of spontaneous activity below 0.5 imp/s or if the activity increased by $\geq 100\%$ in fibres with rates of spontaneous activity >0.5 imp/s.

2.III.6. Experimental procedure

Only filaments which contained A- and/or C-fibres that exhibited at least one of the following functional properties: spontaneous activity or/and mechanical or/and thermosensitivity were analysed. Filaments in which A- or C-fibres were activated by electrical stimulation but could not be excited by natural stimulation or did not exhibit spontaneous activity were discarded. First it was tested whether a filament isolated from the sural nerve contained fibres with one of these functional properties. If a filament contained a spontaneously active fibre, the discharge was recorded on digital tape first for 3-5 minutes in order to determine its rate and pattern. Then a series of mechanical and thermal stimuli were applied along the lesioned sural nerve from 10 mm proximal up to 18 mm distal to the lesion site and to the target tissues of the sural nerve territory in order to localise the receptive fields of the mechanosensitive and/or thermosensitive A- or C-fibres.

2.IV. Data analysis

Neural activity, temperature of the stimulation thermode and arterial blood pressure were simultaneously fed into a computer (IBM-compatible) with ADC and counter interface (Burr-Brown PCI-20000, data acquisition software CARDS by S. Tiedemann). The neural activity (action potentials) was converted in time-activity histograms (Fig. 2.6). The signals were stored also on a digital tape recorder (DTR-2602, Biologic, Claix, France) and used for further off-line analysis using Spike/Spidi software package [Forster and Handwerker, 1990]) which employs a template-matching procedure. Data are expressed as means \pm S.E.M. For statistical analysis the Chi^2 -test, student *t*-test or the non-parametric Wilcoxon signed-rank test were used.

3. RESULTS

3.I. SURAL NERVE LESION FOLLOWED BY REGENERATION

Fifty-eight rats which underwent lesion of the sural nerve (crush respectively section and resuturing) were used in the present study to investigate the spontaneous and evoked (mechanical and thermal) discharge properties of the injured myelinated and unmyelinated afferent nerve fibres. The electrophysiological experiments were performed 1 to 3 weeks post sural nerve lesion (time period in which the injured nerve fibres were in the process of regeneration to the target tissue) and 1 to 4 months post nerve lesion (when the regeneration process is supposed to be completed, assuming a regeneration rate at about 1 to 3 mm/day; Forman et al., 1979; for review see Lisney, 1989).

3.I.1. Characterisation of the lesioned and regenerating fibres 1 to 3 weeks post nerve injury

In 34 electrophysiological experiments performed 1 to 3 weeks post sural nerve crush (N=18) or sural nerve section and resuturing (N=16) 187 filaments were dissected from the proximal stump of the lesioned nerve. A total number of 346 afferent fibres (177 A- and 169 C-fibres) which exhibited at least one discharge property (mechanosensitivity, thermosensitivity and/or spontaneous activity) were investigated. Twenty-nine units (24 A- and 5 C-fibres) had already reinnervated the peripheral target in the original sural nerve territory, 4 of them (3A- units and 1C-unit) having the receptive field both in the target tissue and in the nerve; most of these fibres (23/29 units) were found in rats with sural crush lesion. It might be that these fibres were still in the process of regeneration and could be excited from sural nerve fascicles below the skin. The afferent units which had regenerated and reached already the target tissue are included in section 3.I.2.

3.I.1.1. Conduction velocities of the lesioned and regenerating nerve fibres

In 107 filaments electrical stimulation was used in order to determine the conduction velocities of the lesioned and regenerating myelinated and unmyelinated fibres.

In the total sample, the conduction velocities of the *myelinated* (A) fibres varied between 2.1 and 80 m/s. Figure 3.1A illustrates the mean conduction velocities of the myelinated fibres in the crush group and in the section and resuturing group which were activated by

electrical stimulation from electrodes placed both proximal and distal to the lesion site (see inset Fig. 3.1) and the conduction velocities of the intact A-fibres (control group).

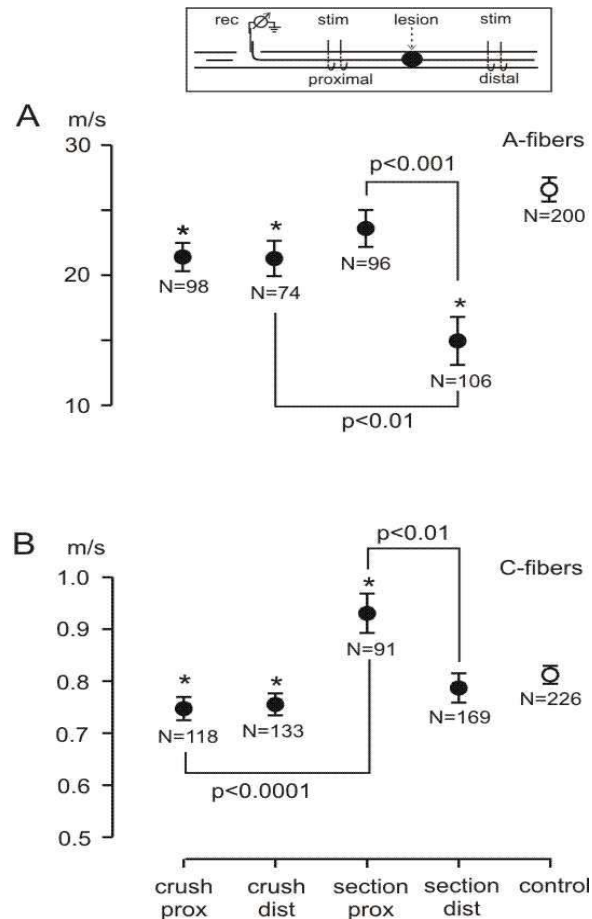


Fig. 3.1. Conduction velocities (mean \pm SEM) of the myelinated (A) and unmyelinated (B) afferent nerve fibres (in meters per second, m/s) in the *crush* and *section and resuturing* group obtained by electrical stimulation via stimulation electrodes placed *proximal* and *distal* to the lesion site. *Control* represents the mean conduction velocities of the A- and C-fibres recorded in the sural nerve of rats without nerve lesion. The asterisks indicate the conduction velocities of the lesioned myelinated (A) and unmyelinated (B) fibres which were statistically significantly different ($p < 0.05$, *t*-test) from the conduction velocities of the intact A- and C-fibres (control group). The inset shows a schematic drawing of the sural nerve and the position of the proximal and distal stimulation electrodes and the recording electrode (rec).

Following *crush* lesion the conduction velocities of the A-fibres activated from electrodes placed distal to the lesion site did not differ significantly from those activated from proximal stimulation electrodes but both of them were significantly lower ($p < 0.01$; *t*-test) than the conduction velocities of A-fibres in the control group.

After *section and resuturing* of the sural nerve the conduction velocities of the A-fibres obtained by electrical stimulation from the distal electrodes were significantly lower

($p < 0.001$; t -test) than those obtained from the proximal stimulation electrodes and than the conduction velocities of the intact A-fibres.

The conduction velocities of the A-fibres activated from proximal stimulation electrodes were not significantly different between the crush group and the section and resuturing group whereas the conduction velocities of the A-fibres activated from distal stimulation electrodes were significantly lower ($p < 0.01$; t -test) following section and resuturing than after crush lesion.

In the *unmyelinated* (C) fibres population the conduction velocities varied between 0.24 and 2 m/s. Figure 3.1B shows that in the *crush* group the conduction velocities of the C-fibres activated from the distal stimulation electrodes did not differ significantly from those activated from the proximal stimulation electrodes but both of them were significantly lower ($p < 0.05$, t -test) than the conduction velocities of the intact C-fibres (control group). In the *section and resuturing* group, it can be seen that the conduction velocities of the C-fibres which were electrically activated from proximal stimulation electrodes were faster than those activated from distal stimulation electrodes and than the conduction velocities of the intact C-fibres ($p < 0.01$; t -test). The conduction velocities of the C-fibres activated from the proximal stimulation electrodes were significantly higher ($p < 0.0001$, t -test) in the section and resuturing group than in crush group whereas the conduction velocities of the C-fibres activated from the distal stimulation electrodes were not significantly different between the two types of nerve lesion.

3.1.1.2. Proportion of A- and C-fibres exhibiting ectopic activity following nerve lesion

In 20 experiments performed 5 to 21 days after sural nerve lesion (crush or section and resuturing) the percentages of ectopically active A- and C-fibres among the total number of electrically activated fibres were investigated. For this purpose only those filaments in which the number of electrically activated A- or C-fibres could be determined were analysed. These filaments contained maximally 7 A-fibres (mean 3 fibres) or maximally 10 C-fibres (mean 5 fibres). Strands in which the number of nerve fibres activated by electrical stimulation could not be determined were discarded. All electrically activated fibres were tested for their spontaneous activity and whether they could be excited by mechanical and/or thermal (cold/heat) stimuli applied to the nerve (Table 3.1).

Table 3.1

Proportion of myelinated and unmyelinated nerve fibres which exhibit ectopic firing properties among the total number of electrically activated fibres

	A-fibres		C-fibres	
	Proximal	distal	proximal	Distal
Crush	34% (31/92)	33% (24/73)	22% (27/125)	27% (29/106)
Section and resuturing	40% (63/157)	37% (45/122)	21% (51/244)	23% (51/222)

Thirty-three to 40% of the electrically activated *A-fibres* exhibited ectopic firing properties. Within the same group of lesion (crush respectively section and resuturing) no significant difference ($p > 0.05$; *Chi*²-test) between the percentage of ectopically active A-fibres activated from proximal versus distal stimulation electrodes was found. Also no significant difference ($p > 0.05$; *Chi*²-test) was found between the percentage of ectopically active A-fibres in the crush versus section and resuturing group.

In the *C-fibres* population, 21 to 27% of the total number of electrically activated units were ectopically active. As in the case of myelinated fibres, no significant difference ($p > 0.05$; *Chi*²-test) in the percentage of ectopically active C-fibres was found between the two types of lesion (crush versus section and resuturing) and within the same group of lesion between proximal versus distal stimulation electrodes.

3.1.1.3. Ectopic discharge properties of A- and C-fibres

One to three weeks post sural nerve crush or section and resuturing 346 ectopically active afferent units which exhibited spontaneous activity and/or could be activated mechanically and/or thermally *from the nerve* were investigated. Among these units 177 were identified as A- and 169 as C-fibres. The numbers of afferent A- and C-fibres with at least one ectopic discharge property were almost equally distributed over the first, second and third week post-lesion in both experimental groups (Table 3.2).

Table 3.2

Distribution of discharge properties of the A- and C-fibres during the first 3 weeks post nerve lesion (crush or section and resuturing)

A-fibres

	Crush				Section and resuturing		
	1 st week	2 nd week	3 rd week		1 st week	2 nd week	3 rd week
N	38	27	7	N	46	33	26
SA	11 29%	8 30%	1 14%	SA	1 2%	1 3%	1 4%
MS	35 92%	19 70%	6 86%	MS	44 96%	31 94%	26 100%
TS	3 8%	3 11%	0	TS	4 9%	3 9%	1 4%

C-fibres

	Crush				Section and resuturing		
	1 st week	2 nd week	3 rd week		1 st week	2 nd week	3 rd week
N	25	20	34	N	25	34	31
SA	18 72%	15 75%	18 53%	SA	13 52%	9 26%	11 32%
MS	9 36%	7 35%	10 29%	MS	9 36%	19 56%	21 68%
TS	16 64%	11 55%	22 65%	TS	8 32%	17 50%	20 68%

With one exception the proportion of myelinated (A) and unmyelinated (C) fibres which exhibited ectopic discharge properties did not differ significantly during the first three weeks of investigation both in crush and in section and resuturing group ($p > 0.05$, Chi^2 -test). In the section and resuturing group the proportion of mechonosensitive C-fibres was significantly lower in the first week than in the third week post lesion.

N- number of fibres; SA - spontaneous activity; MS - mechanosensitivity; TS - thermosensitivity.

The response characteristics of the afferent nerve fibres in these three time periods were similar. Furthermore, the incidence of every ectopic discharge characteristic was, with one exception, not statistically significantly different throughout the first three weeks post nerve lesion ($p > 0.05$, Chi^2 -test). Only the incidence of thermosensitive C-fibres was significantly lower in the first week than in the third week post-lesion in rats with sectioned and resutured sural nerve. *Therefore*, the data obtained in the first three weeks of investigation were pooled together (Table 3.3 and 3.4).

Table 3.3

Discharge properties of the injured A-fibres 1 to 3 weeks following crush or section and resuturing of the sural nerve

Type of fibre	A-fibres	
Type of nerve lesion	Crush	Section and resuturing
Total number of fibres with ectopic properties	72	105
INDIVIDUAL ECTOPIC DISCHARGE PROPERTIES		
Spontaneous activity	20 (28%) ^a	3 (3%) ^a
Mechanosensitivity	60 (83%)	101 (96%)
Thermosensitivity	6 (8%)	8 (8%)
<i>Cold activated</i>	0	1
<i>Heat activated</i>	6	7
<i>Cold and heat activated</i>	0	0
COMBINATION OF ECTOPIC DISCHARGE PROPERTIES		
Mechanosensitive/spontaneously active	49* / 6	97* / 0
Cold sensitive/spontaneously active	0 / 0	0 / 1
Heat sensitive/spontaneously active	0 / 1	2* / 1
Cold and heat sensitive/spontaneously active	0 / 0	0 / 0
Mechanical and cold sensitive/ Spontaneously active	0 / 0	0 / 0
Mechanical and heat sensitive/ Spontaneously active	3 / 2	3 / 1
Mechanical, cold and heat sensitive/ Spontaneously active	0 / 0	0 / 0
Spontaneous activity only	11* (15%)	0

* Afferent fibers with only one ectopic discharge property. Letter *a* indicate the ectopic discharge property which is significant different ($p < 0.05$; χ^2 test) between the two types of nerve lesion (crush versus section and resuturing). In combination of ectopic discharge properties the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active.

3.I.1.3.1 Characterisation of A-fibres

Most (159/177; 90%) of the A-fibres belonging to both groups of lesion exhibited only one ectopic property being either mechanosensitive, thermosensitive or spontaneously active; 8% of the myelinated fibres had two discharge properties and only 2% showed all three ectopic properties together (Fig. 3.2).

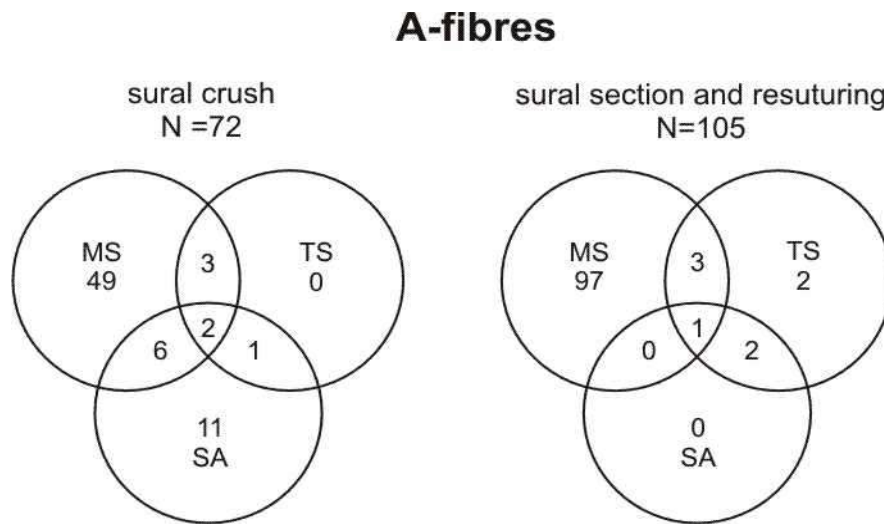


Fig. 3.2. Distribution of mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) in the A-fibres following *crush* or *section and resuturing* of the sural nerve. The overlaps represent the number of fibres which exhibit a combination of 2 respectively 3 of the mentioned discharge properties.

Spontaneous activity

Twenty-eight per cent of the A-fibres in the crush group and 3% of the A-fibres in section and resuturing group exhibited spontaneous activity following nerve lesion ($p < 0.01$, Chi^2 -test; Table 3.3). The rates of spontaneous discharge varied from 0.03 to 10.6 imp/s (mean = 2.4 ± 0.7 ; median = 1 imp/s) in the lesioned A-fibres of both nerve lesion groups. The pattern of spontaneous activity was irregular in most myelinated fibres and only 9 units exhibited regular or bursting discharges (Fig. 3.3A and B). In 11 A-fibres the spontaneous activity was the only ectopic discharge property.

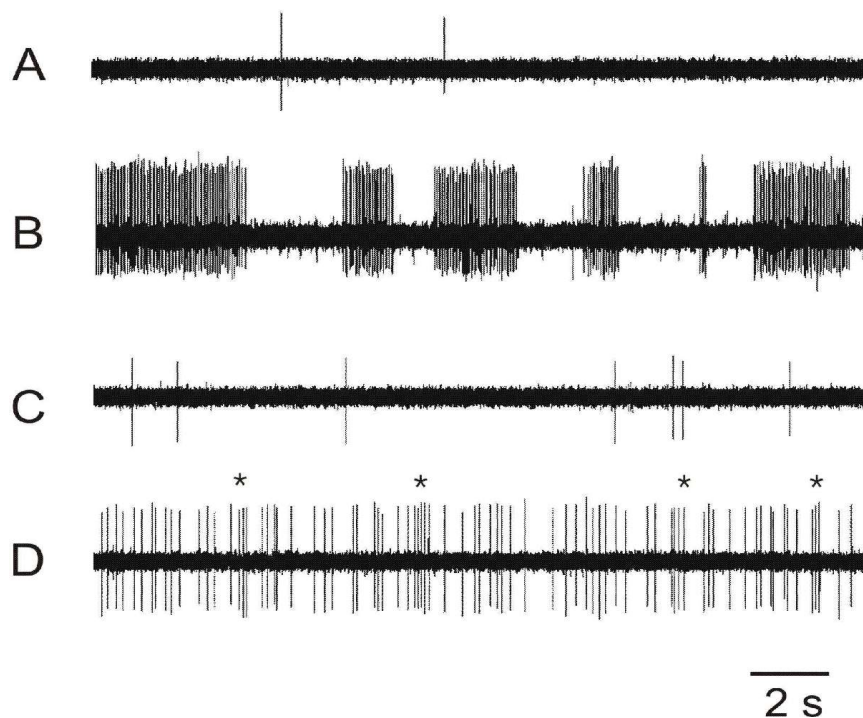


Fig 3.3. Original records of spontaneous activity in myelinated (A,B) and unmyelinated (C,D) nerve fibres. A: Two myelinated nerve fibres (0.01, 0.05 imp/s). B: Bursting activity in a myelinated nerve fibre (10.6 imp/s). The example presents an extreme case of spontaneous activity at a very high rate. C: Unmyelinated nerve fibre (0.4 imp/s). D: Unmyelinated nerve fibre exhibiting some bursting discharges indicated by asterisks (3.2 imp/s)

Mechanosensitivity

Mechanosensitivity was the main discharge characteristic of the lesioned and regenerating myelinated nerve fibres. Mechanical stimuli applied to the lesioned nerve excited 83% of the A-fibres in the crush group and 96% of the A-fibres in the section and resuturing group (Table 3.3). Within the considered time period the mechanosensitive A-fibres belonging to both groups of lesion had the receptive fields distributed along the lesioned sural nerve extending from 6 mm proximal to 15 mm distal to the lesion site depending on the time after nerve lesion and on the type of injury. The main histogram in figure 3.4 shows the distribution along the nerve of the receptive fields of the mechanosensitive A-fibres in both nerve lesion groups.

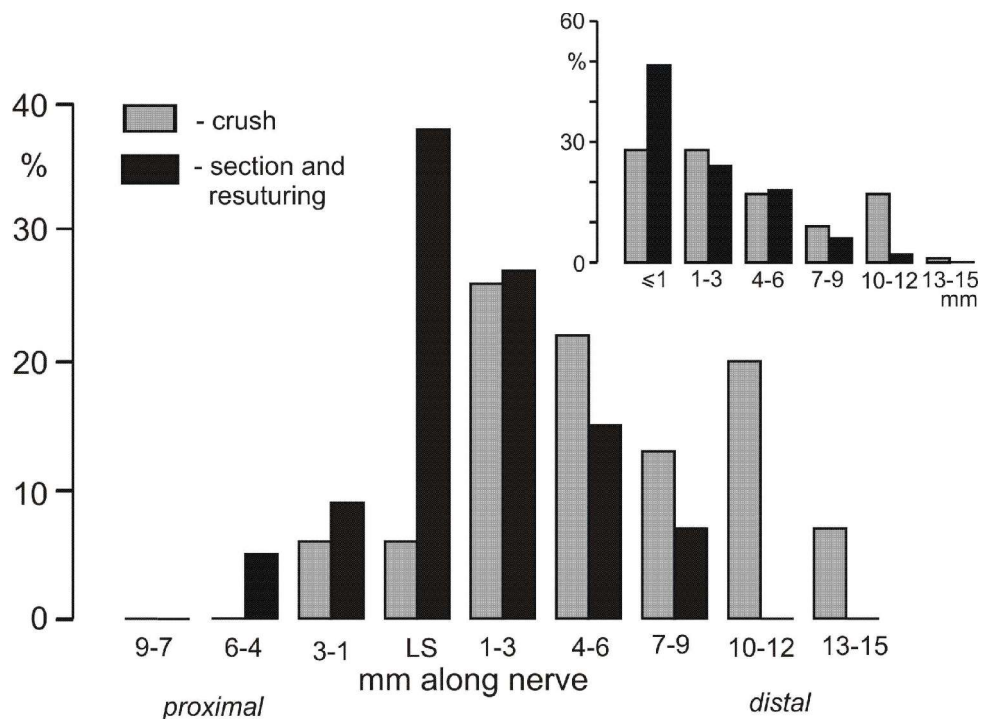


Fig. 3.4. Distribution and size of receptive fields for mechanosensitive A-fibres under regeneration. Mechanical stimuli were applied at different sites along the nerve proximally and distally to the lesion site (LS) using a fine tipped glass rod. The main histogram exhibits the distribution of the most distal/proximal point of the receptive fields (crush N = 53; section and resuturing N = 87). The inset shows the distribution of the receptive field size in mm. The smallest spatial resolution was 0.5 mm, therefore fibres which could be excited only from one site had a receptive field size of < 0.5 mm.

In the *section and resuturing* group 38% of the mechanosensitive A-fibres were trapped at the injury site and did not succeed to regenerate. The rest of the myelinated fibres had the tendency to sprout centripetally or centrifugally and the most remote point on the nerve they could reach within 3 weeks post injury was 9 mm distal to the lesion site. In the *crush* group only 6% of the mechanosensitive A-fibres were trapped at the lesion site, the other fibres being in the process of regeneration along the nerve. Within the considered time period the most remote point on the nerve the lesioned and regenerating mechanosensitive A-fibres could reach following crush lesion was 15 mm distal to the lesion site. Due to the sprouting and arborisation process the length of the receptive fields of the mechanosensitive A-fibres varied considerably (see inset Fig. 3.4A). The sprouting process was more pronounced following crush than after section and resuturing lesion, some mechanosensitive A-fibres could be excited over a distance of 15 mm along the path of regeneration. The receptive fields of the lesioned and regenerating nerve fibres were mostly discontinuous and consisted of punctuate mechanosensitive spots along the nerve.

This is demonstrated for one A-fibre in Fig. 3.5 (large action potential) in which the mechanosensitivity was present 1 mm and 4 to 6 mm but not 2 to 3 mm distal to the nerve lesion.

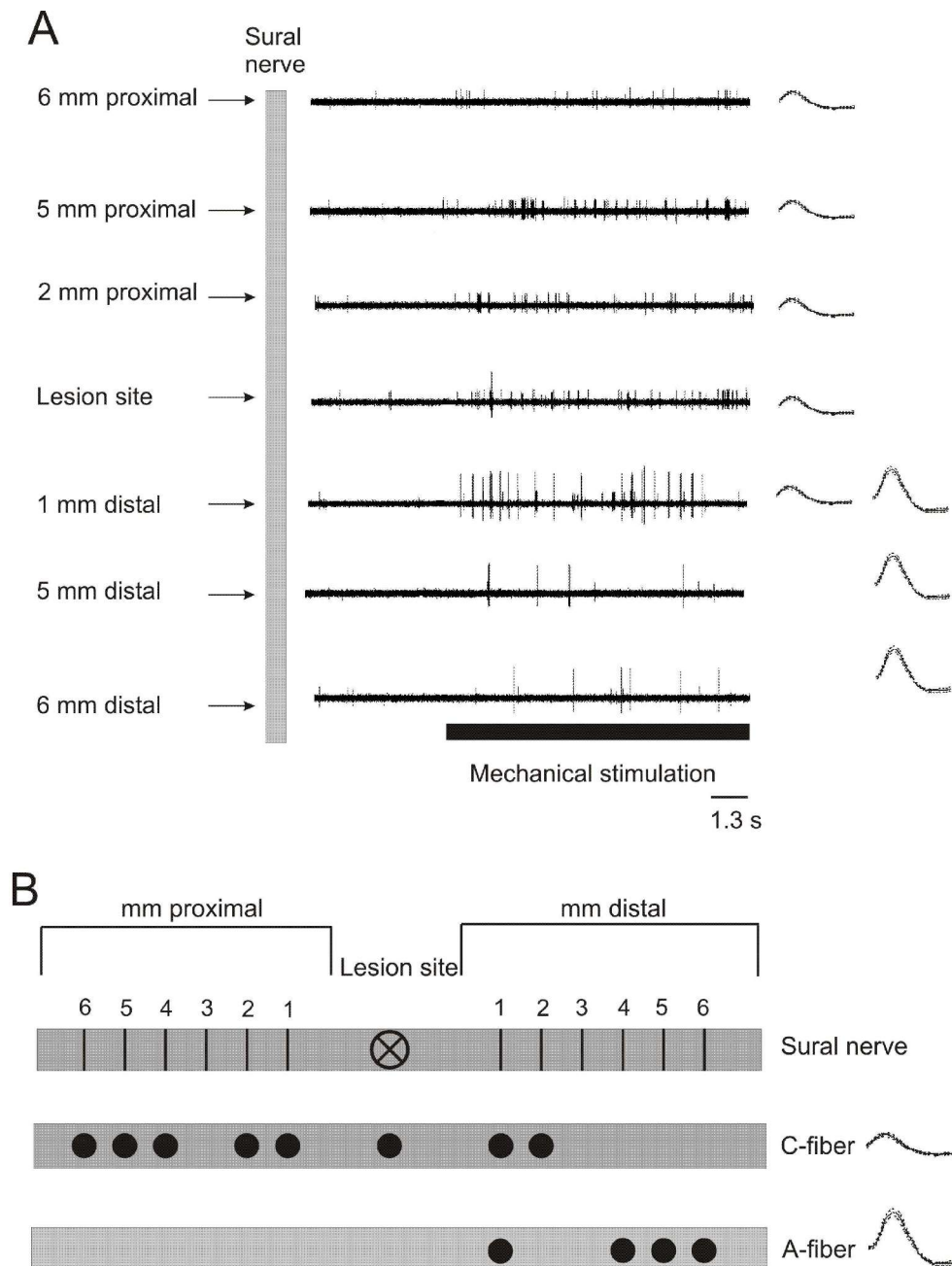


Fig. 3.5 A. Spatial distribution along the nerve of mechanosensitivity of one silent A-fibre (large action potential) and one spontaneously active C-fibre (small action potential) recorded in the same bundle. Repetitive mechanical stimuli were applied with a fine-typed glass rod to the nerve from 6 mm proximal to 15 mm distal to the lesion site. B. Schematic representation of the receptive fields distribution of the same fibres. The A-fibre could be activated from sites 1 mm and 4 to 6 mm but not 2 to 3 mm distal to the lesion site. The mechanosensitive C-fibre had receptive fields from 6 mm proximal up to 2 mm distal to the lesion site. Records on the left show superimposed action potentials.

The mechanosensitive A-fibres responded phasically to mechanical probing of the nerve with a fine-tipped glass stylus (Fig. 3.6A); tonic responses to constant forces were absent. Mechanical thresholds of the A-fibres determined by using calibrated von Frey filaments ranged from 0.45 to 45 mN (Fig. 3.6D, mean = 5.9 ± 0.9 mN; N = 81). These thresholds were not significantly different between the two groups of lesion.

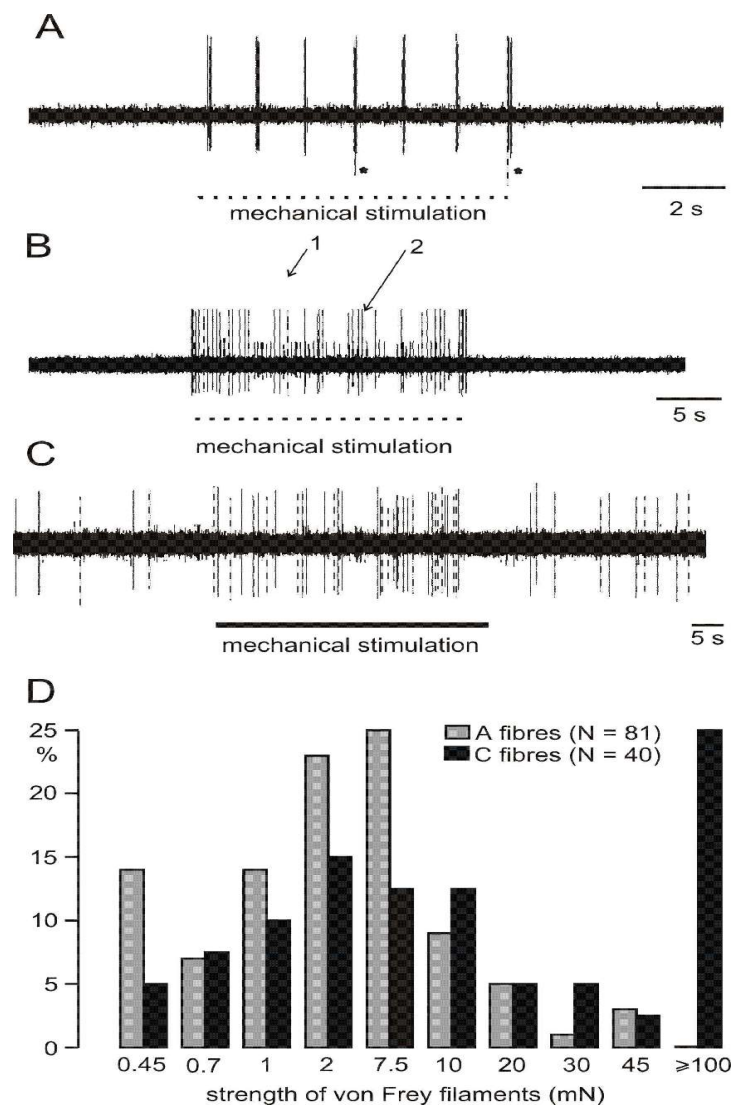


Fig. 3.6. Ectopic mechanical sensitivity of regenerating nerve fibres. A. Two silent A-fibres responding only at the beginning of the stimuli applied at about 1 Hz. Fibre marked by asterisks rarely responded. B. Response of two silent A-fibres to repetitive mechanical stimuli (1 Hz) for 20s. C. Tonic response of a spontaneously active C-fibre to a mechanical constant force stimulus. D. Distribution of mechanical thresholds of A- and C-fibres belonging to both groups of lesion. Mechanical thresholds were measured with calibrated von Frey filaments of different bending forces. C-fibres with thresholds ≥ 100 mN were only excited by stimuli applied with a fine tipped glass rod.

Thermosensitivity

Thermal stimuli applied to the nerve excited 8% of the A-fibres both in the crush and section and resuturing group (Table 3.3). The majority (13/14) of the myelinated fibres belonging to both groups of lesion were activated by heat stimuli applied to the nerve and their activation thresholds ranged from 37° to 45°C. The myelinated fibres responded tonically or phasic-tonically during heat stimulation (Fig. 3.7). Only one A-fibre was activated by cooling.

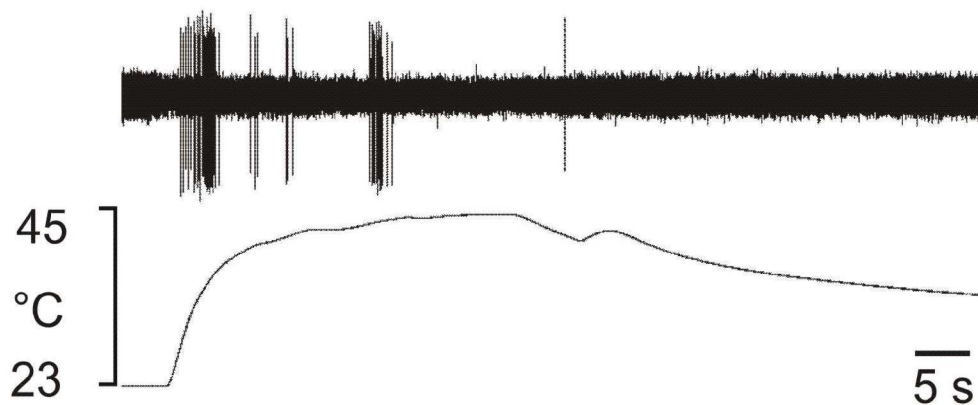


Fig. 3.7. Response of a regenerating afferent A-fibre to heating of the nerve (lower trace). The fibre exhibited some bursting activity in its response to heat stimulation.

3.1.1.3.2. Characterisation of the C-fibres

The pattern of the ectopic discharges in the afferent C-fibres was different from the pattern in the A-fibres as far as the distribution of types of ectopic activity is concerned. Nine per cent (16/169) of the unmyelinated fibres belonging to both groups of lesion were polymodal and exhibited all three ectopic properties, 31% (52/169) of the C-fibres had two firing properties and 60% (101/169) were unimodal being either mechanosensitive, themosensitive or spontaneously active (Fig. 3.8).

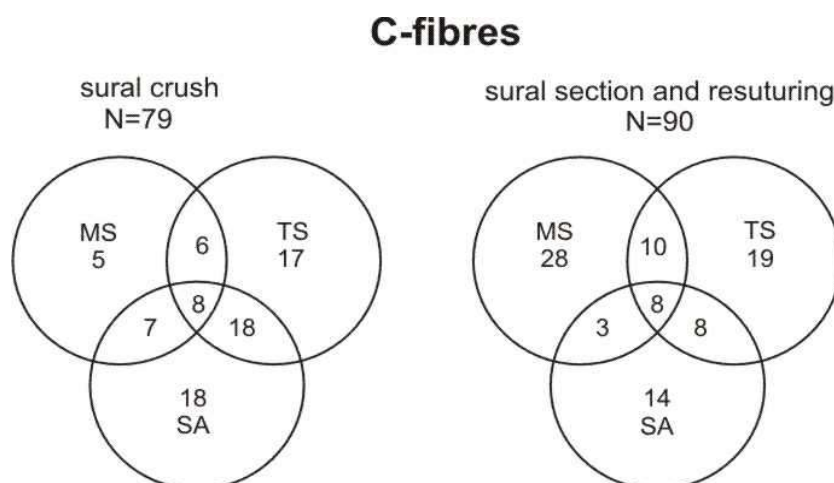


Fig. 3.8. Distribution of mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) in the C-fibres following *crush* or *section and resuturing* of the sural nerve. The overlaps represent the number of fibres which exhibited a combination of 2 respectively 3 of the mentioned discharge properties.

Spontaneous activity

Sixty-five per cent of the unmyelinated units in the crush and in 37% of the C-units in the section and resuturing group exhibited spontaneous activity post nerve lesion ($p < 0.005$, χ^2 -test; Table 3.4). The rate of spontaneous activity ranged from 0.01 to about 8 imp/s (mean = 0.86 ± 0.20 , median = 0.3 imp/s, $N=84$; Fig. 3.9) and it was significantly higher in C-fibres after crush than after section and resuturing lesion (1.23 ± 0.30 vs. 0.36 ± 0.06 imp/s; $p < 0.01$, Wilcoxon test; Fig. 3.9).

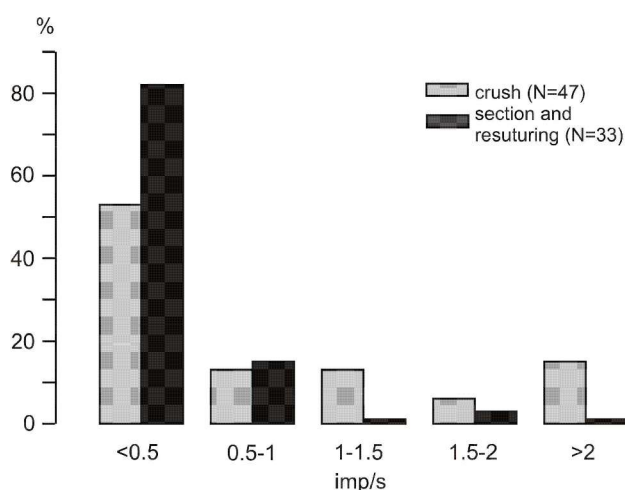


Fig. 3.9. Distribution of rates of spontaneous activity (in impulse per second, imp/s) in lesioned unmyelinated nerve fibres after crush (grey columns) or section and resuturing (black columns) of the nerve. The rates of spontaneous activity in the C-fibres were significantly higher ($p < 0.01$) following crush than following section and resuturing lesion.

Table 3.4

Discharge properties of the injured C-fibres 1 to 3 weeks following crush or section and resuturing of the sural nerve

Type of fibre	C-fibres	
Type of nerve lesion	Crush	Section and resuturing
Total number of fibres with ectopic properties	79	90
INDIVIDUAL ECTOPIC DISCHARGE PROPERTIES		
Spontaneous activity	51 (65%) ^a	33 (37%) ^a
Mechanosensitivity	26 (33%) ^b	49 (54%) ^b
Thermosensitivity	49 (62%)	45 (50%)
<i>Cold activated</i>	29 ^c	16 ^c
<i>Heat activated</i>	12	21
<i>Cold and heat activated</i>	8	8
COMBINATION OF ECTOPIC DISCHARGE PROPERTIES		
Mechaosensitive/spontaneously active	5* / 7 ^e	28* / 3 ^e
Cold sensitive/spontaneously active	9* / 12 ^{d,e}	6* / 6 ^{d,e}
Heat sensitive/spontaneously active	7* / 0 ^d	10* / 1 ^d
Cold and heat sensitive/spontaneously active	1 / 6	3 / 1
Mechanical and cold sensitive/ Spontaneously active	2 / 6 ^d	1 / 3 ^d
Mechanical and heat sensitive/ Spontaneously active	4 / 1 ^d	5 / 5 ^d
Mechanical, cold and heat sensitive/ Spontaneously active	0 / 1	4 / 0
Spontaneous activity only	18* (23%)	14* (16%)

* Afferent fibers with only one ectopic discharge property. Letters *a*, *b*, *c* indicate the ectopic discharge properties which are significant different ($p < 0.05$; χ^2 -test) between the two types of nerve lesion (crush versus section and resuturing). *a* - spontaneous activity in C-fibres ($p < 0.005$); *b* - mechanosensitivity in C-fibres ($p < 0.005$); *c* - cold sensitivity in C-fibers ($p < 0.01$).

d and *e* compare the incidence of spontaneous activity in cold sensitive fibres versus the incidence of spontaneous activity in heat sensitive, respectively mechanosensitive fibres.

d - incidence of spontaneous activity in cold sensitive versus heat sensitive C-fibres (27/45 vs 7/33; $p < 0.005$);

e - incidence of spontaneous activity in cold sensitive versus mechano sensitive C-fibres (18/33 vs 10/43; $p < 0.01$).

In *combination of ectopic discharge properties* the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active.

The spontaneous activity was irregular in most C-fibres (Fig. 3.3C, insets Fig. 3.11) and some C-fibres showed discrete bursts in their spontaneous activity occurring irregularly as shown in Fig. 3.3D (and inset Fig. 3.11B). Sixty-two per cent (52/84) of the C-fibres which exhibited spontaneous activity responded also to mechanical and/or thermal stimuli applied to the lesioned nerve; the remaining 38% (32/84) were only spontaneously active and could not be excited by mechanical or thermal stimuli (Fig. 3.8). The distribution of spontaneous activity of C-fibres exhibited some distinct characteristics (see lower part of Table 3.4): the proportion of spontaneously active cold sensitive C-fibres was significantly higher than the proportion of spontaneously active heat sensitive C-fibres (27/45 vs. 7/33 fibres, $p < 0.005$, Chi^2 -test) and than the proportion of spontaneously active mechanosensitive C-fibres (18/33 vs. 10/43 fibres, $p < 0.01$, Chi^2 -test).

Mechanosensitivity

Mechanical stimuli applied to the nerve excited 33% of the C-fibres in the crush group and 54% of the C-units in the section and resuturing group ($p < 0.005$, Chi^2 -test; Table 3.4). The receptive fields of the lesioned mechanosensitive C-fibres were discontinuously distributed along the nerve and extended, depending on the time post nerve injury and on the type of lesion, from 9 mm proximal to 15 mm distal to the lesion site (Fig. 3.10).

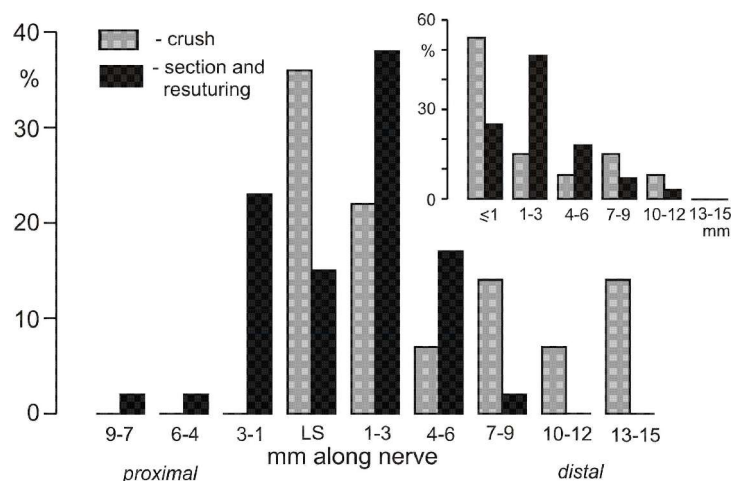


Fig. 3.10. Distribution and size of receptive fields for mechanosensitive C-fibres under regeneration. Mechanical stimuli were applied at different sites along the nerve proximal and distal to the lesion site (LS) using a fine tipped glass rod. The main histogram exhibits the distribution of the location of the most distal/proximal point of the receptive fields (crush N=14; section and resuturing N = 47). The inset shows the distribution of the receptive field size in mm. The smallest spatial resolution was 0.5 mm, therefore fibres which could be excited only from the one site had a receptive field size of < 0.5 mm.

Like the mechanosensitive A-fibres, the injured mechanosensitive C-fibres showed a better regeneration following crush than following section and resuturing lesion. Within 3 weeks post crush injury the lesioned and regenerating C-fibres could be excited from sites 15 mm distal to the injury site while, within the same time period, the most distal point on the nerve from which the injured C-fibres could be excited following section and resuturing lesion was 9 mm distal to the injury site. The size of the receptive fields of the mechanosensitive C-fibres varied between 1 to 12 mm along the nerve (inset in Fig. 3.10). This is demonstrated for one C-fibre in Fig. 3.5 (small action potential) in which the mechanosensitivity was present from 6 mm proximal to 2 mm distal to the lesion site. In 40 C-fibres the mechanical thresholds were determined using calibrated von Frey filaments. The distribution of the mechanical thresholds of the C-fibres was bimodal, 30/40 C-fibres tested had thresholds ranging from 0.45 to 45 mN (mean = 8.4 ± 1.9 mN, median = 4.7) and the remaining 10 C-fibres had mechanical thresholds of ≥ 100 mN (Fig. 3.6D). The C-fibres with low mechanical thresholds responded phasically to mechanical stimuli applied to the nerve while the C-fibres with high thresholds responded with tonic discharges to constant mechanical stimuli (Fig. 3.6C). No difference was found in mechanical threshold of the C-fibres and the response profile to mechanical stimulation between both types of nerve lesion.

Thermosensitivity

Thermal sensitivity was the main characteristic of the lesioned unmyelinated fibres belonging to both groups of lesion. Application of thermal (cold or heat) stimuli to the nerve activated 62% of the C-fibres in the crush group and 50% of the C-fibres in the section and resuturing group (Table 3.4).

Cold sensitivity. Forty-five of 94 (48%) thermosensitive unmyelinated fibres belonging to both groups of lesion were excited by cold stimuli and had their receptive fields distributed in the nerve along the path of regeneration. The proportion of C-fibres which were activated by cooling was higher in nerves with crush lesion than in nerves which were sectioned and resutured (29/79 vs. 16/90 nerve fibres; $p < 0.01$, *Chi*²-test; Table 3.4). Most of the cold sensitive C-fibres (32/35 fibres, 91%) were activated by cooling steps of $< 10^\circ\text{C}$

starting from a baseline temperature of 22-24 °C, some fibres being very sensitive to small temperature changes (see arrow in Fig. 3.11B).

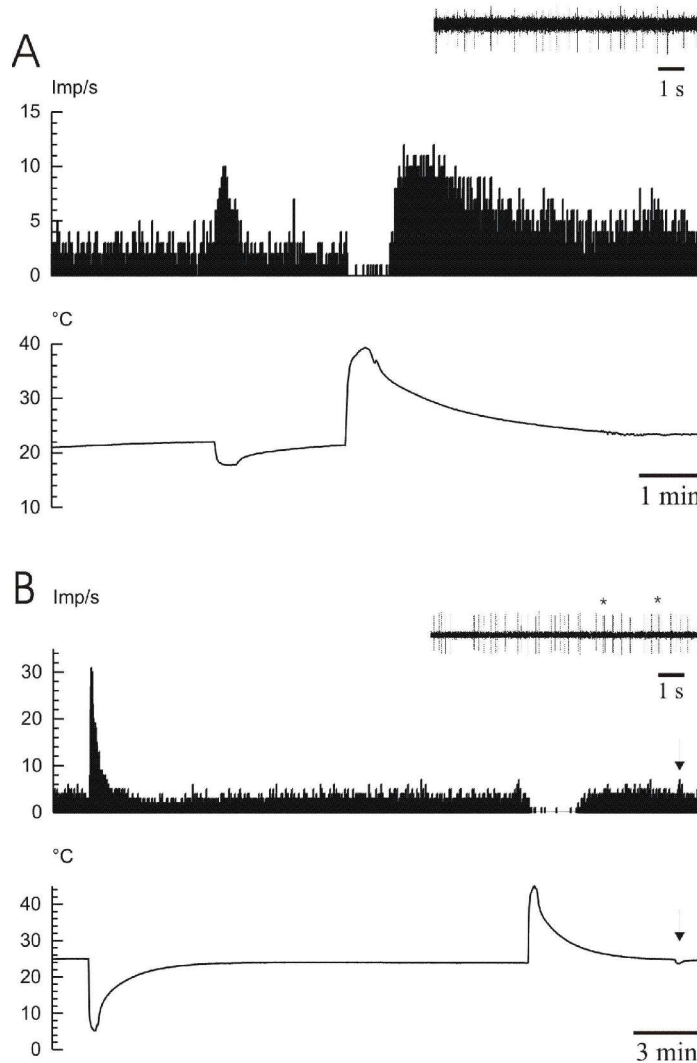


Fig. 3.11. Responses of two spontaneously active C-fibres to cooling or to heating of the nerve. A. The fibre is activated during cooling, depressed in its activity during heating and exhibited rebound activation after heating. Inset: initial spontaneous activity. B. The fibre is activated during cooling, the spontaneous activity is suppressed during heating and exhibit no rebound activation after heating. This fibre exhibited a high sensitivity to cold stimuli (see arrow) and some bursting discharges (see asterisks in inset).

Seventy-one per cent of these cold sensitive C-fibres (32/45 fibres) responded tonically during cold stimulation (Fig. 3.12). The responses of the remaining cold sensitive C-fibres were phasic, i.e. these fibres were only activated during lowering of the temperature. The peak rate of discharge during cooling ranged from 2 to 47 imp/s (mean = 8.1 ± 1.5 imp/s; N = 39).

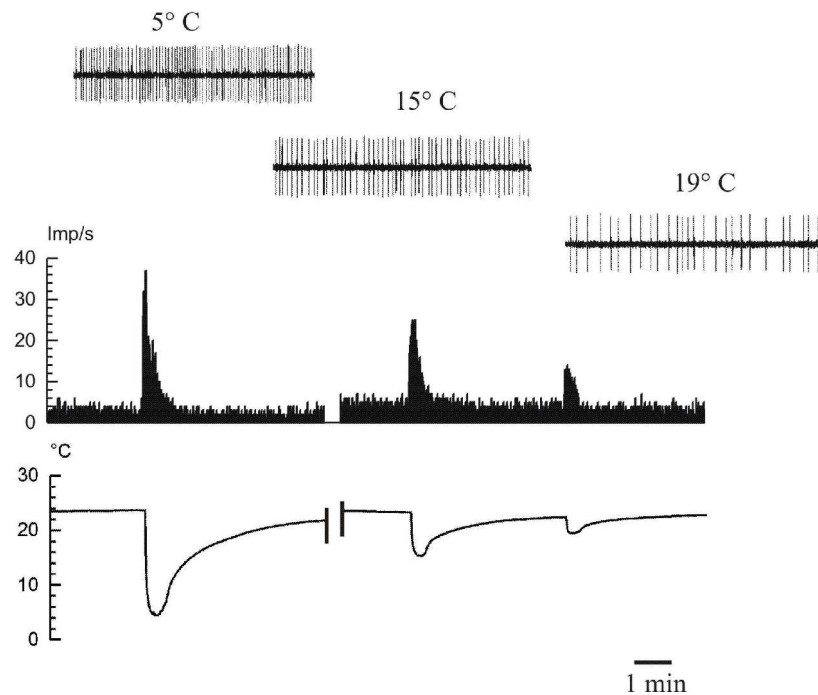


Fig. 3.12. Graded responses of a spontaneously active C-fibre to cooling of the nerve. Insets show original records of 2.5s duration during the peak temperatures of three cooling stimuli of different intensity.

Twenty-seven of 45 cold sensitive C-fibres exhibited also spontaneous activity (Table 3.4). In 11 of 27 spontaneously active cold sensitive C-fibres the ongoing activity was suppressed during heating of the nerve (Fig. 3.11B). Ten of them exhibited rebound excitation after nerve heating starting at temperatures which were in the range of 35 to 40°C (Fig. 3.11A) and this rebound excitation lasted for 2 to 6 minutes. In sixteen of the spontaneously active cold sensitive C-fibres the baseline activity did not change during heating.

Heat sensitivity Thirty-three of 94 thermosensitive unmyelinated fibres belonging to both groups of lesion were activated by heat stimuli and their activation thresholds ranged from 32 to 50°C. Nineteen per cent (6/31) of the heat sensitive fibres had low activation threshold being excited at temperatures of 25-29°C; 32% (10/31) were activated at temperatures of 30-39°C and 48% (15/31) responded when the temperature exceeded 40°C. Figure 3.13 shows an example of a heat sensitive C-fibre with high activation threshold. The peak discharge rates during heating ranged from 2 to 31 imp/s (mean = 8 ± 1.3 imp/s, N = 28).

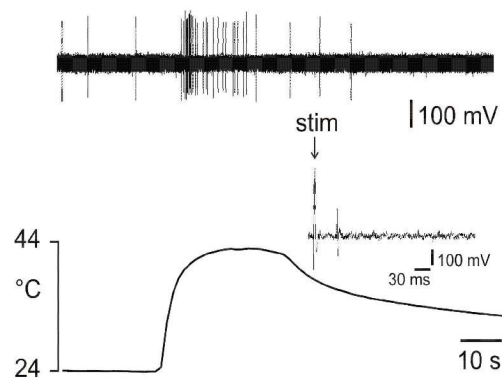


Fig. 3.13. Response of a regenerating afferent C-fibre to heating of the nerve. The C-fibre had spontaneous activity and exhibited a phasic-tonic response during heating. Inset: identification of the C-fibre by electrical stimulation of the sural nerve.

Cold and heat sensitivity. Sixteen of the unmyelinated fibres in both groups of lesion were activated both by cold and heat stimuli and exhibited the same response patterns to cooling or heating as those C-fibres which were activated by one of these stimuli only.

3.I.2. Characterisation of the lesioned and regenerated fibres 1 to 4 months post nerve injury

In 24 electrophysiological experiments performed 1 to 4 months post sural nerve crush (N=14) or sural nerve section and resuturing (N=10) 179 filaments were dissected from the proximal stump of the lesioned sural nerve. A total number of 364 afferent nerve fibres (220 A- and 144 C-fibres) which exhibited at least one discharge property (mechanosensitivity, thermosensitivity and/or spontaneous activity) were investigated.

3.I.2.1. Conduction velocities of the lesioned and regenerating (regenerated) nerve fibres

In 70 filaments, electrical stimulation was used in order to determine the conduction velocities of the lesioned and regenerated myelinated and unmyelinated nerve fibres. In the total sample, the conduction velocities of the *myelinated* fibres ranged from 2.1 to 80 m/s. Figure 3.14A illustrates the mean conduction velocities of the myelinated fibres which could be excited by electrical stimulation of the nerve via electrodes placed proximal or distal to the lesion site both in the crush and in section and resuturing group and the mean conduction velocity of the intact A-fibres (control group). Following *crush* lesion the conduction velocities of the A-fibres activated electrically from the distal stimulation electrodes did not differ significantly ($p>0.05$; *t*-test) from the conduction velocities of the A-fibres activated from the proximal stimulation electrodes but both were significantly lower ($p<0.05$; *t*-test) than the conduction velocities of the intact A-fibres (control group). After *section and resuturing* of the sural nerve the conduction velocities of the A-fibres excited both from proximal and distal stimulation electrodes were significantly lower than the conduction velocities of the intact A-fibres ($p<0.001$; *t*-test). The conduction velocities of the A-fibres activated from proximal or from distal stimulation electrodes were significantly lower ($p<0.01$; *t*-test) in the section and resuturing group than in the crush group.

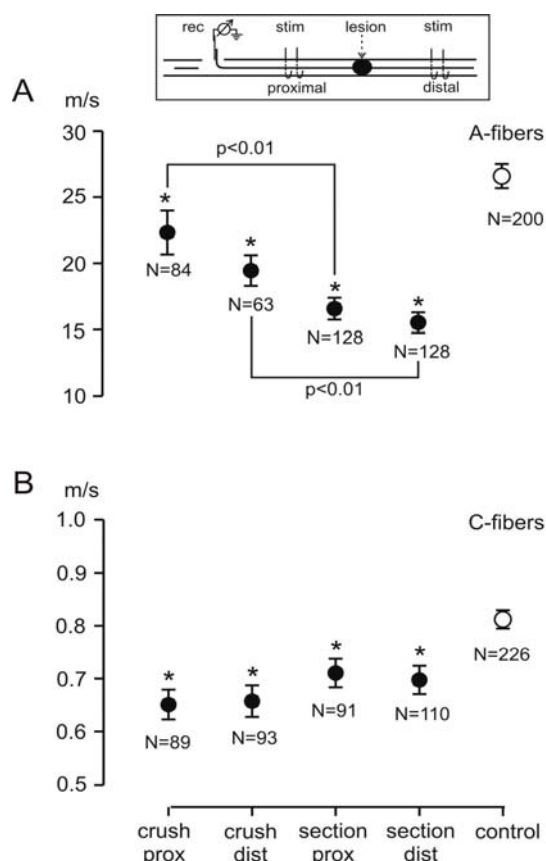


Fig. 3.14. Conduction velocities (mean \pm SEM) of the myelinated (A) and unmyelinated (B) afferent nerve fibres (in meters per second, m/s) in *crush* and *section and resuturing* group obtained by electrical stimulation from the electrodes placed *proximal* and *distal* to the lesion site. *Control* represents the mean conduction velocities of the A- and C-fibres recorded in the sural nerve of rats without nerve lesion. The asterisks indicate the conduction velocities of the lesioned myelinated (A) and unmyelinated (B) fibres which were statistically significantly lower ($p < 0.05$; *t*-test) than the conduction velocities of the intact A- and C-fibres, respectively (control group). The inset shows a schematic drawing of the sural nerve and the position of the proximal and distal stimulation electrodes and the recording electrode (rec).

In the total sample, the conduction velocities of the *unmyelinated* fibres varied between 0.2 and 2 m/s. Figure 3.14B shows that both following *crush* or *section and resuturing* of the sural nerve the conduction velocities of the C-fibres excited from the distal stimulation electrodes did not differ significantly from the conduction velocities of the C-fibres excited from the proximal stimulation electrodes but all of them were significantly lower ($p < 0.001$; *t*-test) than the conduction velocity of the intact C-fibres (control group).

3.I.2.2. Proportion of A- and C-fibres exhibiting discharge properties following sural nerve lesion

In 12 experiments performed 5 to 16 weeks post sural nerve lesion (crush or section and resuturing) the proportion of A- and C-fibres which exhibited discharge properties among the total number of electrically activated fibres was investigated. For this purpose there were analysed only those filaments in which the number of electrically activated A- or C-fibres could be determined. All electrically activated fibres were tested for their spontaneous activity and whether they responded to mechanical and/or thermal stimuli applied along the lesioned sural nerve and to the hind paw target tissues of the sural nerve territory. Among the total number of electrically activated *myelinated* fibres 39% of the units in the crush group and 37% of the units in section and resuturing group exhibited at least one discharge property, i.e. were spontaneously active, mechanosensitive and/or thermosensitive (Table 3.5). In the population of *unmyelinated* fibres, 29% to 41% of the electrically activated C-unit exhibited at least one discharge property (Table 3.5). No significant difference in the proportion of A- and C-fibres which exhibited these discharge properties was found between the two types of nerve lesion (crush versus section and resuturing).

Table 3.5

The proportion of myelinated and unmyelinated nerve fibres which exhibited one or more discharge properties among the total number of electrically activated fibres

	A-fibres	C-fibres
Crush	39% (20/51)	41% (43/104)
Section and resuturing	37% (46/126)	29% (45/156)

3.I.2.3. Discharge properties of A- and C-fibres

Among 364 afferent nerve fibres which exhibited at least one discharge property, 80% (173/215) of the A-fibres belonging to both groups could be activated by natural (mechanical and/or thermal) stimuli applied to the target tissues of the sural nerve territory

(Fig. 3.15A and B) whereas only 29% (37/126) of the C-fibres could be activated from the skin sural nerve territory (Fig. 3.19 A and B) ($p < 0.001$; χ^2 -test). The remaining myelinated and unmyelinated afferent fibres could be activated by natural (mechanical and/or thermal) stimuli applied along the lesioned sural nerve.

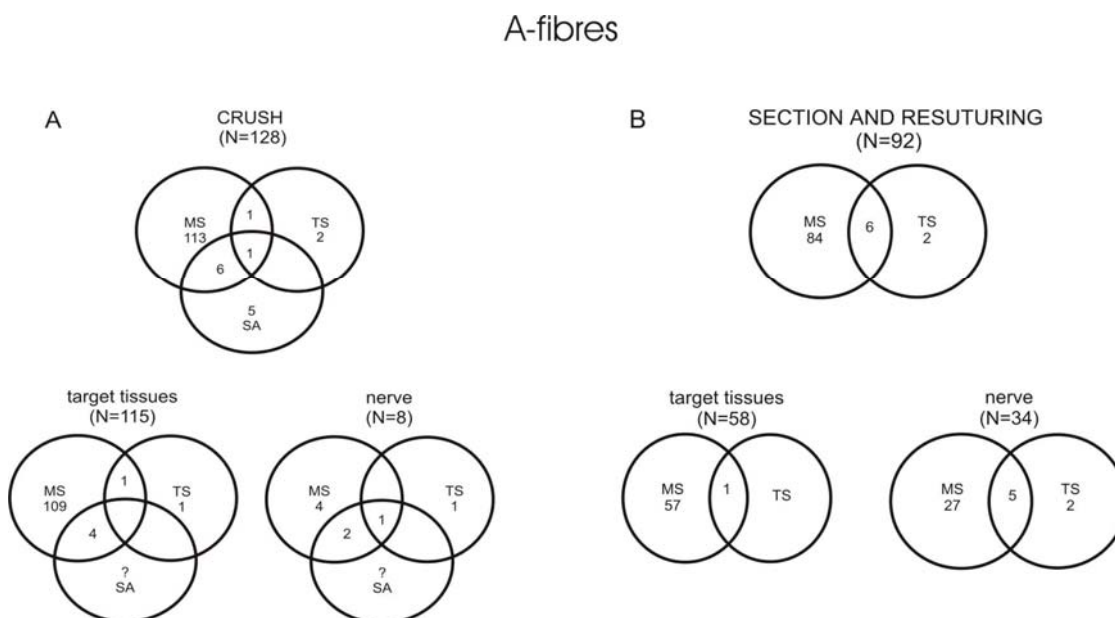


Fig. 3.15. Distribution of mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) of the A-fibres following *crush* (A) or *section and resuturing* (B) of the sural nerve. The overlaps represent the number of fibres which exhibit a combination of 2 respectively 3 of the mentioned discharge properties. The lower figures present the distribution of discharge properties of the A-fibres according to the receptive fields (*target tissues* or *nerve*) from where they could be mechanically and/or thermally activated. In the group *target tissues* are included also the fibres which had their receptive fields both in the lesioned sural nerve and in the target tissues of the sural nerve territory. (?) indicates that the receptive fields of the spontaneously active A-fibres in the crush group could not be identified.

3.1.2.3.1. Characterisation of the A-fibres

Most (94%; 206/220) A-fibres belonging to both groups of lesion exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active; the remaining fibres exhibited a combination of two or three discharge properties. The proportion of A-fibres which had successfully regenerated to the sural nerve territory (as judged by their responses to physiological stimuli applied to skin or subcutaneous tissues) was significantly higher following crush than following section and resuturing lesion (115/123 versus 58/92; $p < 0.001$; χ^2 -test) (Fig. 3.15A and B)

Spontaneous activity

After *crush* lesion 9% of the A-fibres investigated exhibited spontaneous activity. Most (7/12) of these spontaneously active A-fibres were also mechanosensitive and had their receptive fields in the deep somatic tissue (N=1), skin (N=2), nerve (N=3) or both nerve and skin (N=1). Five A-fibres were only spontaneously active and did not respond to mechanical or thermal stimuli applied to the lesioned sural nerve or to the target tissues of the hind paw; their receptive fields were not identified. The rate of spontaneous discharge ranged from 0.05 to 17.1 imp/s (mean = 3.86 ± 1.9 ; median = 0.86 imp/s; N=12). The pattern of discharge was irregular in all spontaneously active A-fibres investigated and showed no bursting activity. Following *section and resuturing* lesion, no spontaneously active A-fibre was found.

Mechanosensitivity

Ninety-five per cent of the A-fibres in the crush group and 98% of the A-fibres in the section and resuturing group responded to mechanical stimuli applied to the target tissues, nerve or both nerve and target tissues (Table 3.6). The mechanosensitive A-fibres that had regenerated and could be excited from the target tissues of the sural nerve territory were classified according to the receptive fields of their peripheral endings as cutaneous (hair follicles mechanoreceptors and skin mechanoreceptors) or subcutaneous (deep somatic tissues) receptors (Table 3.6). Figure 3.16A shows the distribution of the receptive fields of the mechanosensitive A-fibres following crush and section and resuturing lesion. Within 4 months post section and resuturing of the sural nerve 36% of the mechanosensitive A-fibres did not reach the target tissues and had their receptive fields distributed along the lesioned nerve whereas following crush lesion only 6% of the tested mechanosensitive A-fibres did not regenerate to the peripheral targets and could be excited by mechanical stimuli applied to the sural nerve ($p < 0.001$; Chi^2 -test). The remaining mechanosensitive A-fibres belonging to both groups of lesion had successfully regenerated and could be activated from the target tissues.

Table 3.6

Discharge properties of the injured A-fibres 1 to 4 months following crush or section and resuturing of the sural nerve

	CRUSH	SECTION AND RESUTURING		
Fibres with properties	128	92		
INDIVIDUAL DISCHARGE PROPERTIES				
Spontaneous activity	12 (9%)	-		
Mechanosensitivity	121 (95%)	90 (98%)		
Receptive fields				
Deep somatic tissue	31	8		
Skin	39	10		
Hair follicle	43	30		
Nerve	7	32		
Nerve and target tissue	1 [•]	10 [•]		
Thermosensitivity	4 (3%)	8 (9%)		
<i>Cold activated</i>	1	1		
<i>Heat activated</i>	3	7		
COMBINATION OF DISCHARGE PROPERTIES				
	Target tissues [•]	Nerve	Target tissues [•]	Nerve
Mechanosensitive/ Spontaneously active	109* / 4	4* / 2	57* / 0	27* / 0
Cold sensitive/ Spontaneously active	-	-	-	1* / 0
Heat sensitive/ Spontaneously active	1* / 0	1* / 0	-	1* / 0
Mechanical and cold sensitive/ Spontaneously active	1 / 0	-	-	-
Mechanical and heat sensitive/Spontaneously active	-	0 / 1	1 / 0	5 / 0
Spontaneous activity only	5*			-

* Afferent fibers with only one ectopic discharge property. In *combination of ectopic discharge properties* the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active

• The fibres which had their receptive fields both in the target tissues and in the nerve (crush N=1; section and resuturing N=10) had mechanosensitivity as unique discharge property and are included in the column *target tissues*.

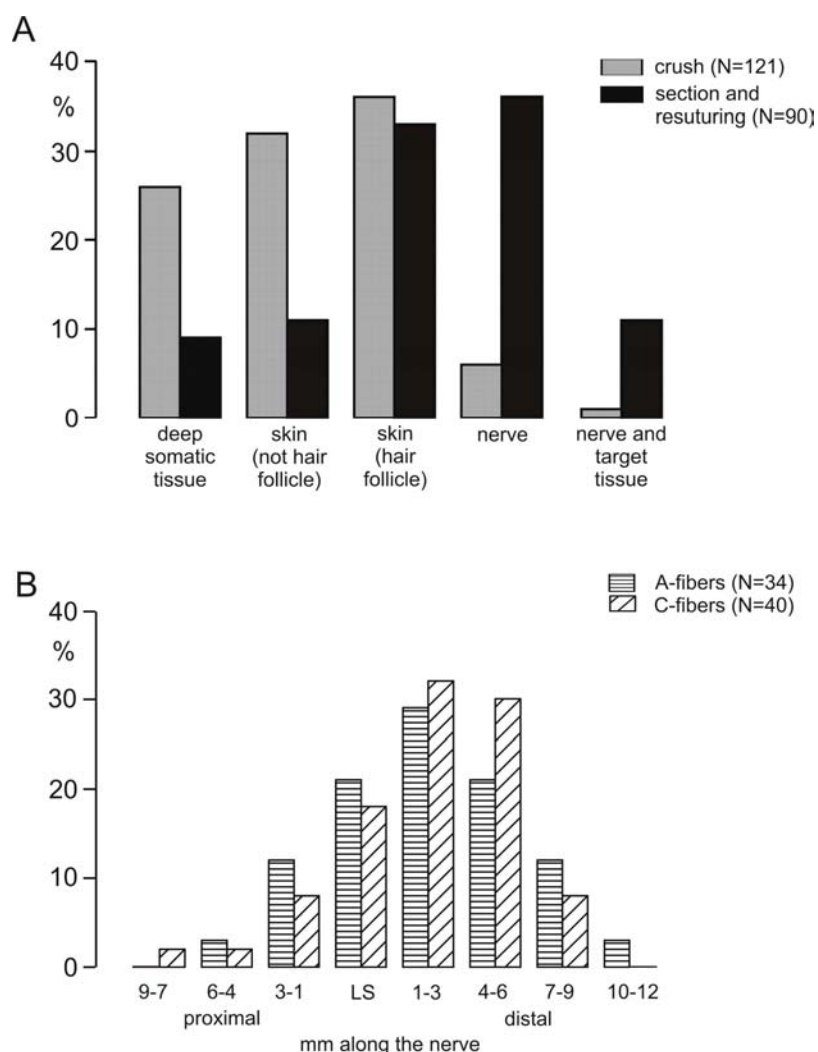


Fig. 3.16. A. Distribution of the receptive fields of the mechanosensitive A-fibers 1 to 4 months following *crush* or *section and resuturing* of the sural nerve. Within the considered time period the receptive fields of the mechanosensitive A-fibers were distributed in the *target tissues* (deep somatic tissue, skin, hair follicle) of the sural nerve territory, in the lesioned sural nerve or both in the nerve and target tissues. B. Distribution of the receptive fields of the injured A- and C-fibers along the lesioned sural nerve. Mechanical stimuli were applied at different sites along the nerve proximally and distally to the lesion site using a fine tipped glass rod. The histogram shows the distribution of the most distal/proximal point of the receptive fields. LS = lesion site

The mechanosensitive A-fibers which could be excited from the nerve had their receptive fields distributed along the path of regeneration from 6 mm proximal to 12 mm distal to the lesion site (Fig. 3.16 B).

Following section and resuturing lesion 11% of the mechanosensitive A-fibers could be excited by mechanical stimuli applied both to the lesioned sural nerve and to the target tissues of the sural nerve territory. This is demonstrated in figure 3.17 for one A-fibre which could be excited by mechanical stimuli applied both to the nerve and to the skin.

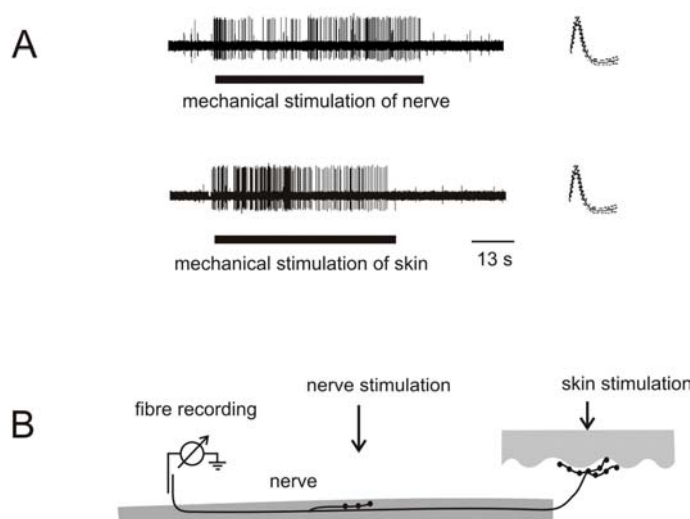


Fig. 3.17. A. Example of one silent mechanosensitive A-fibre which had distinct receptive fields both in the nerve and in the skin. Mechanical stimuli applied with a fine tipped glass rod to the lesioned sural nerve and to the skin of sural nerve territory led to the activation of the A-fibre. Records on the left show superimposed action potentials. B. Schematic presentation of the nerve preparation and stimulation sites.

The mechanosensitive A-fibres which had their receptive fields along the lesioned sural nerve or in the skin of the sural nerve territory responded phasically to mechanical stimulation and their mechanical thresholds ranged from 0.3 to 70 mN (mean = 12.5 ± 2.2 ; median = 7.5 mN; N=62; Fig. 3.18).

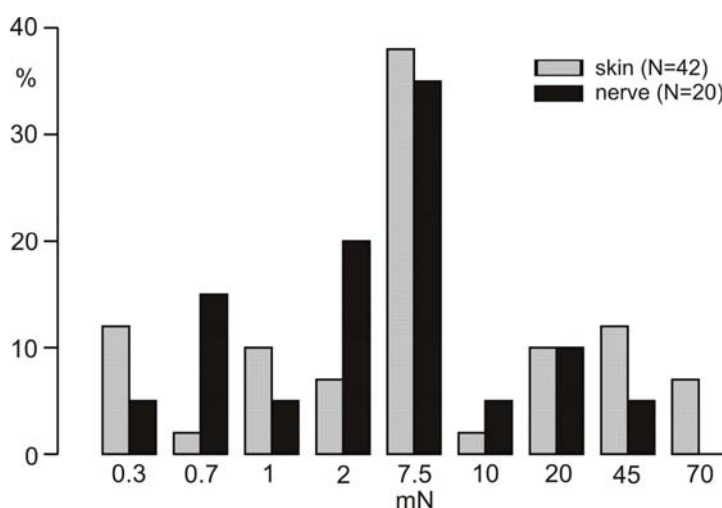


Fig. 3.18. Distribution of mechanical thresholds (in mN) of injured A-fibres following *crush* or *section and resuturing* lesion. Mechanical thresholds were measured with calibrated von Frey filaments with different bending forces. The mechanical thresholds of the injured A-fibres were not significantly different between the two types of nerve lesion. Grey bars: mechanical thresholds of the lesioned mechanosensitive A-fibres which had regenerated and reached the *skin*; black bars: mechanical thresholds of the lesioned mechanosensitive A-fibres which had their receptive fields distributed along the lesioned *nerve*.

No significant difference in mechanical thresholds (sensitivity) of the myelinated fibres was found between the two types of nerve lesion (crush versus section and resuturing). The mechanosensitive A-fibres which had regenerated and reached the skin had a significantly higher threshold than those which had their receptive fields along the lesioned sural nerve (mean = 14.6 ± 2.9 versus 7.9 ± 2.3 mN; $p < 0.05$; Wilcoxon test).

The lesioned A-fibres which had regenerated and reinnervated the hair follicles responded phasically, with brief bursts of discharges to repetitive mechanical stimulation, their pattern of discharge being similar to that of the units in the intact sural nerve territory (control experiments). The hair follicle units had very low activation thresholds being excited by light movements of the hairs.

The mechanosensitive units which had successfully regenerated to the deep somatic tissues of the sural nerve territory exhibited tonic discharges during constant mechanical stimulation. Their discharge pattern was similar to that of the units in the intact sural nerve territory.

Thermosensitivity

Three per cent of the A-fibres in the crush group and 9% of the A-fibres in the section and resuturing group reacted to thermal stimuli applied to the lesioned sural nerve ($n=9$) or to the skin of sural nerve territory ($n=3$). The majority (10/12) of the myelinated fibres belonging to both groups of lesion were excited by heat stimuli and were activated at temperatures exceeding 40 - 44°C. Two A-fibres were cold sensitive.

3.I.2.3.2. Characterisation of the C-fibres

The pattern of discharge properties in afferent C-fibres was significantly different from the pattern in A-fibres as far as the distribution of spontaneous activity, mechanical sensitivity and thermal sensitivity is concerned. Forty-eight per cent (69/144) of the C-fibres belonging to both groups of lesion exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active; the remaining fibres had a combination of two or three discharge properties (37% respectively 15%) (Fig. 3.19). The proportion of C-fibres which succeeded to regenerate and could be activated mechanically and/or thermally from the skin of the sural nerve territory was significantly higher

following crush than following section and resuturing of the sural nerve (23/59 versus 14/67; $p < 0.05$; Chi^2 -test) (Fig. 3.19A and B).

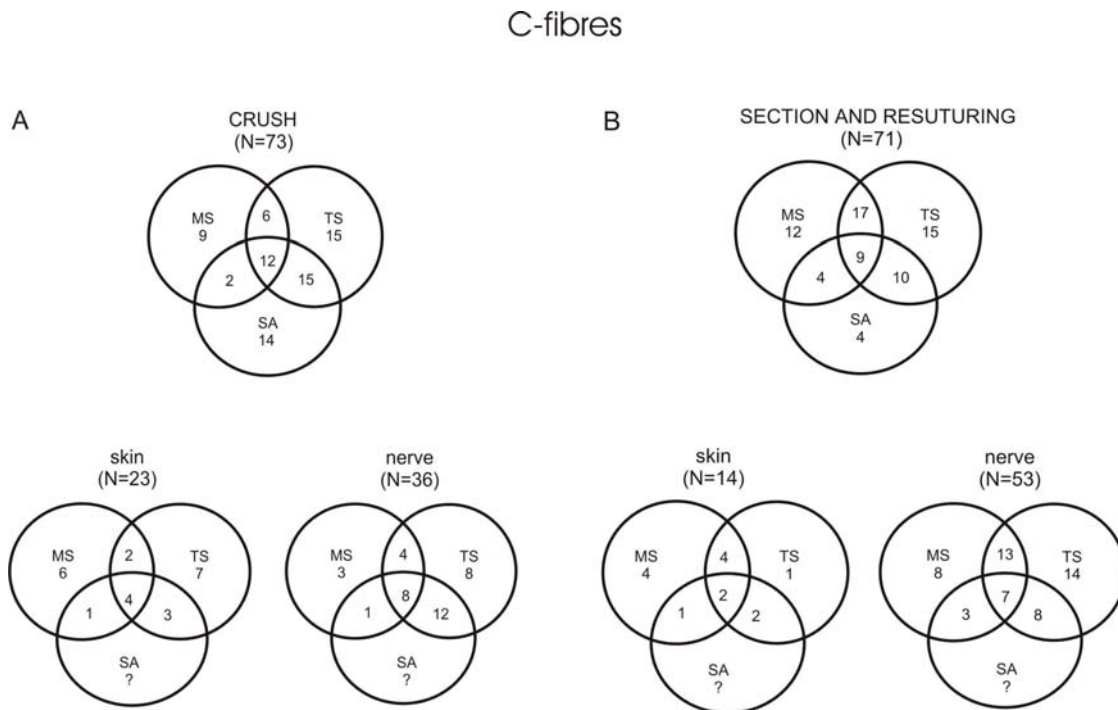


Fig. 3.19. Distribution of mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) of the C-fibres following *crush* (A) or *section and resuturing* (B) of the sural nerve. The overlaps represent the number of fibres which exhibit a combination of 2 respectively 3 of the mentioned discharge properties. The lower figures present the distribution of discharge properties of the C-fibres according to the receptive fields (*skin* or *nerve*) from where they could be mechanically and/or thermally activated. In the group *skin* are included also the fibres which had their receptive fields both in the sural nerve and in the target tissues of the sural nerve territory. (?) indicates that the receptive fields of the spontaneously active C-fibres could not be identified.

Spontaneous activity

Fifty-nine per cent of the C-fibres in the crush group and 38% of the C-units in the section and resuturing group exhibited spontaneous activity ($p < 0.05$; Chi^2 -test). Most spontaneously active C-fibres (52/70) belonging to both lesion groups reacted also to natural (mechanical and/or thermal) stimuli and had their receptive fields along the lesioned sural nerve, in the skin of the sural nerve territory or both in the nerve and skin. The proportion of spontaneously active C-fibres which had their receptive fields in the nerve was significantly higher than the proportion of spontaneously active C-fibres which could be activated from the skin (39/52 versus 13/52; $p < 0.001$; Chi^2 -test).

The rates of spontaneous discharge in the injured C-fibres belonging to both groups of lesion ranged from 0.03 to 9.5 imp/s (mean = 1.01 ± 0.2 ; median = 0.46 imp/s; N=65) (Fig. 3.20).

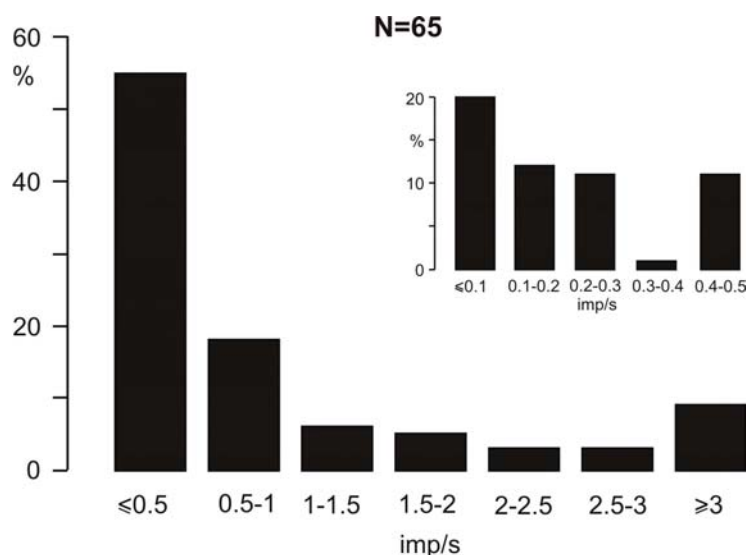


Fig. 3.20. Distribution of rates of spontaneous activity (in impulse per second, imp/s) in the lesioned unmyelinated nerve fibres following *crush* or *section and resuturing* of the sural nerve (N=65). There was no significant difference in the discharge rates of spontaneously active C-fibres between the two types of nerve lesion. The inset shows the distribution of spontaneous activity in the C-fibres which fire at rates of ≤ 0.5 imp/s.

No significant difference in rate and pattern of discharge of the unmyelinated fibres was found between the two types of nerve lesion (crush versus section and resuturing) and between the spontaneously active C-fibres which could be activated by mechanical or thermal stimuli applied to the skin and those which had their receptive fields in the lesioned sural nerve. Eighteen C-fibres (14 of them after crush and 4 after section and resuturing) were spontaneously active only and could not be excited by mechanical or thermal stimuli applied to the lesioned sural nerve or to the cutaneous sural nerve territory of the hind paw.

Mechanosensitivity

Mechanosensitivity was present in 40% of the C-fibres in the crush group and 59% of the C-units in the section and resuturing group ($p < 0.05$; χ^2 -test). The receptive fields of the mechanosensitive C-fibres were distributed along the lesioned sural nerve, in the skin territory of sural nerve innervation or both in the nerve and skin (Table 3.7).

Table 3.7

Discharge properties of the injured C-fibres 1 to 4 months following crush or section and resuturing of the sural nerve

	CRUSH			SECTION AND RESUTURING		
Fibres with properties	73			71		
INDIVIDUAL DISCHARGE PROPERTIES						
Spontaneous activity	43 (59%) ^a			27 (38%) ^a		
Mechanosensitivity	29 (40%) ^b			42 (59%) ^b		
<i>Receptive Fields</i>						
Skin	9			5		
Nerve	16			31		
Skin and nerve	4			6		
Thermosensitivity	48 (66%)			51 (72%)		
<i>Cold activated</i>	13			21		
<i>Heat activated</i>	31 ^c			19 ^c		
<i>Cold and heat activated</i>	4			11		
<i>Receptive Fields</i>						
<i>Skin</i>	9			3		
<i>Nerve</i>	32			42		
<i>Skin and nerve</i>	7			6		
COMBINATION OF DISCHARGE PROPERTIES						
	Skin	Nerve	Skin and nerve	Skin	Nerve	Skin and nerve
Mechanosensitive/ Spontaneously active	6* / 1	3* / 1	-	3* / 0	8* / 3	1* / 1
Cold sensitive/ Spontaneously active	0 / 3	2* / 5	1* / 0	1* / 0	7* / 6	0 / 1
Heat sensitive/ Spontaneously active	4* / 0	6* / 6	1* / 0	-	4* / 2	-
Cold and heat sensitive/ spontaneously active	-	0 / 1	1 / 0	-	3 / 0	0 / 1
Mechanical and cold sensitive/ Spontaneously active	0 / 1	0 / 1	-		2 / 3	1 / 0
Mechanical and heat sensitive/ Spontaneously active	-	4 / 7	1 / 2	1 / 1	6 / 2	2 / 1
Mechanical, cold and heat sensitive/ Spontaneously active	0 / 1	-	1 / 0	-	5 / 2	-
Spontaneous activity only	14*			4*		

* Afferent fibers with only one ectopic discharge property. Letters *a*, *b*, *c* indicate the discharge properties which are significantly different ($p < 0.05$; Chi^2 -test) between the two types of nerve lesion (crush versus section and resuturing).

In *combination of ectopic discharge properties* the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active

Following crush lesion 45% of the mechanosensitive C-fibres had successfully regenerated and reached the skin whereas in the section and resuturing group 26% of the mechanosensitive C-fibres could be excited from the target tissue ($p > 0.05$; Chi^2 -test). The majority (90%) of the mechanosensitive C-fibres which had regenerated and reached the skin could be activated by von Frey filaments with bending forces ≥ 100 mN or by noxious pinch stimulation. They responded phasically, with brief bursts of discharges to pinch stimuli (Fig. 3.21A).

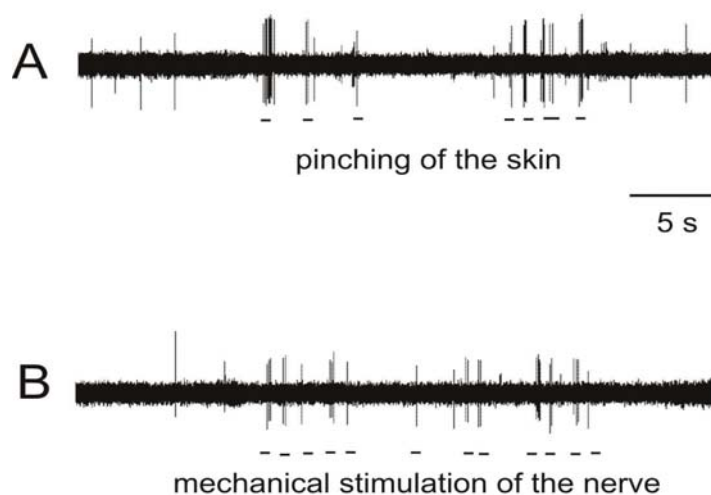


Fig. 3.21. Responses of two mechanosensitive unmyelinated fibres. A. C-fibre which responded with brief discharges to noxious stimulation (pinching) of the skin. B. Silent C-fibre which responded phasically to mechanical stimulation of the nerve at a frequency of 0.5 Hz.

The mechanosensitive C-fibres which could be excited from the lesioned sural nerve had their receptive fields distributed mainly distal to the injury, up to 9 mm remote from the lesion point (Fig. 3.16B). Most (55%) of the mechanosensitive C-fibres activated from the nerve had a high mechanical threshold and could be excited only by stimuli applied with a fine tipped glass rod (Fig. 3.21B). The remaining mechanosensitive C-fibres were activated by von Frey filaments with bending forces ranging from 2 to 10 mN.

Thermosensitivity

Thermosensitivity was the main property of the lesioned and regenerating unmyelinated fibres; 66% of the C-fibres in the crush group and 72% in the section and resuturing group reacted to thermal (cold or/and heat) stimuli.

Cold sensitivity. Thirty-four of 99 thermosensitive C-fibres belonging to both groups of lesion reacted to cold stimuli applied to the lesioned sural nerve (N=26), skin of the sural nerve territory (N=5) or both to the nerve and skin (N=3). The majority (82%) of the cold sensitive C-fibres (which had their receptive fields either in the nerve or in the skin) reacted to cooling steps $<10^{\circ}\text{C}$, some of them being activated when the temperature decreased by $2\text{-}3^{\circ}\text{C}$ from baseline (Fig. 3.22A).

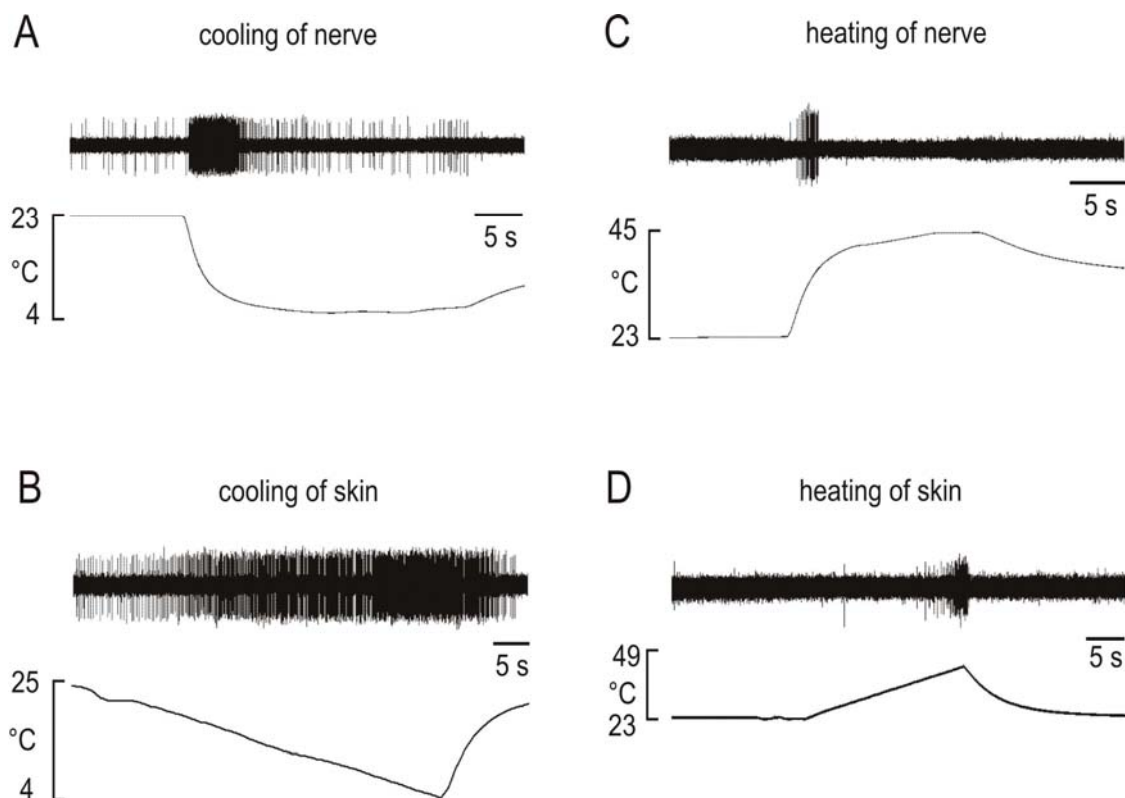


Fig. 3.22. Responses of 4 C-fibres to thermal (cold or heat) stimulation of the nerve or of the skin. A, B: Two spontaneously active C-fibres which are activated by cold stimuli applied to the nerve (A) or to the skin (B). The C-fibre activated from the *nerve* had a high sensitivity (low threshold) and was activated when the temperature on the nerve decreased by $2\text{-}3^{\circ}\text{C}$ from a baseline of 23°C . B. The C-fibre excited from the skin was activated at 17°C . C, D: Two silent C-fibres activated by heat stimuli from the nerve (C) respectively from the skin (D).

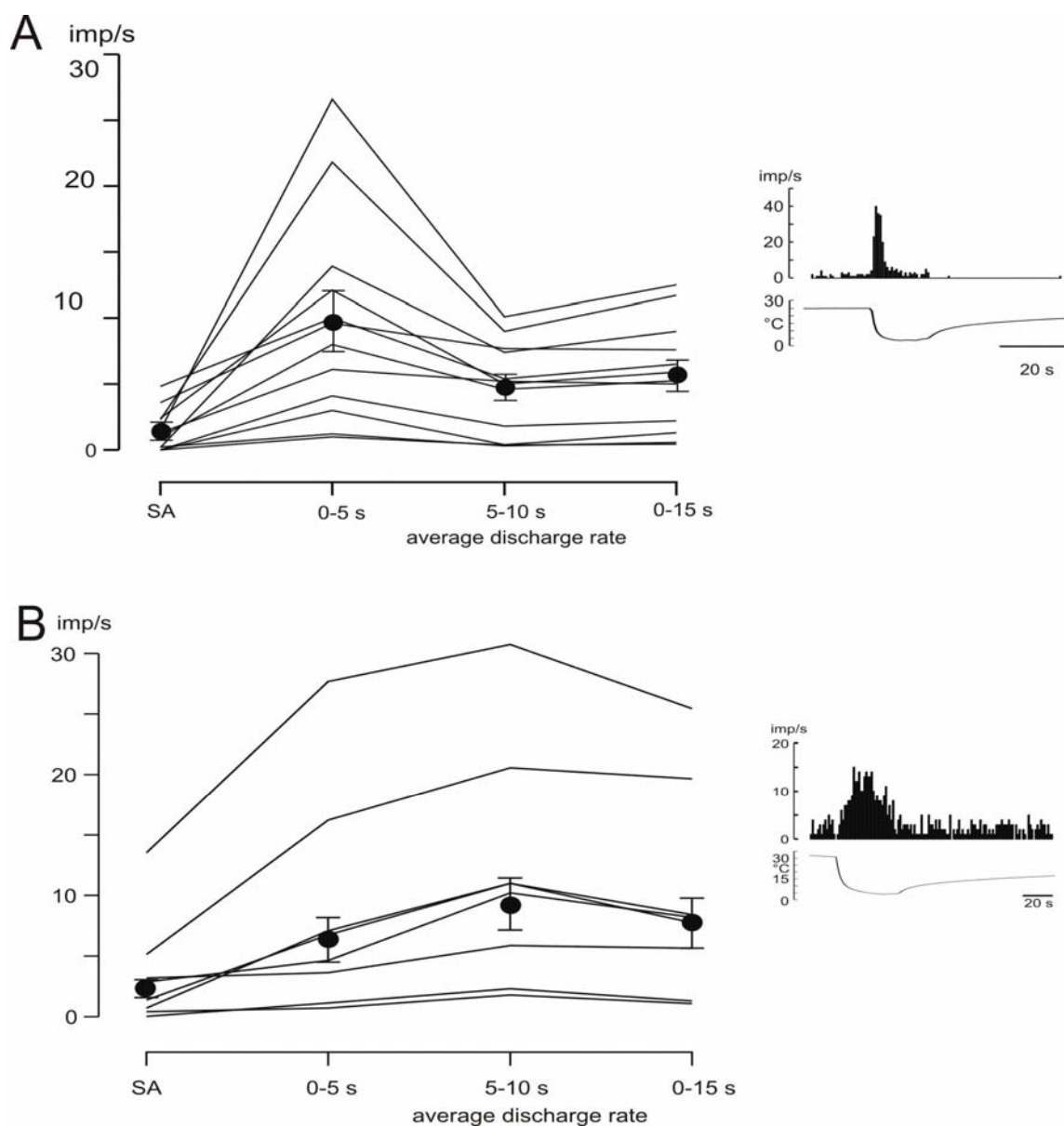


Fig. 3.23. Responses of thermosensitive C-fibres which had their receptive fields along the lesioned sural nerve to cold stimulation. The fibres were divided according to their discharge profiles (see insets) in two categories: A: Fibres which had the maximal discharge rates of response during the first 5s of cold stimulation, before the lowest temperature of the stimulus was reached (N = 12); B: Fibres which had the maximal discharge rates of response 5-10s after beginning of stimulation, when the thermal stimulus reached a plateau (N = 8). The main graphs present the mean discharge rates of individual C-fibres during the first 0-5s of thermal stimulation (dynamic phase), next 5-10s (static phase) and during a period of 15s of thermal stimulation (0-15s). SA (spontaneous activity) represents the initial discharge rates recorded during 30-60 s before application of a thermal stimulus. The insets show the time histogram of two spontaneously active cold sensitive C-fibre which had the maximal discharge frequency during the dynamic phase of stimulation (A) or during the static phase of stimulation (plateau 5°C) (B). The mean discharge rates (\pm SEM) during 0-5s, 5-10s and 0-15s of the thermal stimulation were obtained by averaging the discharge rates of all individual fibres in A and B, respectively.

The discharge profiles of 20 cold sensitive C-fibres which had their receptive fields on the lesioned sural *nerve* were investigated quantitatively (Fig. 3.23 A and B). Twelve of them reacted phasically during cold stimulation of the nerve. In these fibres the responses to cold stimuli started with an initial high frequency of discharge which declined as the stimuli were maintained (see inset Fig. 3.23A). Eight units discharged tonically during cold stimulation and had the maximal discharge rates 5-10s after beginning of stimulation when or after the cold stimulus reached the lowest temperature (Fig. 3.23B).

The cold sensitive C-fibres which had regenerated and reached the *skin* were activated at temperatures ranging from 22 to 10°C, starting from a baseline temperature of 23°C. Figure 3.22B shows an example of a cold sensitive C-fibre which exhibited a graded response during cold stimulation, the rate of discharge increased gradually when the temperature of the stimulus decreased and was maximal when the stimulus reached the lowest temperature.

The maximal discharge rates during cold stimulation ranged between 1 and 39 imp/s (mean = 10.8 ± 1.8 ; median = 10; N=23) and no significant difference in the discharge rate was found between the fibres which were activated from the lesioned nerve and those which had regenerated and were activated from the skin of the sural nerve territory.

Twenty of 34 cold sensitive C-fibres which were activated by cold stimuli exhibited also spontaneous activity and most (15/20) of them had the receptive field on the nerve.

Heat sensitivity. Fifty of 99 thermosensitive C-fibres reacted to heat stimuli, the proportion of C-fibers which were heat sensitive being higher following crush than following section and resuturing lesion (31/48 versus 19/51; $p < 0.01$; Chi^2 -test). The receptive fields of the heat sensitive fibres belonging to both groups of lesion were distributed along the lesioned sural nerve (N=37), in the skin of sural nerve territory (N=6) or both in the nerve and skin (N=7). The activation thresholds of the heat sensitive C-fibres which had their receptive fields on the *nerve* ranged from 25 to 48°C (N=31). Most of them (21/31) were activated at temperatures $\leq 39^\circ\text{C}$ and the remaining fibres reacted when the temperature on the nerve exceeded 40°C (Fig. 3.24B).

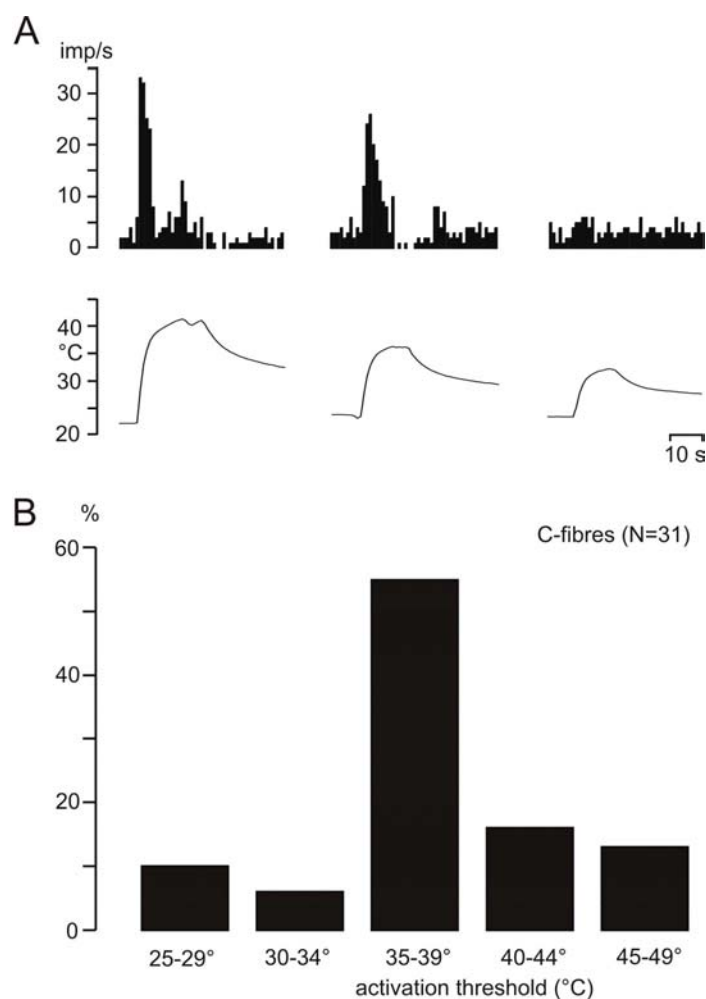


Fig. 3.24. A. Response of a thermosensitive C-fibre to different degrees of heating of the nerve; the fibre is activated by stimuli of 41°C and 36°C but not by a stimulus of 32°C. B. Distribution of activation threshold of the heat sensitive C-fibres which had the receptive fields along the lesioned nerve.

The discharge profiles of 42 heat sensitive C-fibres which were activated by stimuli applied to the *nerve* were investigated. Among these, 19 fibres had the highest frequency of discharge during the dynamic phase of heat stimulation (Fig. 3.25A) and 13 had the maximal discharge during the plateau (Fig. 3.25B). The remaining 10 fibres exhibited constant discharge rates during heat stimulation.

The heat sensitive C-fibres which succeeded to regenerate and had reached the *skin* were activated at temperatures ranging between 41 and 47°C and responded phasically during heat stimulation (Fig. 3.22D). The maximal rate of discharge during heating ranged from 2 to 54 imp/s (mean = 15.2 ± 2.2 ; median = 10; N=43). Twenty-one of 50 heat sensitive C-fibres were also spontaneously active and most (17/21) had their receptive field on the nerve.

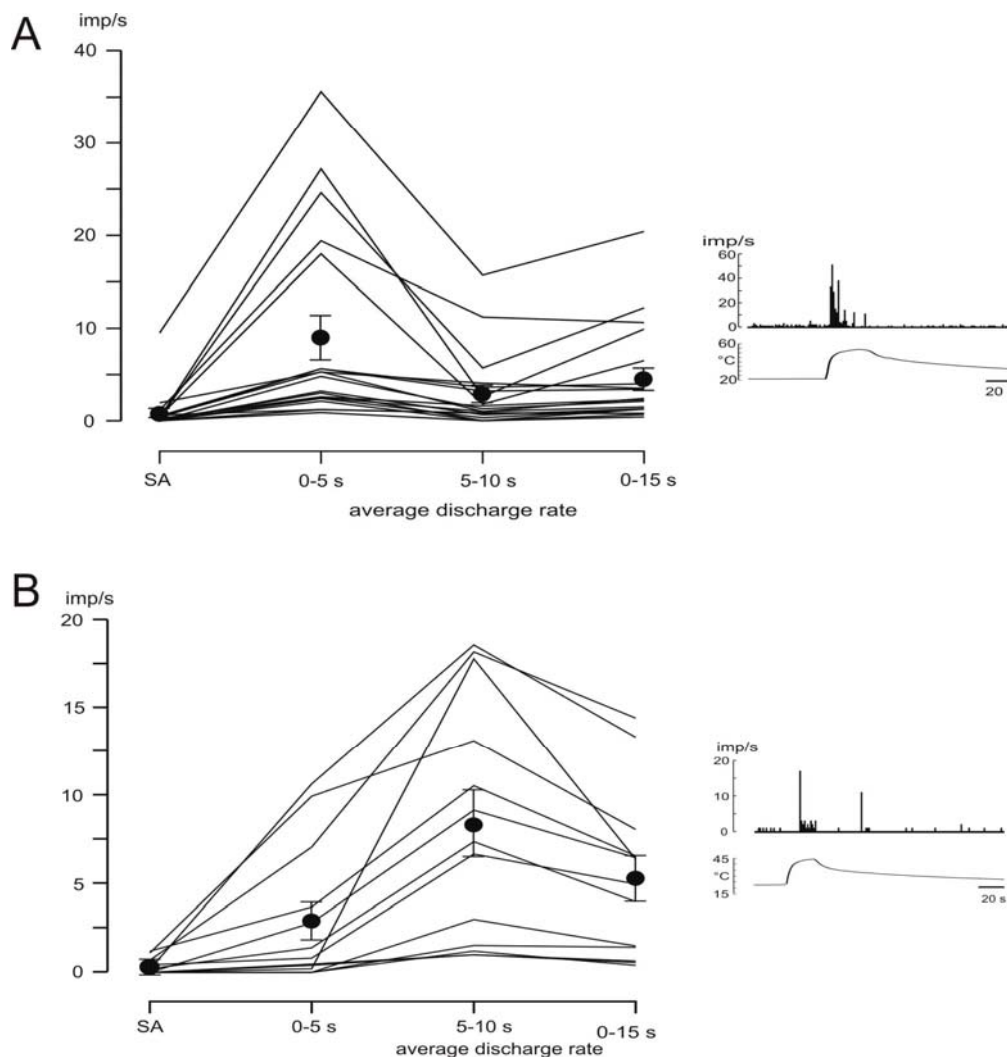


Fig. 3.25. Responses of thermosensitive C-fibers which had their receptive fields along the lesioned sural nerve to heat stimulation. The fibres were divided according to their discharge profiles (see insets) in two categories: A: Fibres which had the maximal discharge rates of response during the first 5s of heat stimulation, before the highest temperature of the stimulus was reached (N = 19); B: Fibres which had the maximal discharge rates of response 5-10s after beginning of stimulation, when the thermal stimulus had reached a plateau (N = 13). The main graphs present the mean discharge rates of individual C-fibers during the first 0-5s of thermal stimulation (dynamic phase), next 5-10s (static phase) and a period of 15s of thermal stimulation (0-15s). SA (spontaneous activity) represent the initial discharge rates recorded during 30-60 s before application of the thermal stimulus. The insets show the time histogram of two heat sensitive C-fibers which had the maximal discharge frequency during the dynamic phase of stimulation (A) or during the static phase of stimulation (plateau 45°C) (B). The mean discharge rates (\pm SEM) during 0-5s, 5-10s and 0-15s of thermal stimulation were obtained by averaging the discharge rates of all individual fibres in A and B, respectively.

Cold and heat sensitivity: Fifteen of 99 C-fibers responded both to cold and heat stimuli applied to the nerve (N=11), skin (N=3) or both nerve and skin (N=1). The C-fibers which were activated by both cooling and heating exhibited the same response pattern to cooling or heating as those C-fibers which were only activated by one of these stimuli.

3.II. SPARED NERVE INJURY MODEL

In the Spared Nerve Injury (SNI) model the tibial and common peroneal nerves were ligated and sectioned sparing the sural nerve. This nerve lesion model was used to study the behavioural changes to mechanical and thermal stimulation of the sural nerve territory and the electrophysiological changes of the intact sural afferent nerve fibres.

3.II.1. Behavioural studies

Signs of "pain-like" behaviour reflected in changes in mechanical and thermal sensitivity were investigated in 23 rats which underwent Spared Nerve Injury. Mechanical and thermal stimuli were applied to the sural and tibial nerve territory of the hind paws ipsi- and contra-lateral to the lesion side. Following SNI the rats exhibited a marked sensory hypersensitivity of the sural nerve territory ipsilateral to the nerve injury to innocuous mechanical and cold stimuli. This increased sensitivity was present in every tested rat. It developed in less than 24 hours after nerve lesion and continued to be present as long as the animals were monitored. Here I will call this increased sensitivity to mechanical and cold stimuli mechanical and cold allodynic behaviour, respectively.

3.II.1.1. Mechanical sensitivity

Paw withdrawal thresholds to mechanical stimuli applied with von Frey filaments to the hind paws ipsi- and contralateral to the lesion side were stable during the 3 days of baseline testing prior to the nerve lesion (mean = 143 ± 3.8 mN). After SNI the paw withdrawal threshold to mechanical stimulation of the *ipsilateral sural* nerve territory decreased significantly from 144 mN before lesion to 20 mN on day one post injury ($p < 0.001$) and remained at a similar decreased level for up to 55 days post injury (15 mN; $p > 0.05$) (Fig. 3.26A).

Mechanical sensitivity of the *contralateral sural* nerve territory did not change significantly from pre-surgery baseline until day 27 post lesion (134 mN versus 110 mN; $p > 0.05$). On day 34 post injury the paw withdrawal threshold was significantly lower in comparison to baseline (69 mN versus 134 mN; $p < 0.05$) and it dropped down to 35 mN until day 55 post lesion (Fig. 3.26A).

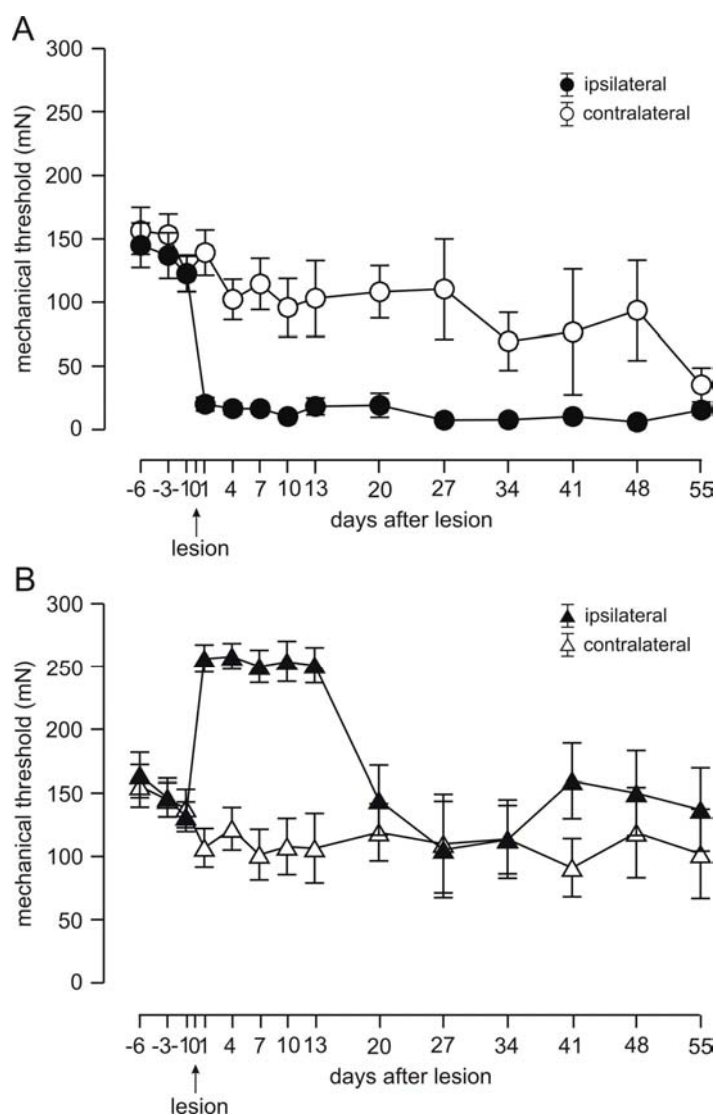


Fig. 3.26. Paw withdrawal thresholds to mechanical stimulation with von Frey filaments of the *sural* (A) and *tibial* (B) nerve territories (see Fig. 2.1) following spared nerve injury (SNI) in rats. A. Paw withdrawal threshold to mechanical stimulation of the *sural* nerve territory on the *ipsilateral* (lesioned) side significantly decreased ($p < 0.001$) starting on day one post lesion. This decrease persisted for 55 days after lesion. Paw withdrawal thresholds to mechanical stimulation of the *sural* nerve territory *contralateral* to the lesion side had a tendency to decrease over the whole period of investigation and at 55 days post lesion reached a value not significantly different ($p > 0.05$) from that measured on the ipsilateral paw. B. SNI induced a significant ($p < 0.005$) increase of paw withdrawal threshold to mechanical stimulation of the *tibial* nerve territory *ipsilateral* to the lesion side starting on day one post lesion and persisting 20 days post nerve injury. Paw withdrawal threshold to mechanical stimulation of the *tibial* nerve territory *contralateral* to the lesion side did not show any significant change from pre-surgery baseline following SNI. Number of rats used for behavioural testing: N=23 (days -6, -3, -1, 1 and 4); N=17 (day 7); N=15 (day 10); N=7 (day 13); N=10 (days 20 and 34); N=12 (day 41) and N=8 (days 48 and 55).

Mechanical sensitivity of the *ipsilateral tibial* nerve territory was significantly reduced starting on day one post lesion due to denervation of the tibial territory following SNI. The paw withdrawal threshold to mechanical stimuli applied to the ipsilateral tibial nerve

territory increased significantly ($p < 0.005$) from 147 mN before lesion to 256 mN on day 1 post injury. This hypo-sensitivity persisted until day 20 post lesion when the mechanical threshold of the middle plantar territory had returned almost to the same value (144 mN) as before lesion and remained at a similar level until the end of the investigation period (Fig. 3.26B).

Mechanical sensitivity of the *contralateral tibial* nerve territory did not demonstrate any statistically significant change from pre-surgery baseline over the whole period of investigation (Fig. 3.26B).

3.II.1.2. Cold sensitivity

Before the SNI application of an innocuous cold stimulus (acetone drop) to the sural or tibial nerve territory evoked no flexor response. Starting on day 1 post SNI the rats developed a marked *cold* hypersensitivity of the *ipsilateral sural* nerve territory reflected in an increased duration (9s) of paw withdrawal in response to acetone application. The duration of paw withdrawal in response to acetone stimulation increased significantly ($p < 0.005$) up to 17s until day 13 post lesion. This cold hypersensitivity was maintained until day 55 post lesion when the acetone continued to elicit a sustained flexor response indicative of an abnormal responsiveness to a normally innocuous stimulus (Fig. 3.27A). Following SNI acetone stimuli applied to the *sural* nerve territory of the hind-paw *contralateral* to the lesion side evoked no flexor response or the response was shorter than 0.5s (Fig. 3.27A).

3.II.1.3. Heat sensitivity

Before SNI radiant heat stimuli applied to the *sural* nerve territory elicited a paw withdrawal reaction with a mean latency of 10s. Following SNI the paw withdrawal latency to heat stimulation of *ipsi-* and *contralateral* sural nerve territory did not show any statistical significant change from pre-surgery baseline over the whole period of investigation (Fig. 3.27B).

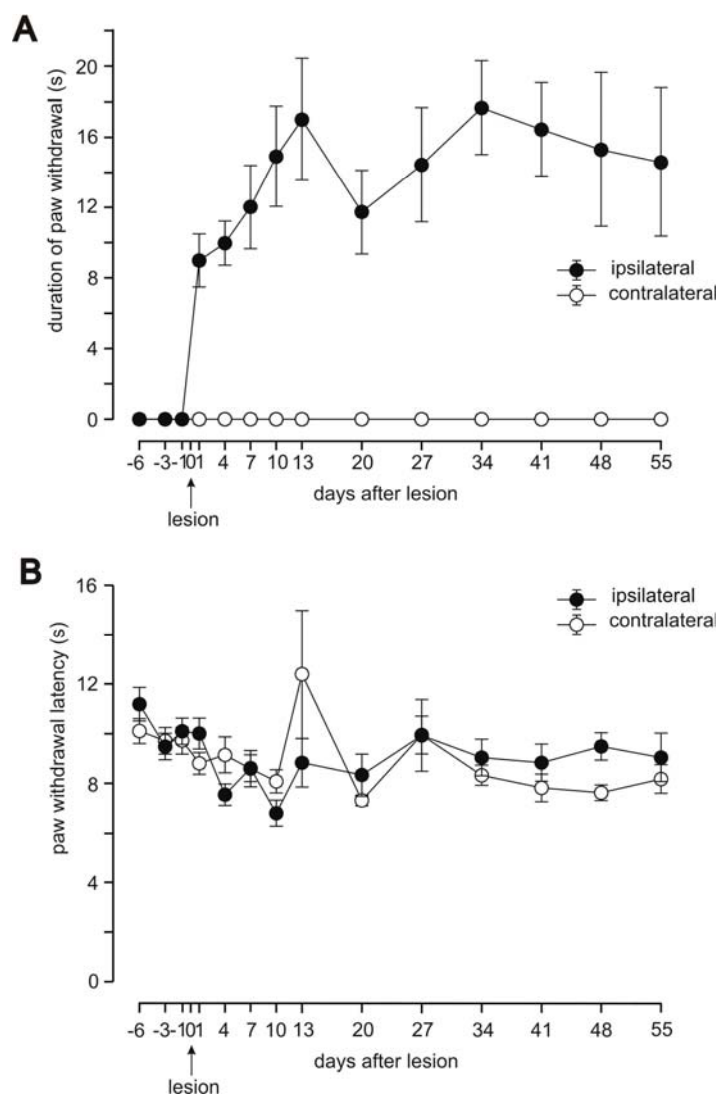


Fig. 3.27. Cold (A) and heat (B) sensitivity of the sural nerve territory ipsilateral (filled symbols) and contralateral (open symbols) to the lesion side following spared nerve injury (SNI) in rats. A. Duration of paw withdrawal following application of an acetone drop. Rats which underwent SNI exhibited an increased cold sensitivity of the *ipsilateral sural* nerve territory which started on day 1 post lesion and persisted during the whole period of testing. Stimulation of the *sural* nerve territory *contralateral* to the lesion site with acetone did not elicit paw withdrawals longer than 0.5s. B. Latency of paw withdrawal in response to heat stimulation of the *ipsi-* and *contralateral sural* nerve territory did not change significantly in comparison to pre-surgery baseline over the whole period of investigation. Number of rats used for behavioural testing: N=23 (days -6, -3, -1, 1 and 4); N=17 (day 7); N=15 (day 10); N=7 (day 13); N=10 (days 20 and 34); N=12 (day 41) and N=8 (days 48 and 55).

3.II.2. Neurophysiological investigations

In order to investigate the neurophysiological changes in the intact sural afferent nerve fibres following the injury of the neighbouring nerves electrophysiological experiments were performed at different time intervals: 1 day to 1 week (I period), 2 to 4 weeks (II

period) and 7 to 10 weeks (III period) post SNI. In total 356 A- and 145 C-fibres which exhibited spontaneous activity and/or could be activated by natural (mechanical and/or thermal) stimuli applied to the sural nerve territory of the hind-paw were investigated.

3.II.2.1. Conduction velocities of the myelinated and unmyelinated sural nerve fibres following SNI

In the total sample, conduction velocities of the A-fibres ranged from 2.7 to 87 m/s (mean = 27.4 ± 0.8 ; median = 25.7; N= 329). The conduction velocities of the sural nerve A-fibres following SNI were not statistically different among the three time periods of investigation and did not differ significantly from the conduction velocities of the A-fibres in the control (no lesion) group (Fig. 3.28A).

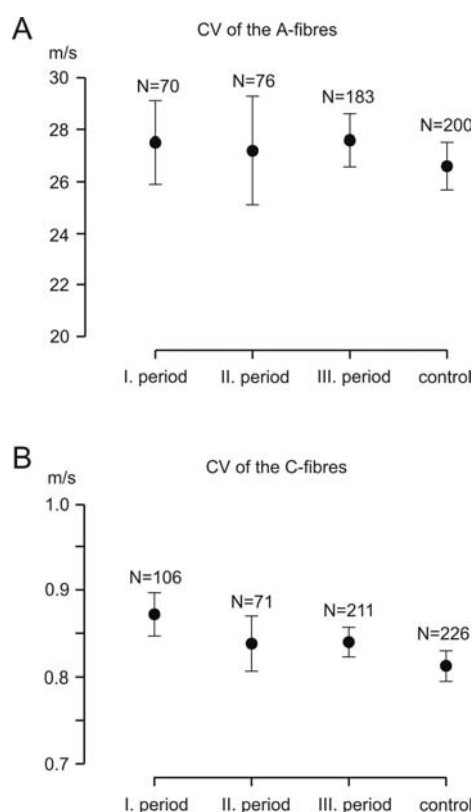


Fig. 3.28. Mean conduction velocities (\pm SEM) of the myelinated (A) and unmyelinated (B) sural nerve fibres (in meters per second, m/s) 1 day to 1 week (I. period), 2 to 4 weeks (II. period) and 7 to 10 weeks (III. period) after SNI. Control represents the mean conduction velocities of the A- and C-fibres recorded in the sural nerve of rats without nerve lesion. No significant difference in conduction velocity of the A- respectively C-fibres was found among the three time periods of investigation; m/s = meters per second

The conduction velocity of the unmyelinated fibres ranged from 0.32 to 1.8 m/s (mean = 0.84 ± 0.01 ; median = 0.8; N = 388). No significant difference in the conduction velocities of the sural nerve C-fibres was found among the three time periods post nerve lesion. Furthermore, in each period of investigation the conduction velocities of the C-fibres did not differ significantly from those in the control (no lesion) group (Fig. 3.28B).

3.II.2.2. Proportion of sural nerve A- and C-fibres exhibiting discharge properties following SNI

In filaments in which the number of electrically activated myelinated respectively unmyelinated fibres could be determined the proportion of A- and C-fibres which exhibited discharge properties among the total number of electrically activated fibres was investigated. All electrically activated fibres were tested for their spontaneous activity and whether they respond to mechanical and/or thermal stimuli applied to the hind paw area innervated by the sural nerve. Thirty-six to 50% of the electrically activated A-fibres and 15 to 20% of the electrically activated C-fibres exhibited at least one of the tested discharge properties, i.e. were spontaneously active and/or responded to mechanical or thermal stimuli applied to the target tissues of the hind paw innervated by the sural nerve (Table 3.8). No significant difference in the proportion of A- respectively C-fibres which exhibited discharge properties was found among the three time periods of investigation.

Table 3.8

The proportion of the sural nerve A- and C-fibres which exhibited discharge properties among the total number of electrically activated fibres

	I period (1 day - 1 week)	II period (2 - 4 weeks)	III period (7 - 10 weeks)
A- fibres	50% (48/96)	36% (30/84)	41% (96/235)
C-fibres	18% (27/146)	20% (29/144)	15% (63/425)

3.II.2.3. Characterisation of the myelinated and unmyelinated sural nerve fibres following SNI

3.II.2.3.1. A-fibres

Within the three time periods post nerve injury the majority (314/356; 88%) of the A-fibres investigated exhibited only one discharge property being either mechanosensitive or spontaneously active. The remaining A-fibres had a combination of two (11%) or three (1%) discharge properties (Fig. 3.29).

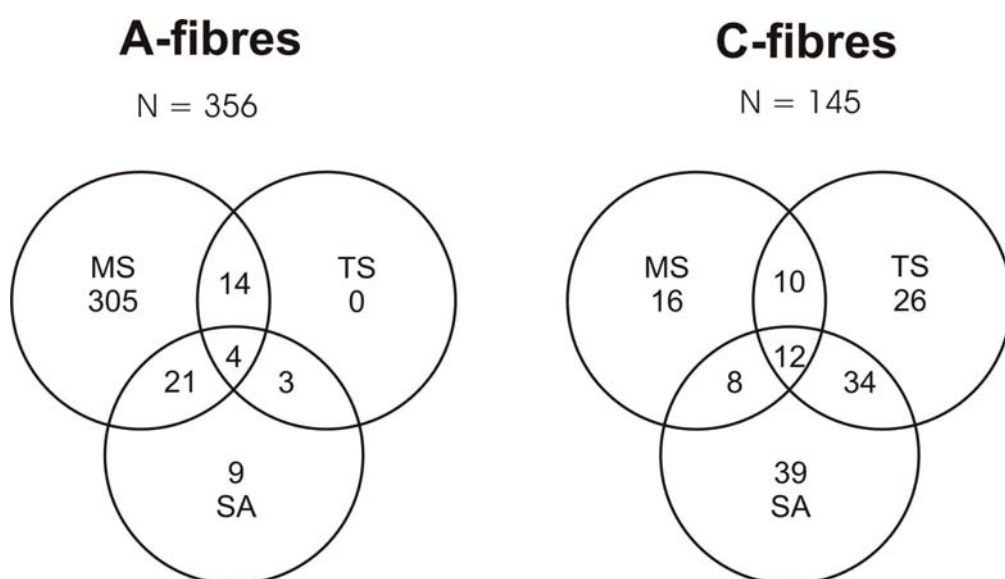


Fig. 3.29. Distribution of mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) of the sural nerve A- and C-fibres following SNI. The overlaps represent the number of fibres which exhibit a combination of 2 respectively 3 of the mentioned discharge properties. The data from all three time periods of investigation were pooled together. For further details see tables 3.9 and 3.10.

Spontaneous activity

Thirty-seven of 356 A-fibres investigated exhibited spontaneous activity, the proportion of spontaneously active A-fibres being higher in the first and second time period than in the third time period post nerve lesion (Table 3.9).

Table 3.9

Discharge properties of the intact sural nerve A-fibres following SNI

TYPE OF FIBRE	I period	II period	III period
FIBRES WITH PROPERTIES	121	83	152
INDIVIDUAL PROPERTIES			
Spontaneous Activity	15 (12%)	15 (18%)	7 (5%)
Mechanosensitivity	118 (98%)	76 (92%)	150 (98%)
<i>Excited from (by)</i>			
Deep somatic tissue	33	25	56
Hair follicle	51	37	86
Skin	26	13	6
Stretching	3	1	1
Pinching	2	-	1
?????	3	-	-
Thermosensitivity	8 (7%)	5 (6%)	8 (5%)
<i>Cold activated</i>	8	5	2
<i>Heat activated</i>	-	-	6
COMBINATION OF PROPERTIES			
Mechanosensitive/spontaneously active	102* / 9	65* / 7	138* / 5
Cold sensitive/spontaneously active	0 / 1	0 / 1	-
Heat sensitive/spontaneously active	-	-	0 / 1
Mechano- and cold sensitive/spontaneously active	4 / 3	3 / 1	2 / 0
Mechano- and heat sensitive/spontaneously active	-	-	5 / 0
Spontaneous activity only	2	6	1

Afferent fibers with only one ectopic discharge property. In *combination of properties* the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active.

(?) -the receptive fields could not be identified

The rates of discharge in the intact sural A-fibres following SNI ranged from 0.02 to 15.3 imp/s (mean = 1.88 ± 0.6 ; median = 0.33; N = 32 ; Fig. 3.30) and were significantly higher in the first two time periods than in the third time period post lesion (mean = 1.63 ± 0.8 respectively 2.63 ± 1.2 versus 0.26 ± 0.06 imp/s; $p < 0.05$; Wilcoxon test).

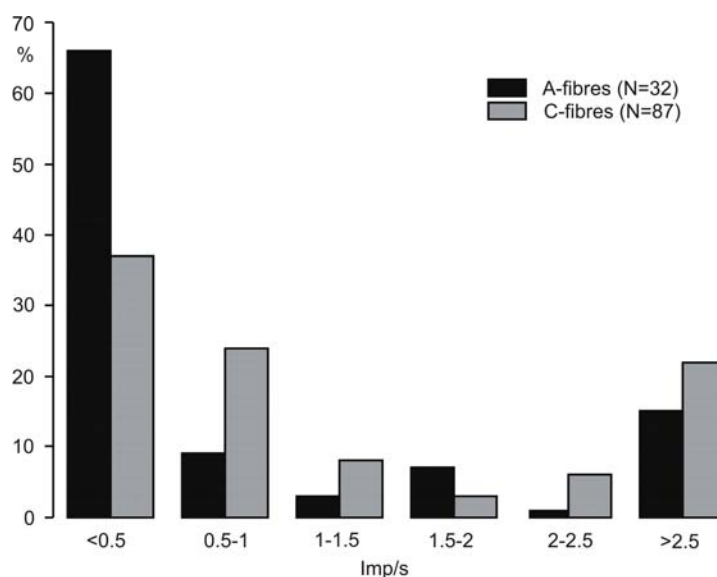


Fig. 3.30. Distribution of rates of spontaneous activity (impulse per second, imp/s) in the intact sural nerve A-fibres (black columns) and C-fibres (grey columns) following lesion of tibial and common peroneal nerves. The data from all three time periods of investigation are pooled together.

Twenty-five of the spontaneously active A-fibres were also mechanosensitive and could be excited from deep somatic tissues (n=10), hair follicles receptors (n=11) or other skin mechanoreceptors (n=4). Six spontaneously active A-fibres were cold-sensitive, four of them being excited by both mechanical and cold stimuli. Nine A-fibres were only spontaneously active and did not respond either to mechanical or to thermal stimuli applied to the target tissues of the hind paw.

Mechanosensitivity

Mechanical sensitivity was the main characteristic of the sural nerve A-fibres. Three hundred forty-four of 356 myelinated fibres investigated were activated by mechanical stimuli. The proportion of A-fibres which exhibited mechanical sensitivity was not significantly different among the three time periods post nerve lesion (Table 3.9). The mechanosensitive A-fibres were classified according to the receptive fields of their peripheral endings as cutaneous (hair follicle mechanoreceptors and skin mechanoreceptors) or subcutaneous (deep somatic tissues) receptors. Hair follicle mechanoreceptors were most frequently (174/344 fibres) encountered in the area innervated by the sural nerve. They had high sensitivity being excited by light movements of the hairs and responded phasically to mechanical stimulation. Eleven of the hair units

had also spontaneous activity (Fig. 3.31A) and nine of the silent hair units exhibited after-discharges post mechanical stimulation (Fig. 3.31B). Short mechanical stimulation (displacement) of the hair follicles evoked trains of action potentials in the afferent A-fibres which lasted 3 to 10 s post stimulation.

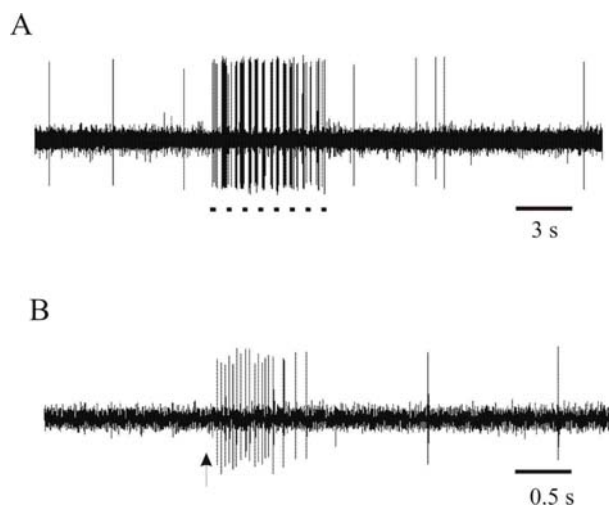


Fig. 3.31. Responses of two mechanosensitive A-fibres activated from hair follicles. A. Spontaneously active A-fibre which responded phasically to repetitive mechanical stimulation of the hair follicle at a frequency of about 2 Hz (see interrupted bar). B. Single mechanical stimulation of the hair follicle (arrow) evoked after-discharges in the afferent A-fibre which lasted for 3s post mechanical stimulation.

Fifteen per cent (53/344) of the mechanosensitive A-fibres had the receptive endings in the skin (but could not be excited from hair follicles). Among the skin mechanoreceptors 33 units had activation thresholds ranging from 0.45 to 45 mN (mean = 15.2 ± 2.2 ; median = 10) (Fig. 3.32); 4 units had high mechanical thresholds and could be activated only by von Frey filaments with bending forces ≥ 100 mN or by noxious pinch stimulation; 5 units were excited by stretching of the skin. No significant difference in mechanical sensitivity (threshold) of the myelinated fibres was found among the three time periods of investigation. One hundred fourteen of 344 mechanosensitive A-fibres had their receptive fields in subcutaneous (deep somatic) tissues. They innervated the joints of the 5th and 4th toes and could be activated by the flexion or extension of the toe. Deep somatic units responded tonically during constant mechanical stimulation.

The receptive fields of three mechanosensitive A-fibres could not be determined.

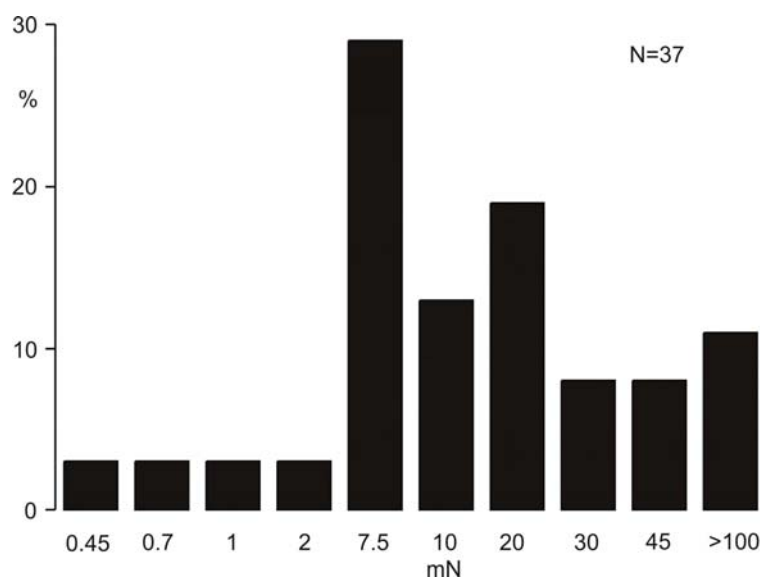


Fig. 3.32. Distribution of mechanical thresholds (in mN) of A-fibres which could be activated by mechanical stimulation of the skin (but not hair follicles). Mechanical thresholds were measured with calibrated von Frey filaments of different bending forces (abscissa ; values in mN).

Thermosensitivity

Thermal stimuli applied to the skin of the sural nerve territory excited 21 of 357 A-fibres investigated. Most (15/21) of the thermosensitive A-fibres were cold sensitive units which were activated at temperatures ranging from 21° to 14°C. The remaining 6 units were activated by heat stimuli.

3.II.2.3.2. C-fibres

Discharge properties of the unmyelinated fibres differ from those of the myelinated ones as far as the distribution and overlap of their functional properties are concerned. Fifty-six per cent (81/145) of the C-fibres exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active; 36% (52/145) of the units exhibited a combination of two discharge properties and 8% (12/145) had all three properties (Fig. 3.29).

Spontaneous activity

Ninety-three of 145 (64%) C-fibres investigated were spontaneously active; the proportion of C-fibres which exhibited spontaneous activity was significantly higher in the first and second time period than in the third time period post lesion (Table 3.10).

Table 3.10

Discharge properties of the intact sural nerve C-fibres following SNI

TYPE OF FIBRE	I period	II period	III period
FIBRES WITH PROPERTIES	38	41	66
INDIVIDUAL PROPERTIES			
Spontaneous Activity	28 (74%)	32 (78%)	33 (50%)
Mechanosensitivity	13 (34%)	12 (29%)	21 (32%)
Thermosensitivity	18 (47%)	20 (49%)	43 (65%)
<i>Cold activated</i>	11	17	26
<i>Heat activated</i>	2	2	15
<i>Cold and heat activated</i>	5	1	3
COMBINATION OF PROPERTIES			
Mechanosensitive /spontaneously active	3 [*] /2	4 [*] /5	9 [*] /1
Cold sensitive /spontaneously active	3 [*] /4	3 [*] /12	8 [*] /12
Heat sensitive /spontaneously active	-	1 [*] /0	7 [*] /4
Cold and heat sensitive /spontaneously active	2 /1	0 /1	2 /0
Mechano- and cold sensitive /spontaneously active	0 /4	0 /2	3 /3
Mechano- and heat sensitive /spontaneously active	2 /0	1 /0	4 /0
Mechano, cold and heat sensitive /spontaneously active	0 /2	-	0 /1
Spontaneous activity only	15 [*]	12 [*]	12 [*]

* Afferent fibres with only one ectopic discharge property. In *combination of properties* the second part of the term represent the fibres which are mechanical respectively thermosensitive *and* spontaneously active

The rates of discharge in the intact sural nerve C-fibres following lesion of neighbouring nerves ranged from 0.02 to 10.5 imp/s (mean = 1.63 ± 0.2 ; median = 0.8; N = 87; Fig. 3.30). The discharge rates of the spontaneously active C-fibres were not significantly different between the first two time periods of investigation but were significantly lower than the discharge rates of the C-fibres in the third time period post injury (1.42 ± 0.4 and 1.32 ± 0.3 versus 2.1 ± 0.4 imp/s; $p < 0.05$, Wilcoxon test). Fifty-four of 93 spontaneously active C-fibres responded also to natural (mechanical and/or thermal) stimuli and among these most (42/54) of the units were cold sensitive. Thirty-nine of the C-fibres were spontaneously active only and could not be activated by mechanical or thermal stimuli applied to the target tissues of the hind paw.

Mechanosensitivity

Mechanosensitivity was present in 46 of 145 C-fibres investigated. The proportion of C-fibres which exhibited mechanosensitivity was similar throughout the whole period of investigation. Most (34/38; 89%) of the mechanosensitive C-fibres investigated were mechanonociceptors which could be activated by von Frey filaments with bending forces ≥ 100 mN or by noxious pinch stimulation. Figure 3.33A shows an example of a C-mechanonociceptor activated by pinch stimulation.

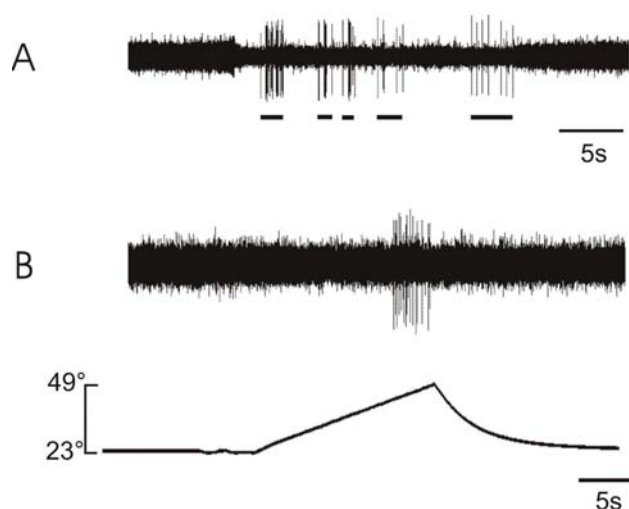


Fig. 3.33. Example of one C-fibre activated both by noxious mechanical and heat stimuli. A. Five pinch stimuli applied to the skin; B. Heat stimulation of the skin. A ramp heat stimulus increasing at a rate of 1.5°C/s from 23°C to 49°C led to activation of the C-fibre at $\geq 45^{\circ}\text{C}$.

The remaining 11% (4/38) of the fibres were C-mechanoreceptors which had activation thresholds ranging from 10 to 30 mN. Twenty of the mechanosensitive C-fibres exhibited spontaneous activity.

Thermosensitivity

Eighty-one of 145 (56%) C-fibres investigated were activated by thermal (cold, heat or both cold and heat) stimuli applied to the skin of the sural nerve territory. The proportion of C-fibres which could be excited by thermal stimuli was similar in the first and second time period post lesion (47% respectively 49%), but significantly lower ($p < 0.05$; Chi^2 -test) than the proportion of thermally activated C-fibres in the third period post injury (65%) (Table 3.10).

Cold sensitivity: Among the thermosensitive C-fibres investigated most (54/81) of the units were activated by cold stimuli. The activation thresholds of the cold sensitive fibres, determined at cooling rates of 0.5, 1 and 1.5°C/s and starting from a baseline temperature of 23°C, ranged from 22° to 8°C. The mean activation thresholds determined at cooling rates of 0.5°C/s and 1°C/s were not significantly different (mean = 19.3 versus 18.5°C; $p>0.05$) but were higher than the mean activation thresholds determined at 1.5°C/s (mean = 16.3°C). The cold sensitive C-fibres were divided according to their activation temperature into low and high threshold. The majority (19/33; 58%) of the cold activated units had low activation thresholds and were sensitive to small changes in temperature being excited when the temperature on the paw decreased by $\leq 2-4^\circ\text{C}$ from a baseline temperature of 23°C (Fig. 3.34B).

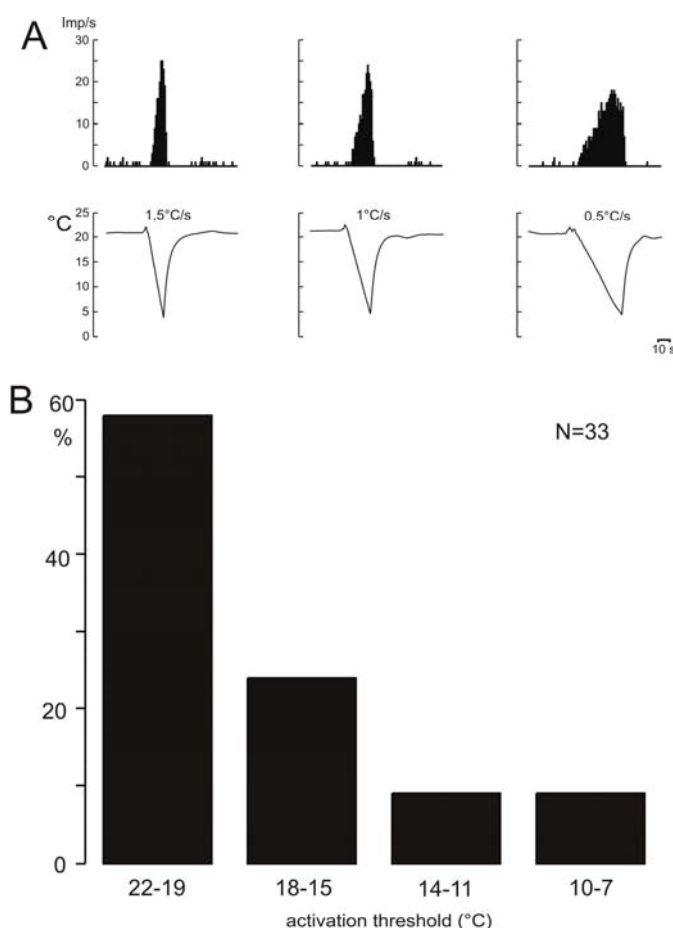


Fig. 3.34. A. Response of a spontaneously active low threshold cold sensitive C-fibre to cold stimuli. Ramp cold stimuli decreased from a baseline temperature of 23°C up to 4°C and were applied to the sural nerve territory at rates of 1.5°C/s, 1°C/s or 0.5°C/s. B. Distribution of activation thresholds of the cold sensitive C-fibres.

Figure 3.35 A shows an example of a cold sensitive fibre with high sensitivity, i.e. responding to small changes in temperature. The broken arrow indicates the increase of discharge with small decrease in temperature whereas the continuous arrows indicate the decrease of discharge when the temperature on the skin slightly increased. The remaining 42% of the cold sensitive C-fibres had high activation threshold and were excited at temperatures ranging from 18 to 8°C, thus the temperature had to decrease by $\geq 5^\circ\text{C}$ in order to excite these fibres (Fig. 3.35C).

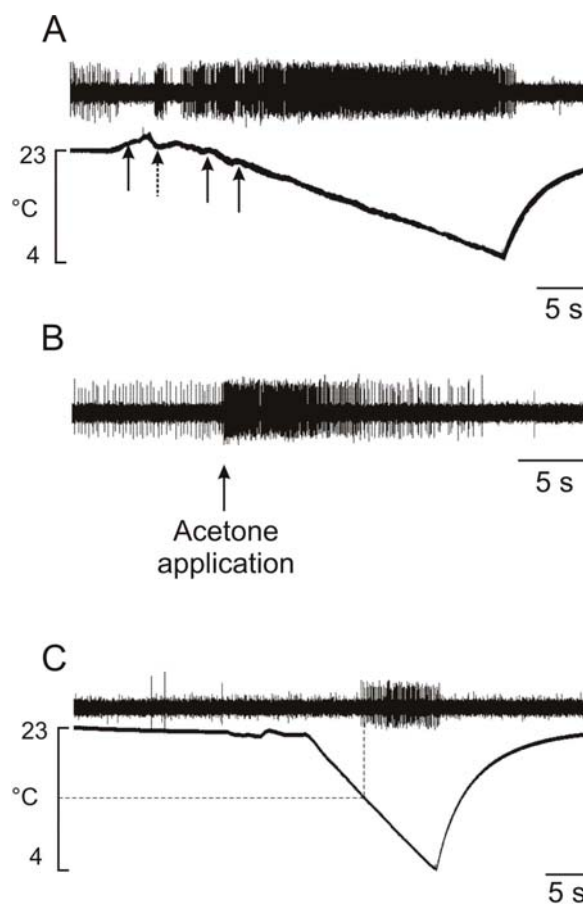


Fig.3.35. Examples of cold sensitive C-fibres. A, B: Responses of a spontaneously active low threshold cold sensitive C-fibre both to cold and acetone stimulation. A. A ramp cold stimulus was applied to the skin at a rate of $0.5^\circ\text{C}/\text{s}$ starting from a baseline temperature of 23°C to 4°C . The spontaneously active C-fibre had a very high sensitivity to small changes in temperature; less than 1°C increase in temperature led to suppression of spontaneous activity (continuous arrows) and less than 1°C decrease in temperature led to activation of the spontaneously active C-fibre (broken arrow). B. Application of an acetone drop to the receptive field activated the spontaneously active cold sensitive fibre. C. A ramp cold stimulus applied to the skin at a rate of $1^\circ\text{C}/\text{s}$ led to activation of a cold sensitive C-fibre at 15°C . This was a high threshold cold sensitive C-fibre.

In 22 cold sensitive fibres the stimulus response functions during cold stimulation were investigated quantitatively (Fig. 3.36). Eleven of these had low activation threshold and were activated when the temperature on the paw decreased by 2 - 4°C from a baseline of 23°C (Fig. 3.36A).

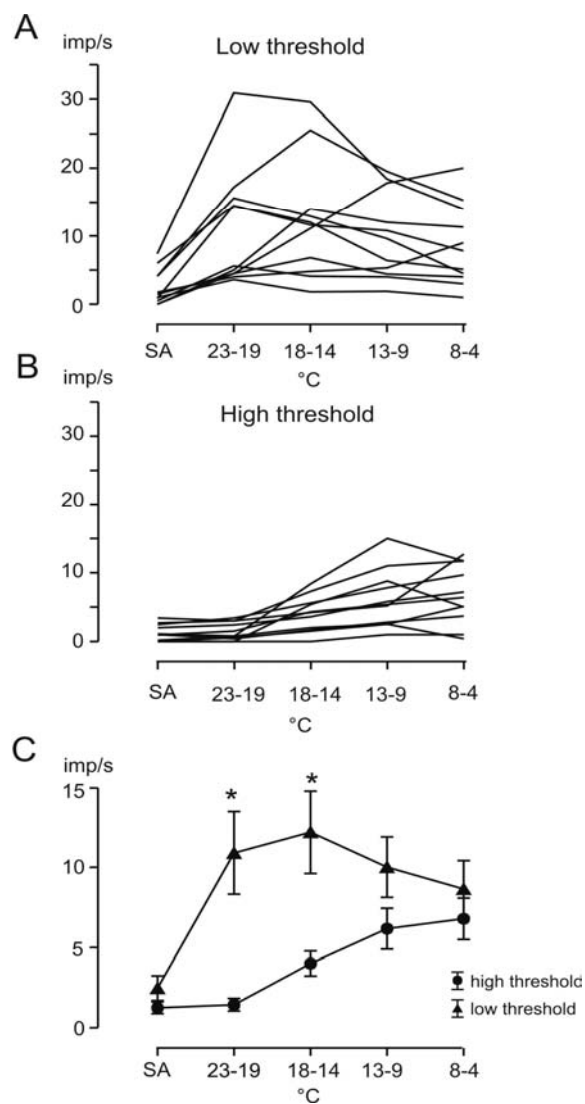


Fig. 3.36. Stimulus-response relations obtained in 22 cold sensitive C-fibers. Cold stimuli, applied to the skin at three different ramps (1.5°C/s, 1°C/s or 0.5°C/s), decreased from a baseline temperature of 23°C up to 4°C. The time of thermal stimulation: 38s (for the ramp of 0.5°C/s); 19s (for the ramp of 1°C/s), and 12.5s (for the ramp of 1.5°C/s) was divided in 4 intervals corresponding to the following temperature intervals 23-19°; 18-14°; 13-9° and 8-4°C. The responses of individual cold sensitive C-fibers were calculated as mean discharge rates (imp/s) during these 4 time intervals. A. Low threshold cold sensitive fibres activated when the temperature of the stimulus decreased by less than 4°C starting from a baseline temperature of 23°C (N=11). B. High threshold cold sensitive fibres which were activated at temperatures $\leq 18^\circ\text{C}$ (N=11). C. Mean stimulus response relations of the low threshold (▲) and high threshold (●) cold sensitive C-fibers obtained when the stimulus response relations of individual fibres from A and B, respectively were averaged. The asterisks indicate the mean discharge rates which are significantly higher in low threshold than in high threshold cold sensitive C-fibers. SA (spontaneous activity) represent the initial discharge rates recorded during 30-60 s before application of the thermal stimulus.

In 6 of the low threshold cold sensitive fibres the response to cold stimuli started with an initially high frequency of discharge which showed a gradual decline when the temperature of the stimulus decreased further. In the remaining 5 fibres the rate of discharge increased when the temperature of the stimulus went down.

Eleven cold sensitive fibres had high activation thresholds and were not activated during decrease in temperature by less than 4°C (Fig. 3.36B). These fibres exhibited graded response to cooling: in 8 of them the discharge was highest at the lowest temperature of 4 - 8°C and 3 fibres had maximal discharge rates at temperature of 9-13°C.

The mean discharge rates of the low threshold cold sensitive fibres were significantly higher than those of the high threshold cold sensitive C-fibres (mean = 10.4 versus 4.6 imp/s; $p < 0.01$, Wilcoxon; Fig. 3.36C).

The peak discharge rates of the cold sensitive C-fibres during cooling ranged from 4 to 38 imp/s (mean = 13.6 ± 1.5 ; median = 12; N=26). Thirty-seven of 54 cold sensitive units were also spontaneously active and in 22 of them the spontaneous activity was suppressed during heat stimulation (Fig. 3.37).

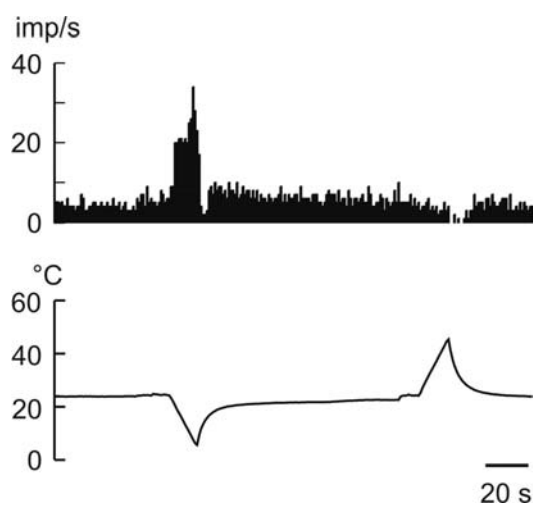


Fig. 3.37. Response of a spontaneously active C-fibre to cold and heat stimulation of the paw. Cooling of the paw led to activation of the C-fibre while heating of the paw suppressed the spontaneous activity.

Application of an acetone drop to the skin activated 37 of 51 cold sensitive C-fibres tested. The maximal discharge rates evoked by acetone stimulation in the cold sensitive fibres ranged from 6.2 to 40.2 imp/s (mean = 17.9 ± 1.8 ; median = 15) and were not significantly different ($p > 0.05$, Wilcoxon test) from the discharge rates during cold stimulation.

Heat sensitivity: Nineteen of 81 thermosensitive C-fibres investigated were excited by heat stimuli applied to the skin. The activation threshold of the heat sensitive units determined at heating rates of 0.5°, 1° respectively 1.5°C/s ranged from 35° to 49°C. No significant difference in the mean activation thresholds of the heat sensitive C-fibres determined at the above mentioned heating rates was found (mean = 44.6; 44.3 and 45.4°C, respectively). Most heat sensitive (12/14) fibres were activated at temperatures higher than 40°C (Fig. 3.38 and Fig. 3.33B). The maximal rates of discharge in the heat sensitive fibres ranged from 2 to 24 imp/s (mean = 8.4; median = 5; N=14).

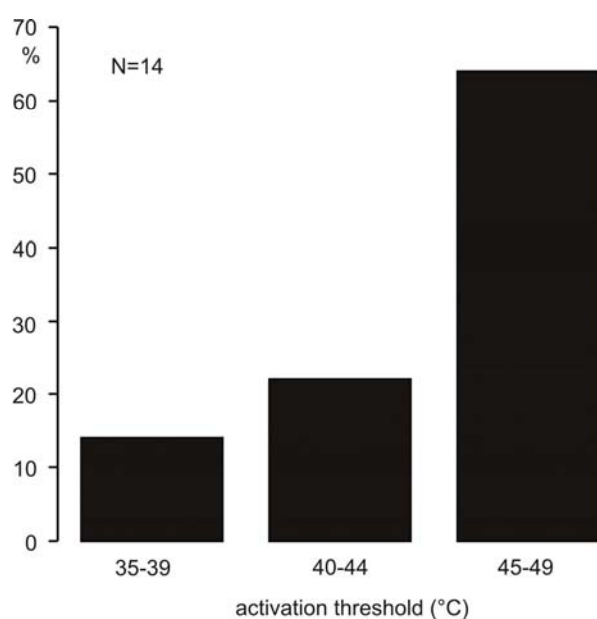


Fig. 3.38. Distribution of activation thresholds of heat sensitive C-fibres. The ramp heat stimuli increased from 23°C to 52°C and were applied to the sural nerve territory at rates of 0.5°, 1° and 1.5°C/s. No difference in the activation thresholds of the heat sensitive fibres among these 3 heating rates was found.

Cold and heat sensitivity: Nine C-fibres responded both to cold and heat stimuli and among these 5 were spontaneously active.

3. III. CONTROL EXPERIMENTS

The receptive properties of the sural nerve primary afferent neurones with myelinated (A-fibres) or unmyelinated (C-fibres) axons were investigated in 17 control rats with no nerve lesion. Electrophysiological recordings were performed in 120 filaments dissected from the peripheral end of the sural nerve in order to identify and characterise (quantitatively and qualitatively) the composition and sensory properties of mechano- and thermo- (cold, heat) receptors in the hind paw area innervated by the sural nerve. Two hundred twenty one afferent nerve fibres (124 A- and 97 C-fibres) isolated from the intact sural nerve were investigated and classified, according to the type of physiological stimulus to which their peripheral receptors respond, in mechanical, cold or heat sensitive units.

3.III.1. Conduction velocities of the myelinated and unmyelinated fibres

The conduction velocities of 426 afferent nerve fibres (200 A- and 226 C-units) recorded from the intact sural nerve were determined. The histograms in Figure 3.39 show the distribution of the conduction velocities of the myelinated and unmyelinated fibres. Conduction velocity of the A-fibres ranged from 2.86 to 80 m/s (mean = 26.6 ± 0.91 ; median = 25.7) whereas the C-fibres conducted between 0.31 and 2 m/s (mean = 0.81 ± 0.02 ; median = 0.79).

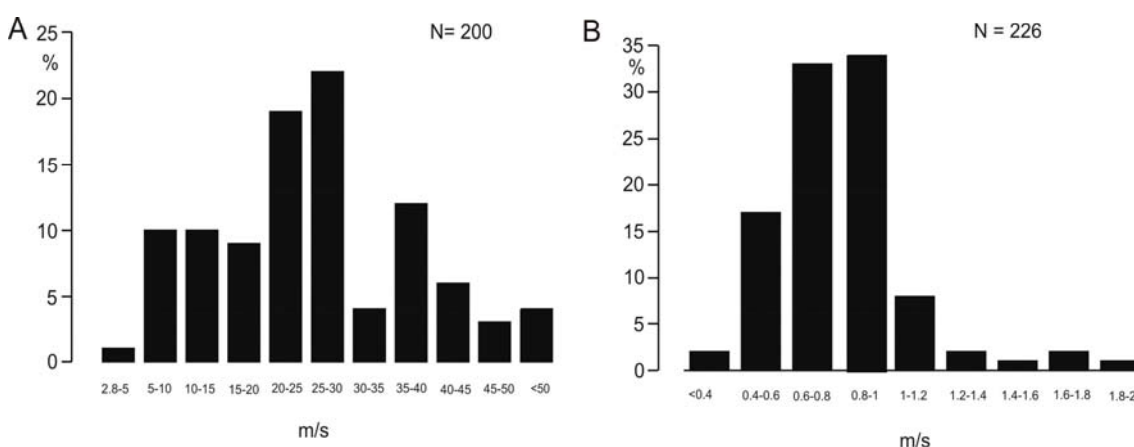


Fig. 3.39. Distribution of conduction velocities of the myelinated (A) and unmyelinated (B) fibres recorded in the intact sural nerve from animals with no nerve lesion. m/s = meters per second

3.III.2. Proportion of sural nerve A- and C-fibres which exhibit discharge properties

In filaments in which the number of electrically activated myelinated respectively unmyelinated fibres could be determined the proportion of sural nerve A- and C-fibres which exhibited discharge properties among the total number of electrically activated fibres was investigated. All electrically activated fibres were tested for their spontaneous activity and whether they respond to mechanical and/or thermal stimuli applied to the hind paw area innervated by the sural nerve. Twenty three per cent of the electrically activated C-fibres and 44% of the electrically activated A-fibres exhibited at least one of the tested discharge properties, i.e. were spontaneously active and/or responded to mechanical or thermal stimuli applied to the sural innervation territory of the hind paw. The innervation territory of the sural nerve consists of the hairy skin of the calf, ankle and lateral hind paw (foot) and 59% of the afferent fibres in the sural nerve innervate the target territories of the calf and ankle (Lewin and McMahon, 1991; Handwerker et al., 1991). A possible explanation for the relatively low proportion of electrically activated A- and C-fibres which could be also excited by natural stimulation from the target tissues could be the fact that in our preparation the hairy skin of the calf and ankle was not accessible for natural stimulation because of the positioning of the pool (see Fig. 2.5A).

3.III.3. Characterisation of the myelinated and unmyelinated nerve fibers

3.III.3.1 .A-fibres

The majority 115/124 (94%) of the A-fibres investigated exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active. The remaining A-fibres had a combination of two discharge properties (Fig. 3.40).

Spontaneous activity

Seven of 124 A-fibres exhibited spontaneous activity; the rates of spontaneous discharge in the myelinated fibres ranged from 0.23 to 2.42 imp/s (mean = 1.9 ± 0.6 ; median = 2.2 imp/s). Among the spontaneously active A-fibres five units were also mechanosensitive and could be excited from deep somatic tissues; one was cold sensitive and one A-fibre

exhibited spontaneous activity only and did neither respond to mechanical nor to thermal stimuli applied to the target tissues of the hind paw.

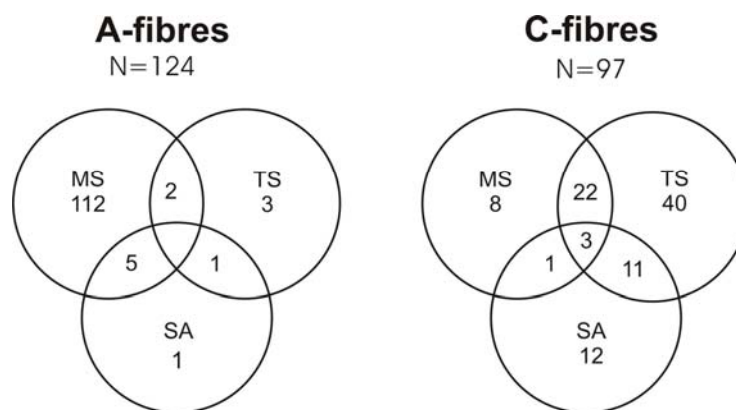


Fig. 3.40. Distribution of discharge properties: mechanosensitivity (MS), thermosensitivity (TS) and spontaneous activity (SA) of the sural nerve A- and C-fibres recorded in the intact sural nerve from control animals (no nerve lesion). The overlaps represent the number of fibres which exhibit a combination of 2 or 3 discharge properties.

Mechanosensitivity

In control animals (no nerve lesion) mechanosensitivity was the main sensory characteristic of the primary afferent A-fibres. Ninety-six per cent of the myelinated fibres investigated were activated by mechanical stimuli and according to the receptive fields of their sensory terminals were classified as cutaneous (hair follicle mechanoreceptors and skin mechanoreceptors) or subcutaneous (deep somatic tissue) receptors (Table 3.11). A large proportion (54%) of the sural A-fibres innervated hair follicles. The hair units had high sensitivity to mechanical stimuli; light displacements of the hairs could elicit action potential discharges in the afferent A-fibres. Hair follicle mechanoreceptors were rapidly adapting and responded with brief bursts of discharges to mechanical stimulation (Fig. 3.41A).

The mechanoreceptors which had their receptive fields in the skin (but were not associated with hair follicles) represented 11% of the population of mechanosensitive A-fibres. Their activation threshold ranged from 1 to 45 mN (mean = 14.7; median = 7.5; N= 6); four units had high mechanical thresholds and could be activated only by intense mechanical stimulation (≥ 100 mN).

Table 3.11
Discharge properties of sural nerve A- and C-fibres in control rats

TYPE OF FIBRE	A-fibres	C-fibres
FIBRES WITH PROPERTIES	124	97
INDIVIDUAL PROPERTIES		
Spontaneous Activity	7 (6%)	27 (28%)
Mechanosensitivity	119 (96%)	34 (35%)
<i>Excited from</i>		
Deep somatic	42 (35%)	
Hair follicles	64 (54%)	
Skin	13 (11%)	34
Thermosensitivity	6 (5%)	76 (78%)
<i>Cold activated</i>	3	20
<i>Heat activated</i>	3	43
<i>Cold and heat activated</i>	0	13
COMBINATION OF PROPERTIES		
Mechanosensitive /spontaneously active	112 [*] / 5	8 [*] / 1
Cold sensitive /spontaneously active	1 [*] / 1	12 [*] / 7
Heat sensitive /spontaneously active	2 [*] / 0	22 [*] / 1
Cold and heat sensitive /spontaneously active	0 / 0	6 / 3
Mechano- and cold sensitive /spontaneously active	1 / 0	0 / 1
Mechano- and heat sensitive /spontaneously active	1 / 0	18 / 2
Mechano, cold and heat sensitive /spontaneously active	0 / 0	4 / 0
Only spontaneous activity	1 [*]	12 [*]

* Afferent fibers with only one ectopic discharge property. In *combination of properties* the second term represent those fibres which are mechanical respectively thermosensitive *and* spontaneously active

Thirty five per cent of the A-fibres in the intact sural nerve responded to mechanical probing of deep somatic tissues. They innervated the joints of the 5th toe and were excited by flexion or extension of the toe. These fibres discharged tonically during mechanical stimulation (Fig. 3.41B). Five of the mechanosensitive A-fibres activated from deep somatic tissue were spontaneously active. No spontaneous activity was encountered in the A-fibres innervating hair follicle mechanoreceptors or other skin mechanoreceptors.

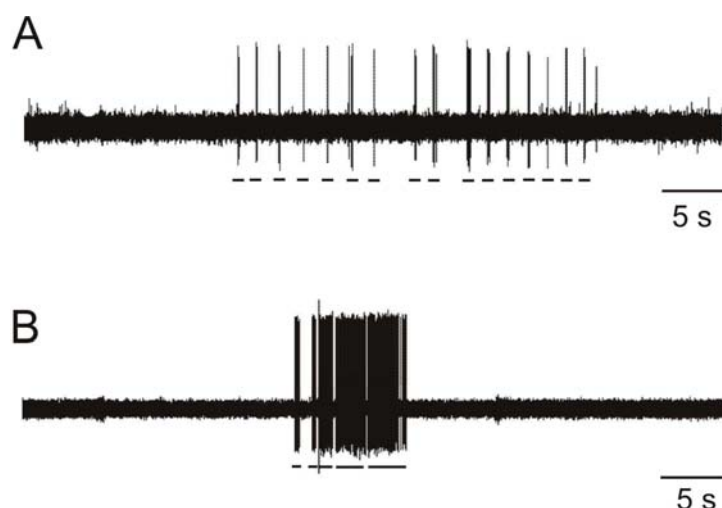


Fig. 3.41. Responses of mechanosensitive A-fibres which innervate hair follicles (A) or deep somatic tissues (B) to mechanical stimulation. A. Silent A-fibre which responded phasically to repetitive mechanical stimulation of the hair follicle. B. Silent A-fibre which discharged tonically during bending of the 5th toe.

Thermosensitivity

Six of 114 A-fibres investigated responded to thermal stimuli applied to the sural nerve territory, 3 of them were activated by cold and 3 by heat stimulation (Table 3.11). The activation threshold was around 47 - 48°C for the heat sensitive fibres and ranged from 10 to 20°C for the cold sensitive fibres. One cold activated A-fibre exhibited spontaneous activity.

3.III.3.2. C-fibres

The distribution of discharge properties of the sensory neurones with unmyelinated fibres differs from that of the sensory neurones with myelinated fibres as far as the overlap of their functional properties is concerned. Fifty-six per cent of the C-fibres exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active. The remaining units had a combination of two (34%) or three (10%) discharge properties.

Spontaneous activity

Twenty-seven of 97 C-fibres investigated exhibited spontaneous activity and discharged at rates ranging from 0.08 to 6.9 imp/s (mean = 1.9 ± 0.5 ; median = 0.9; Fig. 3.42). Eleven of the spontaneously active C-fibres were cold sensitive and 4 units responded to

mechanical and/or heat stimuli. Twelve unmyelinated fibres exhibited only spontaneous activity and could not be activated by mechanical or thermal stimuli. These units could belong to the population of C-fibres whose receptive fields are in the target territories of the calf and ankle and were not available for natural (mechanical and thermal) stimulation because of the positioning of the pool.

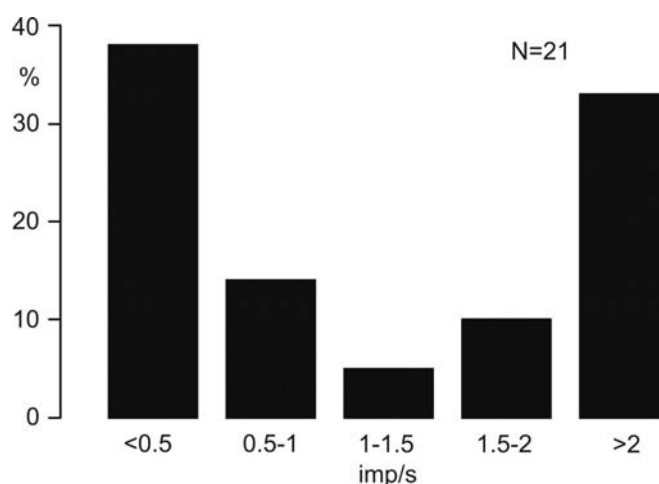


Fig. 3.42. Distribution of rates of spontaneous activity in the intact sural nerve C-fibres in control rats (no nerve lesion).

Mechanosensitivity

Mechanosensitivity was observed in 34 of 97 C-fibres investigated (Table 3.11). Most (32/34) of the mechanosensitive C-fibres were mechanonociceptors which could be activated by von Frey filaments with bending forces of ≥ 100 mN or by noxious pinch stimulation. Two units were mechanoreceptors and had activation thresholds of 7.5 respectively 45 mN. Four of the mechanosensitive C-fibres exhibited spontaneous activity.

Thermosensitivity

Most of the sural nerve C-fibres investigated 78% (76/97) were thermosensitive and could be activated by cold, heat or both cold and heat stimuli applied to the sural nerve territory of the hind paw.

Cold sensitivity: Twenty of 76 thermosensitive C-fibres were activated by cold stimuli (Table 3.11). The activation thresholds of the cold sensitive fibres determined at cooling rates of 0.5, 1 or 1.5°C/s, starting from a baseline temperature of 23°C, ranged from 21° to 7°C (N=16). No significant difference in the mean activation thresholds of the cold sensitive fibres determined at cooling rates of 0.5, 1 and 1.5°C/s was found (mean = 15.2; 14.5 and 14.7°C, respectively). The cold sensitive C-fibres were divided according to their activation temperature in low and high threshold fibres. Thirty-seven per cent (6/16) of the cold sensitive C-fibres had a low activation threshold and were excited when the temperature on the paw decreased by 2 - 4°C from a baseline temperature of 23°C. The remaining 63% of the cold sensitive C-fibres had a high activation threshold, the temperature had to decrease by $\geq 5^\circ\text{C}$ in order to excite these fibres. Figure 3.43 shows the distribution of the activation thresholds of the cold sensitive C-fibres.

4. DISCUSSION

Peripheral nerve lesion leads to impairment of sensory function in the area served by lesioned nerve(s). At the same time, nerve injury leads to development of ectopic spontaneous and evoked activity in the lesioned myelinated and unmyelinated fibres which might be involved in injury induced sensory disorders such as distorted mechanical sensitivity, thermal sensitivity and pain sensation. When the regeneration of the lesioned nerve fibres to the peripheral target tissues is obstructed, as in case of neuroma lesion, the neurophysiological changes in the lesioned nerve fibres can last up to 245 days post injury (Blumberg and Janig, 1984) and the sensory disturbances are persistent. When the lesioned nerve fibres are free to regenerate to the periphery, as in case of crush or section and resuturing lesion, the sensory function of the denervated territory can be partially or completely restored and the functional changes in the injured neurones can be reversed. Functional recovery after peripheral nerve injury is dependent on the efficacy of axonal outgrowth of the lesioned neurones and on their ability to form functional connections in the target tissues.

4.1. Sural nerve lesion followed by regeneration

Spontaneous and evoked discharge properties and the ability of lesioned cutaneous afferent nerve fibres to regenerate to their peripheral target tissues were investigated in two time periods: 1 to 3 weeks (period I) and 1 to 4 months (period II) after crush or section and resuturing of the sural nerve. Within the first time period post lesion most lesioned myelinated and unmyelinated fibres were in the process of regeneration in the nerve, some were trapped at the injury site and only a few lesioned nerve fibres had already regenerated and reached the target tissues of the hind paw. In our preparations the distance from the lesion site to the most proximal target tissue which was available for natural (mechanical and thermal) stimulation was ~20 mm. Assuming a regeneration rate of the lesioned fibres of about 1 to 3 mm/day (Forman et al., 1979; for review see Lisney, 1989) it is supposed that 1 to 4 months post sural nerve lesion the regeneration process is completed and the lesioned nerve fibres could have reached the peripheral hind paw's targets. However, we have found that within 1 to 4 months post lesion 20% of the injured A-fibres and 71% of

the injured C-fibres were still only ectopically excitable from the nerve as it was the case in the first 3 weeks post lesion.

4.I.1. Regeneration of A- and C-fibres following crush or section and resuturing of the sural nerve

In our study we estimated the degree of regeneration of the lesioned myelinated and unmyelinated sural nerve fibres by the proportion of units which could be activated by natural (mechanical and/or thermal) stimuli applied to the target tissues of the hind paw among the population of fibres with functional properties. One to four months post nerve injury, the regeneration was more complete for the lesioned A- than for the C-fibres and both myelinated and unmyelinated fibres showed a better regeneration following sural nerve crush than following sural nerve section and resuturing lesion.

Eighty per cent of the A-fibres which were mechano- and/or thermosensitive regenerated and could be naturally activated from the target tissues of the hind paw whereas only 29% of the naturally activated C-fibres could be excited by stimuli applied to the skin territory of the hind paw within 1 to 4 months post nerve lesion. The remaining mechano- and thermosensitive A- and C-fibres had their receptive fields distributed along the lesioned sural nerve from 7 mm proximal to 12 mm distal to the lesion site. This raises the question why, in spite of the regenerative response initiated, a substantial proportion of the C-fibres with evoked discharge properties failed to re-establish functional contacts with their peripheral targets? Nerve lesion (crush or section and resuturing) disrupts the normal supply of trophic factors (NGF and GDNF) from the peripheral target tissues but is followed by increase of synthesis and supply of neurotrophins (e.g., NGF) in the distal nerve stump (Heumann et al., 1987; Lindholm et al., 1987). Following nerve injury, Schwann cells dedifferentiate and switch from production of myelin to synthesis of growth factors which are thought to facilitate regeneration of the lesioned neurones. Despite this, within four months post injury, 71% of the C-fibres did not succeed to reinnervate the target tissues of the hind paw and could be excited only by mechanical and/or thermal stimuli applied along the lesioned sural nerve. The lack of regeneration may be due to the deficiency of glial cell (Schwann cells) to provide quantitatively enough

trophic factors to promote regeneration of the unmyelinated nerve fibres to the peripheral target tissues.

Both lesioned myelinated and unmyelinated fibres showed a better regeneration following sural nerve crush than following sural nerve section and resuturing. The Schwann cell tubes which remain intact following nerve crush may guide the regenerating axon sprouts of damaged axons more successfully to the peripheral targets than after section and resuturing lesion where the endoneurial tubes are interrupted. The presence of the intact Schwann cell tubes at the lesion site may also facilitate the action of neurotrophic factors of distal origin which stimulate the growth of the injured fibres. Although the continuity of the Schwann cell tubes seems to be restored 4 days post section and resuturing of the endoneurial tubes and the growing (sprouting) conditions of the lesioned nerve fibres are apparently similar in the two types of nerve lesion, the regeneration ability and discharge properties of the injured C-fibres are different between crush and section and resuturing lesion both 1 to 3 weeks and 1 to 4 months post injury.

4.I.2. Proportion of fibres which exhibit discharge properties versus electrically activated fibres

One to four months post sural nerve injury 37- 39% of the A-fibres and 29 - 41% of the C-fibres which could be electrically activated exhibited at least one of the tested discharge properties. These percentages are comparable to those found in intact sural nerve (control group). The relative low proportion of electrically activated A-fibres which exhibit discharge properties and could be excited by natural stimulation from the target tissues could be due to the fact that 59% of the afferent fibres in the sural nerve innervate the hairy skin of the calf and ankle (Lewin and McMahon, 1991) and in our preparation these receptive fields were not accessible for natural stimulation because of the positioning of the pool (see Fig. 2.5A).

One to 3 weeks post sural nerve injury 33- 40% of the A-fibres and 21-27% of the C-fibres which could be electrically activated exhibited spontaneous activity and/or could be activated by natural (mechanical or thermal) stimulation. These percentages are comparable to those of the lesioned fibres trapped in the neuroma 20 to 30 hours after ligation and cutting of the sural nerve (Michaelis et al., 1995, 1999). About 15% of the C-

fibres in the sural nerve are sympathetic postganglionic (Baron et al., 1988) which are unresponsive to mechanical stimuli at intensities known to excite afferent cutaneous C-fibres and do not exhibit spontaneous activity generated in the periphery. It is unclear why approximately 60% of the A- and C-fibres either regenerating in the nerve (1 to 3 weeks post nerve injury) or trapped at the lesion site (neuroma lesion) could not be excited by physiological stimuli and/or did not exhibit spontaneous activity although the terminals of these lesioned nerve fibres were readily accessible for stimulation. One explanation could be that the axotomized afferent neurones may decrease synthesis of transducer proteins as well as channels proteins following the lesion due to the lack of appropriate amounts of trophic factors. This could result in conditions that physiological stimuli produce small generator potentials in the afferent terminals leading to the low percentage of afferent nerve fibres which can be naturally activated. Up to now we cannot explain why the percentage of activated and/or spontaneously active fibres under regeneration are the same with those trapped into a neuroma, although the supply of trophic factors of distal origin ought to be different for these two models of nerve lesion.

4.1.3. Conduction velocities of the lesioned A- and C-fibres

Nerve lesion had a profound effect on the conduction velocities of the injured fibres. One to 4 months following crush respectively section and resuturing lesion myelinated and unmyelinated fibres activated electrically either from proximal or distal stimulation electrodes had lower conduction velocities than the corresponding intact fibres. Moreover, the conduction velocities of the A-fibres tended to be lower across the injury site (distal) than proximal to the lesion site. The decrease in conduction velocities of the myelinated and unmyelinated fibres could be due to the fact that following nerve lesion axons and cell bodies of the injured neurones shrink (Jänig and McLachlen, 1984) and this reduction in diameter results in slowing of conduction velocity (Blumberg and Jänig, 1982).

4.I.4. General characteristics of the lesioned and regenerating (regenerated) myelinated and unmyelinated fibres

Both in the first and second time period post sural nerve injury the distribution of the sensory properties of the lesioned and regenerating A- and C-fibres following *crush* and *section and resuturing* compare in some aspects to those of the lesioned cutaneous afferent nerve fibres which are trapped at the lesion site and prevented to regenerate (Blenk et al., 1996; Michaelis et al., 1995, 1999) and with the distribution of sensory properties of the intact sural nerve fibres in control (unlesioned) animals. Ninety to 94% of the *myelinated* fibres belonging to both lesion groups and to the control group exhibited only one discharge property being either mechanosensitive, thermosensitive or spontaneously active; the remaining 6 to 10% presented a combination of two or three discharge properties. In the *unmyelinated* fibre population the overlap between two or three functional discharge properties was more frequent than in the myelinated fibres in both groups of lesion and in the control group.

The lesioned and regenerating nerve fibres exhibited some specificity of their discharges evoked by physiological (mechanical, cold, heat) stimuli, as do the lesioned nerve fibres which are not regenerating (neuroma) (Blenk et al., 1996; Michaelis et al., 1995, 1999) and the intact afferent nerve fibres which have their sensory endings in the skin (control: unlesioned nerve). Mechanical and thermal (cold, heat) sensitivity of the lesioned and regenerating and non-regenerating nerve fibres are probably intrinsic properties of the afferent neurones which are dependent on distinct membrane proteins for mechano-, cold- and heat- transduction. These transduction proteins are synthesised in the cell bodies of the sensory neurones and continuously carried by the axonal transport into the peripheral terminals of the afferent nerve fibres (Devor and Govrin-Lippmann, 1983). When the regeneration of the injured fibres is obstructed (e.g. by ligation and section of the nerve leading to neuroma formation), the transducer molecules are incorporated in the membrane of the proximal end of the injured axons at the lesion site. When the lesioned fibres are free to regenerate to the periphery (e.g. after nerve crush or section and resuturing) the transducer molecules for mechanical and thermal stimuli are integrated in the regenerating nerve terminals.

In the sensory neurones of intact nerves the phenotypic expression of transduction proteins and voltage gated Na^+ and K^+ channels is dependent on continuous supply of growth factors (NGF and GDNF) from the peripheral target tissues. Following nerve lesion the afferent nerve fibres are disconnected from the peripheral source of trophic factors which might be partially replaced by the trophic factors produced by Schwann cells distal to the lesion site. The changes in availability of trophic factors after lesion can lead to changes in expression of transduction proteins and voltage-gated channels.

Modulation in the expression of voltage-dependent Na^+ and K^+ channels (McCleskey and Gold, 1999) and their abnormal spatial distribution along the axonal trajectory following nerve lesion lead to changes in membrane excitability and generation of spontaneous activity. After nerve injury Na^+ channels accumulate in excess number in the axonal membrane at the neuroma end bulbs (Novakovic et al., 1998) and this renders the injured axons hyperexcitable so that they become an ectopic source of spontaneous discharge (Matzner and Devor, 1994).

4.1.4.1. Spontaneous activity

Spontaneous activity which appears in some lesioned afferent fibres following nerve injury impinge on second order neurones in the spinal cord. This increases the excitability of the dorsal horn neurones which may lead to altered processing of the information which come along the nociceptive and non-nociceptive fibres (Woolf CJ, 1983; Woolf and Thompson, 1991; Dubner and Ruda, 1992).

In the control animals (no nerve lesion) spontaneous activity was present in 27/97 of the sural nerve C-fibres; similar data were reported also by Leem et al., (1993) who showed that 21% of the intact sural nerve fibres were spontaneously active and most of them were cold sensitive units. Spontaneous activity in mechanical and/or heat nociceptors is practically absent (Kress et al., 1992; Leem et al., 1993). Following lesion of the sural nerve by crush or section and resuturing a significantly higher proportion of C-fibres exhibited spontaneous activity. The increase was more pronounced following crush than following section and resuturing lesion: 65% versus 37%, within the first three weeks post lesion. This high incidence of spontaneous activity in the C-fibres was persistent also 1 to 4 months post nerve injury when 59% of the C-fibres in the crush group and 38% of the C-

fibres in the section and resuturing group were spontaneously active. Thus, within the same group of lesion the proportion of C-fibres which exhibit spontaneous activity was similar 1 to 3 weeks and 1 to 4 months post nerve injury. Within the first three weeks post lesion the rates of spontaneous activity in the unmyelinated nerve fibres belonging to the crush group were significantly higher than those belonging to the section and resuturing group mean: 1.23 versus 0.36 imp/s ($p < 0.01$; Wilcoxon test) whereas in the second time period post lesion the rates of discharge were similar for both groups of lesion (mean = 1.21 versus 0.71 imp/s; $p > 0.05$, Wilcoxon test).

In the population of myelinated fibres, sural nerve section and resuturing led to no significant change in proportion of spontaneously active fibres either in the first or the second time period post nerve injury compared to the control group. Within the first three weeks post sural nerve crush a significantly higher proportion of A-fibres exhibited spontaneous activity than in control group (26% versus 6%; $p < 0.001$, χ^2 -test) and the percentage of spontaneously active A-fibres dropped to 9% within 1 to 4 months post crush lesion.

There is a general agreement that one factor involved in generation of spontaneous activity following nerve lesion is the change in expression of voltage-gated Na^+ channels due to alterations in the availability of trophic factors (NGF, GDNF). The nociceptive neurones co-express rapidly inactivating TTX-sensitive (TTX-S) Na^+ channels and slowly activating and inactivating TTX-resistant (TTX-R) Na^+ channels (Caffrey et al., 1992). TTX-R Na^+ channels require high depolarisation for activation, thus C-fibres containing TTX-R Na^+ channels have a high threshold for action potential generation and are normally silent. The expression of two sensory neurone specific TTX-R Na^+ channels (SNS and NaN - for nomenclature see Trends in Pharmacological Science, Receptor and ion channel nomenclature, Supplement 1999) is dependent on the continuous uptake of NGF or GDNF from the peripheral target tissue. Disconnection of the afferent neurones from the peripheral source of trophic factors following axotomy leads to down-regulation of the TTX-R Na^+ channels and up-regulation of a TTX-S Na^+ channel with fast recovering from inactivation (Dib-Hajj et al., 1996, Cummins and Waxman, 1997) which shortens the refractory period of the injured neurones and make them able for abnormal firing. Overall, the excitability of the afferent cell bodies in dorsal root ganglia increases in all groups of afferent neurones after axotomy and practically all currents are affected, the largest

increase in excitability occurring in afferent C-neurones and the smallest one in afferent A-neurones (Abdulla and Smith, 2001). Experiments done by Dib-Hajj et al., (1998), Cummins et al., (2000) and Sleeper et al., (2000) showed that both NGF and GDNF can prevent or attenuate the down-regulation of the TTX-R Na⁺ channels in the axotomized C-neurones *in vivo* and *in vitro*.

Lesion of a peripheral nerve leads to local increase in synthesis of NGF produced by the non-neuronal cells distal to the lesion site (Stoll and Müller, 1999) which might partially compensate for the interrupted NGF supply from the peripheral target. Although in case of nerve lesion followed by regeneration (crush or section and resuturing) the injured nerve fibres grow in an environment rich in neurotrophines (NGF, GDNF [?]) produced by Schwann cells distal to the lesion site, the spontaneous activity was still encountered in a certain proportion of the myelinated and unmyelinated fibres. One possible explanation could be that the synthesis and release of trophic factors by non-neuronal cells distal to the lesion site are quantitatively too low to compensate for the lack of trophic factors from the peripheral target tissues. Another explanation for the presence of spontaneous activity in the lesioned and regenerating nerve fibres could be that during the regeneration the growing nerve fibres are in close contact with a rich network of proinflammatory cytokines (IL-1; TNF α) released during Wallerian degeneration distal to the lesion site (Shamash et al., 2002). TNF α , that is believed to elicit spontaneous discharges in sensory fibres when applied along the nerve (Sorkin et al., 1997), is produced by Schwann cells in response to nerve lesion (Wagner and Myers, 1996). The intimate contact of the regenerating nerve fibres with this inflammatory cytokine may lead to development of local changes which result in generation of spontaneous firing.

Following both crush and section and resuturing lesion the injured nerve fibres are free to regenerate to the periphery and the sprouting conditions for the lesioned nerve fibres seem to be similar for both types of nerve lesion. However, we can not explain why the proportion of spontaneously active fibres is higher following crush than following section and resuturing lesion.

4.I.4.2. Evoked discharge characteristics of the myelinated fibres

Mechanosensitivity was the main discharge property of the A-fibres investigated both in the lesioned and regenerating afferent nerve fibres and in the intact ones (control group). The proportion of myelinated fibres which exhibited mechanosensitivity was similar in crush and section and resuturing group both 1 to 3 weeks (period I) and 1 to 4 months (period II) post injury. Within the first 3 weeks post injury the receptive fields of almost all mechanosensitive A-fibres were found in the lesioned sural nerve. Within 1 to 4 months post sural nerve lesion 80% of the mechanosensitive A-fibres had regenerated and reinnervated the target tissues of the hind paw; the remaining fibres had their receptive fields along the lesioned nerve. The mechanical thresholds of the A-fibres which had regenerated and reached the skin were similar to those in the intact sural nerve in control animals but significantly higher than the mechanical thresholds of the A-fibres which had receptive fields distributed along the lesioned nerve. Mechanical transduction at low threshold was particularly prominent in the A-fibres excited from the nerve.

Within both time periods post injury only a few A-fibres reacted to thermal stimulation, almost all of them being activated by heat stimuli.

In the A-fiber population mechanical and thermal sensitivity of the lesioned nerve fibers were similar distributed following crush and section and resuturing lesion both in first and second time period post-injury.

4.I.4.3. Evoked discharge characteristics of the unmyelinated fibres

Distribution of discharge properties of the injured unmyelinated fibres was significantly different between the crush and the section and resuturing group within both time periods post nerve injury. However, within the same group of nerve lesion (crush respectively section and resuturing) the distribution of discharge properties was not significantly different between both time periods (1 to 3 weeks and 1 to 4 months) post nerve lesion.

- *Mechanical sensitivity*

In the intact sural nerve 34/97 (35%) of the unmyelinated fibres could be activated by mechanical stimuli applied to the skin and among these the great majority (32/34) were high threshold mechanonociceptors; the remaining 2 units were low threshold

mechanoreceptors. Crush lesion did not lead to a significant change in the proportion of C-fibres which exhibited mechanosensitivity either in the first or in the second time period of investigation whereas following section and resuturing lesion both 1 to 3 weeks and 1 to 4 months post nerve injury, a significantly higher proportion of lesioned C-fibres were responsive to mechanical stimulation. Within the first time period post lesion almost all mechanosensitive C-fibres had the receptive fields along the lesioned nerve whereas in the second time period 66% (47/71) of the mechanosensitive C-fibres responded to mechanical stimuli applied to the nerve, the remaining 34% (24/71) had already reinnervated the skin. The mechanosensitive fibres which had their receptive fields in the nerve exhibited an increased excitability indicated by low mechanical thresholds (in the innocuous range) and increased incidence of spontaneous activity compared to mechanosensitive fibres in control (unlesioned) nerves. Among the mechanosensitive C-fibres which could be excited from the nerve 75% of the units in the first time period post lesion and 45% of the units in the second time period post lesion had mechanical thresholds ranging from 0.45 to 45 mN (mean = 7.6 mN). Within both time periods post nerve lesion 57% (24/42) of the mechanosensitive C-fibres in the crush groups and 26% (21/80) of the C-fibres in the section and resuturing group which had their receptive fields on the nerve were spontaneously active while in the intact sural nerve spontaneous activity was present only in 4 of 34 mechanosensitive C-fibres.

Among the regenerated mechanosensitive C-fibres which had reinnervated the skin 90% were mechanonociceptors which could be activated by von Frey filaments with bending force of ≥ 100 mN or by noxious pinch stimulation. The regenerated afferents had receptive field properties similar with those found in control animals and appeared quite similar to the control receptors.

- *Heat sensitivity*

The response to heat stimulation is mediated by the vanilloid receptor type 1 (VR1) and vanilloid receptor like type 1 (VRL1) which are expressed in a subpopulation of neurones with unmyelinated and thinly myelinated fibres and have activation thresholds around 43° respectively 52°C (Caterina et al., 1997, 1999). In naive animals (no nerve lesion) 44% (43/97) of the sural nerve C-fibres responded to heat stimuli applied to the skin of the hind paw sural nerve territory and most of them (92%) were activated at temperatures higher

than 40°C. Within the first three weeks following sural nerve injury a significant lower proportion of C-fibres investigated responded to heat stimuli than in the control group (15% in the crush group respectively 23% in the section and resuturing group). In the second time period post nerve injury the reduction in the proportion of heat sensitive fibres was significant only following section and resuturing lesion.

Michael and Priestley (1999) reported that axotomy of the sciatic nerve leads to down-regulation of VR1 in the corresponding lesioned DRG cells which might be correlated with the loss of target derived neurotrophines (NGF respectively GDNF). Although in case of nerve lesion followed by regeneration (as in crush or section and resuturing) the injured fibres come in contact with local trophic factors produced by Schwann cells distal to the lesion site (Heumann et al., 1987; Lindholm et al., 1987) a significantly lower proportion of the C-fibres investigated were responsive to heat stimulation. The reduction in proportion of heat sensitive fibers might be due to downregulation of VR1 as a result of lack of appropriate amounts of neurotrophic factors produced and released by Schwann cells following the lesion.

On the other hand, both following crush and section and resuturing lesion in the first and second time period post injury, the activation thresholds of the heat sensitive C-fibres which could be excited from the lesioned nerve were significantly lower than those of the heat sensitive units in the control group. Around 60% (37/62) of the heat sensitive neurones with the receptive fields on the nerve were excited at $\leq 39^{\circ}\text{C}$ whereas in the control group only 8% of the heat receptors with peripheral endings in the skin were excited at these intensities of heat stimulation. The reduction in the activation threshold of the lesioned and regenerating heat sensitive neurones might be related to the processes which take place in injured nerves during regeneration. The injured and regenerating nerve fibres grow in an environment rich in growth factors and cytokines produced by Wallerian degeneration distal to the nerve lesion and it is known that the surrounding milieu modulate the transduction characteristics of the sensory neurones. *In vivo* observations show that inflammation leads to changes in receptive properties of nociceptors so that the affected tissues become sensitive to normally non-noxious stimuli. NGF appears to play an important role in this process (Koltzenburg, 1999). Investigations done *in vitro* on DRG neurones showed that NGF can sensitise the heat sensitive neurones acting directly by phosphorylation of the VR1 receptor (Shu and Mendell, 1999). Sensitisation implies an

increased sensitivity of the heat-responsive neurones so that they are activated at lower temperatures. The cytokines and growth factors produced by Wallerian degeneration distal to the lesion site (for review see Sommer, 2001) can simulate the inflammatory conditions which occur in the peripheral tissues during inflammation and cause changes in sensitivity of the lesioned and regenerating nociceptive fibres. Another hallmark of an altered excitability of heat nociceptors is the development of ongoing activity. Both in the first and second time period post nerve lesion a significant higher proportion of heat-sensitive C-fibres exhibited spontaneous activity (21% respectively 42%) compared to the intact sural nerve where 3 of 43 (7%) of the heat sensitive fibres were spontaneously active.

The lesioned heat sensitive fibres which had successfully regenerated and reached the skin were activated at temperatures higher than 40°C which correspond to the activation temperature of the intact heat sensitive neurones but the sample size of regenerated heat C-fibres was too small for definitive comparison.

- *Cold sensitivity*

Twenty one per cent (20/97) of the the intact sural nerve C-fibres reacted to cold stimuli applied to the skin. One to 3 weeks following crush lesion a significantly higher proportion of C-fibres exhibited cold sensitivity compared to the intact (control) nerve (29/79 versus 20/97; $p < 0.01$, Chi^2 -test). Section and resuturing lesion did not lead to a significant change in the proportion of C-fibres which exhibited cold sensitivity compared to the intact sural nerve either in first or in second time period post lesion.

Recent *in vitro* studies done on intact DRG neurones showed that the cold receptors belong to the same family of receptor proteins (TRP channels) like VR1 (McKemy et al., 2002). While the thermal thresholds for the heat sensitive fibres are well defined, the literature regarding the activation thresholds of the cold sensitive fibres shows a wide variability. Thus, the receptor types that transduce sensations of innocuous cold versus noxious cold are not so firmly established. Following sural nerve lesion 54/62 (87%) of the cold sensitive fibres in both periods of investigation reacted to cooling steps lower than 10°C and some of them being activated when the temperature on the nerve decreased by 2-3°C. Besides the increased sensitivity of the cold sensitive fibres to small changes in temperature, following sural nerve crush a significantly higher proportion of cold sensitive C-fibres exhibited spontaneous activity compared to the intact sural nerve.

4.I.4.4. Combination of discharge properties in the myelinated and unmyelinated nerve fibres

Both following crush and section and resuturing lesion the majority (365/397; 92%) of the myelinated fibres investigated 1 to 3 weeks and 1 to 4 months post lesion exhibited only one discharge property. The remaining 8% showed a combination of 2 or 3 discharge properties. Most (372/397) A-fibres were mechanosensitive. In some myelinated fibres mechanosensitivity was associated with thermosensitivity (N=13), spontaneous activity (N=12) or both (N=4). Distribution of discharge properties of the lesioned and regenerating nerve fibers was similar following crush and section and resuturing and did not differ from distribution of discharge properties of the intact nerve fibres in control animals.

In the unmyelinated fibre population 46% (143/313) of the C-fibres belonging to both groups of lesion exhibited a combination of 2 or 3 discharge properties. No significant difference in the proportion of fibres with 2 respectively 3 discharge properties was found between the lesioned and regenerating C-fibres and the intact C-fibres in control animals. Thermosensitivity was the main discharge characteristic of the intact and lesioned C-fibres. In the intact sural nerve 43 of 97 C-fibres were heat sensitive and 20 of them reacted also to mechanical stimuli. Only 4 of the mechanical and/or heat sensitive fibres were spontaneously active. In the control nerve spontaneously activity was mainly found in the cold sensitive fibres. Following nerve lesion the incidence of spontaneous activity increased significantly both in the cold sensitive but also mechano and heat sensitive fibres.

4.II. Spared Nerve Injury model

Peripheral nerve lesion leads in humans to loss of sensory function in the denervated area whereas the territories adjacent to the area served by the lesioned nerves may develop hypersensitivity to innocuous mechanical and/or thermal stimuli. Decosterd and Woolf (2000) developed a nerve lesion model which consists of ligation and section of 2 branches of the sciatic nerve (namely tibial and common peroneal nerves) sparing the sural nerve. They called this model spared nerve injury (SNI) model. The SNI model enables to test the sensory changes in the skin territories neighbouring the denervated area. Following SNI the rats develop an increased sensitivity to normally innocuous mechanical and cold stimuli applied to the sural nerve territory of the hind paw ipsilateral to the lesion side whereas heat sensitivity remains unchanged. Sural afferent neurones (which are spared from axotomy) encode the peripheral stimuli and transfer the information to the spinal cord. In the present study the electrophysiological changes in the intact sural nerve fibres following lesion of the neighbouring nerves were investigated and correlated with the abnormal sensory reactions which are displayed by rats following SNI.

4.II.1. Possible mechanisms leading to abnormal behavioural reactions developed by rats following peripheral nerve lesion.

Lesion of a peripheral nerve leads to pathophysiological changes in the injured primary afferent neurones, in the intact neurones neighbouring the lesioned neurones and in the second order neurones in the spinal cord. Under normal conditions there is a permanent centripetal and centrifugal transport of molecules between the peripheral terminals and the cell bodies of the primary afferent neurones. Trophic factors are continuously taken up from peripheral targets and transported to the cell bodies of the afferent neurones where they regulate the synthesis of a variety of molecules such as neuropeptides, transduction molecules (mechanical and thermal receptors) and ionic (Na^+ , K^+ , Ca^{2+}) channels (for review see McMahon and Bennett, 1999). The receptor proteins and ionic channels are then transferred via axoplasmic transport to the periphery where they are integrated in the receptive terminals of the afferent neurones serving as transducers for a variety of sensory stimuli and determining the excitability of the sensory terminals. Figure 4.1 addresses the

different mechanisms which might be important for the development of the abnormal behavioural reactions and which I will discuss now.

1. Following peripheral nerve lesion the afferent neurones become disconnected from the target tissues. The interruption of peripheral supply of trophic factors leads to *transcriptional* changes which consists in alterations in expression of genes encoding neuropeptides, receptor proteins and ionic channels in the injured neurones. There is a switch in the type of Na⁺ channels which are expressed. The slowly inactivating TTX-Resistant (TTX-R) Na⁺ channels are down-regulated while a TTX-Sensitive (TTX-S) Na⁺ channel with fast recovery from inactivation is up-regulated (Cummins and Waxman, 1997). The rapid repriming from inactivation of the TTX-S Na channel shortens the refractory period of the injured neurones and predispose them to spontaneous firing. Overall the excitability of injured afferent neurones with both myelinated and unmyelinated nerve fibres is increased and both populations of neurones can develop spontaneous activity following nerve lesion (Abdulla and Smith, 2001).

2. Peripheral nerve lesion may also lead to neurophysiological changes in the intact neurones innervating territories adjacent to a denervated area. In partially denervated tissues, molecules associated with degeneration of the injured myelinated and unmyelinated axons and neuroactive substances released from reactive Schwann cells, fibroblasts and keratinocytes expose the peripheral terminals of the intact afferent nerve fibres to an altered environment. This altered milieu may potentially act on the intact afferent nerve terminals and induce *post-translational* changes of receptor proteins and ionic channels. Post-translational changes of the receptor proteins (i.e., by phosphorylation) alter their activation thresholds so that they are excited by stimuli of lower intensity. This modulation increases the sensitivity of peripheral terminals and contributes to *peripheral sensitisation*. Post-translational changes of the ionic (Na⁺) channels may alter their activation characteristics (modulate their gating properties) and lead to an increased current through these channels, increased depolarization and development of spontaneous activity in the intact afferents.

3. Spontaneous activity developed in some of the lesioned and unlesioned myelinated and unmyelinated fibres (Michaelis et al., 1995, 1999; Blumberg and Janig, 1984; Rivera et al., 2000; Wu et al., 2001; Ali et al., 1999) impinge continuously on the spinal cord. This leads to depolarisation of the second order neurones in the dorsal horn and increases their

excitability (Woolf CJ, 1983; Woolf and Thompson, 1991; Dubner and Ruda, 1992). The increased excitability of second order neurones is expressed in an increase of the receptive field, decrease of the activation threshold, and increase or development of spontaneous activity. This state is commonly called *central sensitisation* or *central hyperexcitability*.

4. Following peripheral nerve lesion cell bodies of axotomized neurones and of intact neurones co-exist in the same Dorsal Root Ganglion (DRG). This situation can permit a cross-communication between the cell bodies of the two populations of neurones. Michaelis et al., (2000) showed that after transection of the 3 major branches of the sciatic nerve about 20% of the intact afferent neurones projecting to the gastrocnemius soleus muscle developed spontaneous activity and the spontaneous activity originated in the DRG. Because there was no spontaneous activity in the muscle afferents originating in the DRG in control animals before any previous nerve lesion they hypothesised that a paracrine signal induced or generated by axotomized DRG neurones triggered the ectopic spontaneous activity in non-axotomized afferent neurones. The nature of this paracrine signal which induces ectopic activity after nerve injury is unknown; it might be produced by afferent cell bodies or by the satellite cells. Tsuzuki et al., (2001) reported that the expression of mRNA for the ATP-P₂X₃ receptor is up-regulated in intact primary afferent neurones after lesion of neighbouring nerves. These *transcriptional* changes in the intact afferent neurones could be explained by a model of non-synaptic cross-communication in sensory ganglia. Impulse activity in the lesioned neurones leads to release of a variety of neuropeptides and excitatory amino-acids (references in Shinder and Devor, 1994) from the cell soma of lesioned neurones into the extracellular space of the DRG. These chemical mediators can diffuse and have access to receptors on the soma membrane of adjacent intact DRG neurones where they may induce transcriptional changes and alter the expression of genes encoding transducer proteins and ionic channels.

5. The transducer molecules and ionic channels synthesised in the DRG cells are then transferred by axoplasmic transport anterograde to periphery where they are incorporated in the receptive terminals of the intact neurones.

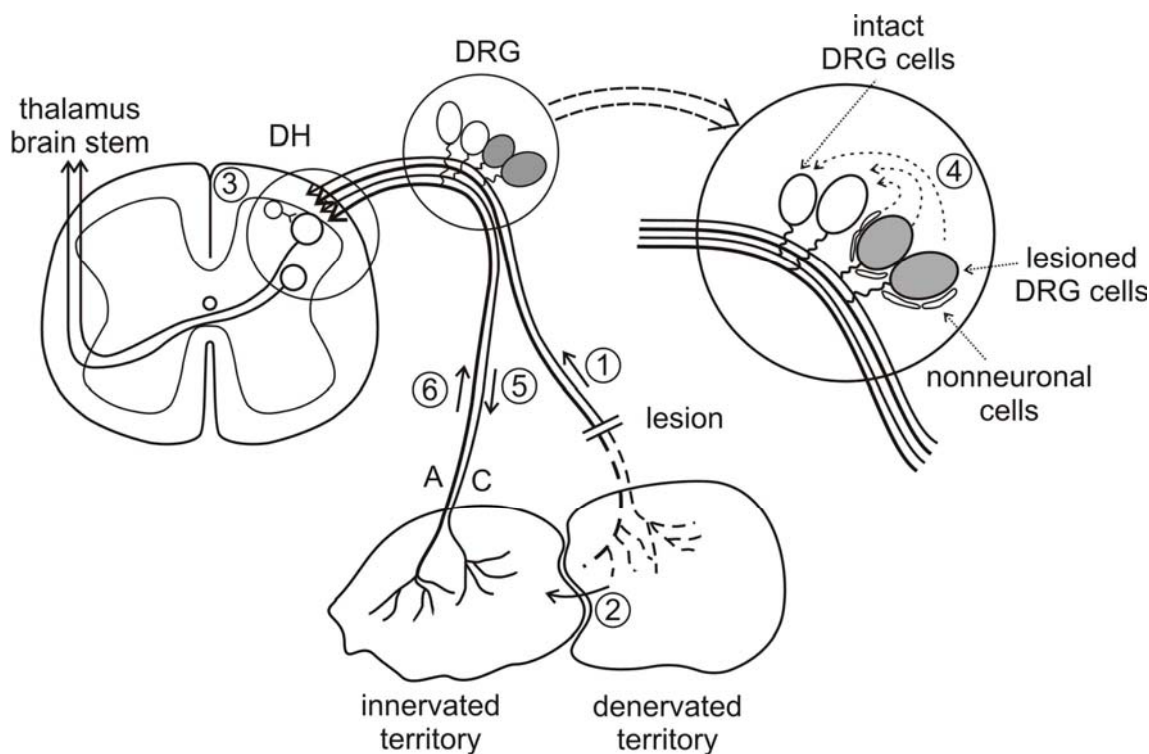


Fig. 4.1. Changes in the lesioned and intact afferent neurones following peripheral nerve lesion

1. Nerve lesion leads to translational and transcriptional changes in the injured afferent neurones which may result in development of spontaneous activity in some of the myelinated and unmyelinated fibres. **2.** Molecules released in the denervated territory may diffuse in the adjacent innervating territory and produce post-translational changes of the pre-existing receptor proteins and ionic channels in the peripheral terminals of the intact neurones. Post-translational changes of Na^+ channels may lead to development of spontaneous activity in the intact afferent fibres. **3.** Spontaneous activity which appear in some of the injured and intact A- and C-fibres following peripheral nerve lesion impinge on the spinal cord where it may increase the excitability of the second order neurones in the dorsal horn (DH). **4.** Following peripheral nerve lesion cell bodies of axotomized neurones and of intact neurones co-exist in the same Dorsal Root Ganglion (DRG) which enables a cross-communication between the two populations of afferent neurones. Paracrine signals produced and released by axotomized DRG cells or by satellite cells may induce transcriptional changes in the cell soma of intact neurones. **5.** The transducer molecules and ionic channels synthesised in the intact DRG cells are then transported anterograde to the periphery where they are incorporated in the receptive terminals of the intact neurones. **6.** The increase in expression of transduction molecules and ionic channels can cause an exaggerated sensory transmission by the intact neurones. Abbreviations: A = myelinated fibres; C = unmyelinated fibres; DH = dorsal horn; DRG = dorsal root ganglion

6. The increase in expression of transduction molecules together with the increased membrane density of Na^+ channels can cause an exaggerated sensory transmission through the intact neurones by facilitation of transduction mechanisms in the peripheral terminals and increased release of neurotransmitters at the central terminals in the dorsal horn.

The mechanisms presented above may operate either alone or together and with different time courses to produce the complex picture of behavioural reactions in response to peripheral innocuous stimulation .

4.II.2. Electrophysiological changes in the intact afferent nerve fibres following SNI and its correlation with signs of pain-like behaviour developed by rats after SNI

4.II.2.1 Spontaneous activity

In the sural nerve of control animals (no nerve lesion) spontaneous activity was encountered in 7/124 of the A- and 27/97 of the C-fibres, respectively. These data are in accordance to those reported by Leem et al. (1993) who showed that in the rats without nerve lesion 21% of the C-fibres in the intact sural nerve were spontaneously active and spontaneous activity was mainly present in the cold sensitive C-fibres; the unmyelinated mechanical and/or heat sensitive C-fibres exhibited only rarely spontaneous activity. In the myelinated fibre population spontaneous activity was present only in the mechanosensitive A-fibres which innervate deep somatic tissue.

Lesion of tibial and common peroneal nerves was followed by a significant increase in proportion of sural nerve afferent C-fibres which exhibit spontaneous activity compared to sural nerve C-fibres from normal animals (93/145 versus 28/97; $p < 0.001$; χ^2 -test). This increase was present within the first week post injury and persisted during the whole period of investigation. The rates of spontaneous discharge in the sural nerve C-fibres were not significantly different between SNI group and control group (mean = 1.63 versus 2.30 imp/s; $p > 0.05$).

Since we recorded centripetally conducted action potentials in afferent fibres the spontaneous activity must originate either along the course of peripheral sural nerve axons or at their peripheral terminals. Wu et al., (2001) reported that lesion of the L5 spinal nerve leads to development of spontaneous activity in afferent C-fibres of the intact L4 spinal nerve. The spontaneous activity could be abolished by application of lidocain to the cutaneous receptive field, suggesting that the receptive terminal of the afferent axon is a likely site for the generation of spontaneous activity (see 2 in Fig. 4.1). Lesion of tibial and common peroneal nerves leads to denervation of the target territories innervated by these nerves. The peripheral terminals of the intact sural nerve fibres are exposed to an altered environment which may potentially induce post-translational changes of the pre-existing Na^+ channels. These post-translational changes of the Na^+ channels alter their activation

characteristics which may potentially lead to an increased current through these channels and hyperexcitability of the uninjured afferents.

Following SNI the intermingling of injured and intact neurones in the periphery is restricted to the border territory between the lesioned tibial nerve and intact sural nerve. In this case the signals from the denervated target tissues and degenerating axons are unlikely to be the sole factors involved in generation of increased excitability of sural nerve C-fibres. A cross-communication between adjacent cell bodies of injured (axotomized) neurones and cell bodies of intact neurones can take place in DRG (see 4 in Fig. 4.1).

The cell bodies of the primary afferent neurones projecting in the sciatic nerve are located in L3, L4 and L5 DRGs (Baron et al., 1988). Following SNI the L4 and L5 DRGs have afferent cell bodies with intact axons (projecting in the sural nerve) and afferent cell bodies with lesioned axons (projecting in the tibial and common peroneal nerves). The situation of co-existence of injured and intact cells in a single DRG could permit a cross-communication between the two populations of neurones (see 4 in Fig. 4.1). Paracrine signals induced and released by axotomized DRG neurones can diffuse and act on neighbouring non-injured cells where they may modulate the expression of genes encoding ionic (Na^+) channels. These channels synthesised in the DRG cells are then transferred to the peripheral endings, where they are incorporated in the receptive terminals of the intact neurones and render them more excitable leading to spontaneous firing.

4.II.2.2. Mechanical sensitivity

Spared nerve injury (SNI) leads in rats to the development of increased sensitivity of the sural nerve territory of the ipsilateral hind paw to innocuous mechanical stimuli. This hypersensitivity was present starting on day 1 post lesion and lasted for at least 2 months following nerve injury. These data are in accordance with those published by Decosterd and Woolf (2000) who showed that following SNI the rats developed an early and long lasting mechanical allodynic-like behaviour. Mechanical allodynia, which is a painful sensation elicited by a non-noxious mechanical stimulus, can be produced either 1. by activation of sensitised peripheral nociceptive terminals which, following nerve lesion, have low activation thresholds as a consequence of *peripheral sensitisation*; (see 2 in Fig. 4.1) or 2. by activation of low threshold mechanoreceptors ($\text{A}\beta$ fibres) whose input is

processed by altered (sensitised) central neurones (e.g., in the dorsal horn ; see 3 in Fig. 4.1).

Following SNI, 97% (344/356) of the A-fibres investigated could be activated by mechanical stimulation. The activation threshold of the mechanosensitive A-fibres with the receptive endings in the skin did not differ significantly from those of the mechanosensitive A-fibres in the sural nerve of control animals (mean = 15.2 versus 14.7 mN; $p > 0.05$; Wilcoxon test). In the unmyelinated fibre population 46 of 145 sural nerve C-fibres following SNI could be activated by mechanical stimuli and most (89%) of them were mechanonociceptors which responded only to intense (≥ 100 mN) mechanical stimuli. Both the proportion of mechanosensitive C-fibres and their activation threshold did not change significantly following SNI compared to the control group. The present data indicate that lesion of the tibial and common peroneal nerves did not lead to a significant reduction of the activation threshold of both mechanosensitive A- and C- fibres which have their receptive endings in the sural nerve territory. Thus, the sensitisation of nociceptive terminals in the intact sural nerve cannot account for the mechanical allodynic behaviour developed in rats following SNI. _

This implies that the "central" component plays an important role in the development of the mechanical allodynic behaviour which appears in rats following SNI. The increase in incidence of spontaneous activity in the sural nerve C-fibres parallels the development of mechanical allodynia shortly (less than 24 hours) after SNI and might be a basis for the increased central sensitivity to normally innocuous mechanical stimuli. Wide dynamic range (WDR) neurones in the spinal cord receive convergent synaptic input both from A β fibres (connected with low threshold mechanoreceptors) and from C-fibres (connected to high threshold mechano-nociceptors) (Menetrey et al., 1977). Following SNI a significantly higher proportion of mechano-nociceptors displayed spontaneous activity compared to those in the control group (20/46 versus 3/34; $p < 0.001$; *Chi*²-test). Spontaneous activity developed by some of the injured and uninjured afferent neurones with C-fibres following peripheral nerve lesion depolarise the WDR (convergent) second order neurones and increase their excitability. The sensory input from low threshold mechanoreceptors carried centrally along residual intact A β afferents is abnormally processed by sensitised spinal second order neurones and leads to abnormal reactions to normally innocuous stimuli.

Other hypothesis such as functional reorganisation within the spinal cord due to collateral sprouting of A β fibres from their terminal area in laminae III-V into laminae II (Woolf et al., 1992; Woolf and Mannion, 1999) or spinal disinhibition due to degeneration of GABA-ergic inhibitory inter-neurons in lamina II (Moore et al., 2002) are difficult to reconcile with the rapid onset of the mechanical allodynia observed here. These mechanisms could account for the abnormal reactions to mechanical stimuli observed in later periods post nerve injury.

4.II.2.3 Cold sensitivity

Application of acetone to normal human skin elicits a non-painful cold sensation. Normal rats (without nerve lesion) did not respond to acetone applied to the plantar surface of the foot indicating that this stimulus is probably innocuous for them, too. Following SNI all rats developed an increased sensitivity to acetone stimulation (cold allodynic-like behaviour) which was present in less than 24 hours post nerve lesion and maintained over the whole period of investigation.

The sense of cold is elicited by activation of cold sensitive C-fibres. In the sural nerve territory the proportion of C-fibres which responded to cold stimuli was significantly higher following SNI compared to control animals (54/145 versus 20/97; $p < 0.005$; χ^2 -test). Moreover, the activation threshold of the cold-sensitive fibres was significantly increased following SNI lesion compared to the control group (mean activation threshold = 18.1 versus 14.6°C; $p < 0.01$).

Both in the SNI group and control group two populations of cold-sensitive C-fibres were present: low threshold fibres (activated when the temperature on the skin decreased by 2 - 4°C) and high threshold fibres (excited by reduction in temperature by $\geq 5^\circ\text{C}$). The proportion of low threshold cold sensitive C-fibres was higher following SNI than in the control group (19/33 versus 6/16) although the difference did not reach the level of significance. The low threshold cold sensitive C-fibres in the SNI group exhibited higher mean discharge rates during cooling than the low threshold fibres in the control group (mean = 10.4 versus 4.7 imp/s; $p < 0.005$; Wilcoxon test). Following lesion of tibial and common peroneal nerves a significant higher proportion of cold sensitive fibres in the intact sural nerve exhibited spontaneous activity compared to the sural nerve of the naive

animals (44/63 versus 10/33; $p < 0.001$; Chi^2 -test); however the rates of discharge did not differ significantly between SNI and control group (mean = 2.4 versus 2.7 imp/s ; $p > 0.05$). The peripheral changes - reflected in reduction of the mean activation threshold of the cold sensitive fibres and the increased discharge rate of the low threshold fibre - together with the increased incidence of spontaneous activity in cold sensitive fibres might be one basis for the abnormal reactions to innocuous cold stimuli developed by rats following SNI.

4.II.2.4. Heat sensitivity

Following SNI the rats exhibited a hypersensitivity to innocuous mechanical and cold stimuli applied to the sural nerve territory of the ipsilateral hind paw, while the heat sensitivity did not change in comparison with the pre-surgery values over the whole period of investigation. Lesion of tibial and common peroneal nerves leads to denervation of the target territories served by these nerves and consequently to an abnormal paw exposure so that only a part of the hind paw was in touch with the floor. Since the heat sensitivity, measured by the latency of paw withdrawal to a standard heat stimulus, is related to the heat transfer and may vary with the degree of paw contact, extreme care must be applied in interpreting the results obtained using this thermal test.

The response to heat stimuli is mediated by the vanilloid receptor type 1 (VR1) and vanilloid receptor-like type 1 (VRL 1) which are present on small (C) and medium size (A δ) primary afferent neurones. Experiments done *in vitro* in isolated DRG cells showed that VR1 is activated at $\geq 43^\circ\text{C}$ while VRL1 responds at $> 50\text{-}53^\circ\text{C}$ (Caterina et al., 1997, 1999). In the animals without nerve lesion, 92% of the heat sensitive fibres in the sural nerve were activated at temperatures higher than 40°C . The activation threshold of the sural nerve heat sensitive fibres was not significantly influenced following lesion of the neighbouring nerves, 84% of heat sensitive C-fibres in the SNI group being activated at temperatures higher than 40°C . The absence of change in threshold temperature of heat nociceptors reflect the lack of peripheral sensitisation and might be correlated with the lack of change in heat sensitivity.

While in the control group 44% (43/97) of the C-fibres responded to heat stimuli, following SNI the proportion of C-fibres which were excited by heat stimuli dropped significantly to 5% in first and second time period post injury and increased again to 23%

in the third period post lesion. Up to now we can not explain why the proportion of heat sensitive C-fibres dropped significantly within the first week post SNI.

4.III. Synopsis

Peripheral nerve lesion leads to impairment of sensory function in the area innervated by the lesioned nerve(s). In most clinical situations of nerve trauma the sensory function of the denervated territory is restored by regeneration of the lesioned nerve fibres to the peripheral target tissues. The recovery of sensory functions depends on the ability of the lesioned fibres to regenerate to the periphery and to form functional connections in the peripheral targets. The regenerative process may be accompanied by distortion in mechanical and thermal sensitivity and/or clinical symptoms of pain. Less than 5% of the patients who sustain a nerve injury followed by partial or complete regeneration suffer from neuropathic pain. Investigation of the potential peripheral and central mechanisms involved in generation and maintenance of neuropathic pain will be the basis for a mechanism-based classification of neuropathic pain (and therefore also of diagnostic criteria) and for the development of mechanism-based therapeutic approaches.

The present data show that within 4 months post sural nerve crush or sural nerve section and resuturing 80% of the A-fibres with functional properties had regenerated and reached the target tissues while only 29% of the mechano- and/or thermosensitive C-fibres had reached the peripheral tissues of the hind paw. The remaining A- and C-fibres which responded to mechanical or thermal stimuli were ectopically excited from the nerve. The impaired regeneration of the C-fibres leads to an incomplete reinnervation of the peripheral targets which may result in a loss of sensory function in the affected territory. Besides the impairment of the sensory function nerve lesions may be accompanied by abnormal sensations evoked by normally innocuous mechanical and/or thermal stimuli. Distortion of the sensory perception is due to: 1. neurophysiological changes which take place in the injured afferent fibres while they regenerate in the nerve and 2. central changes which are triggered by the peripheral changes. The lesioned and regenerating nerve fibres may develop abnormal spontaneous activity and ectopic mechanical and/or thermal sensitivity which might be involved in initiation of injury induced sensory disorders.

Forty-nine per cent of the lesioned and regenerating C-fibres investigated within both time periods post nerve lesion exhibited spontaneous activity. Spontaneous activity acquired by the nociceptive afferent C-fibres, which are normally concerned with signalling of noxious stimuli, leads to a tonic input in the absence of stimulation and might be responsible for symptoms of spontaneous pain reported by some patients with peripheral nerve lesion. Spontaneous activity which appears in some of the myelinated and unmyelinated afferent fibres impinge on the spinal cord neurones and increase the excitability of the second order neurones in the dorsal horn. This will lead to abnormal processing of the afferent impulses generated by the nociceptive and non-nociceptive afferent fibres.

Mechanical and thermal sensitivity of the lesioned and regenerating nerve fibres are probably intrinsic properties of the afferent neurones which are dependent on different transduction proteins in their peripheral terminals. These transduction proteins are modulated by post-translational and transcriptional mechanisms due to interactions with growth factors and products of Wallerian degeneration produced distal to the lesion site. Interaction of the growing nerve sprouts with these molecules leads to peripheral sensitisation of the sensory nerve endings so that they can be activated by stimuli in the non-noxious range. Within both time periods of investigation 65% of the mechanosensitive C-fibers with receptive fields in the nerve had low activation thresholds (0.45 to 45 mN; mean = 7.6 mN). This ectopic mechanosensitivity of injured and regenerating afferent C-fibres can account for pain during movement and increased sensitivity to stimuli applied to the site of axotomy or along the nerve trunk in patients with peripheral nerve lesion.

These changes in sensitivity of the lesioned and regenerating nerve fibres are caused by sensitisation of the peripheral terminals of the sensory neurones due to interactions of the fibres with molecules of regeneration and degeneration released after nerve injury in the distal stump of the nerve (growth factors, interleukines). Further studies of the mechanisms underlying the changes in the afferent neurones during regeneration may help to understand comparable clinical situations of sensory disorders and neuropathic pain during nerve regeneration. The ability to manipulate the receptive properties of the afferent nerve fibres can be an important experimental tool and may lead to new strategies in the therapy of neuropathic pain developed after peripheral nerve lesion.

Lesion of peripheral nerves leads in some patients to development of mechanical and/or thermal allodynia in the territories adjacent to the denervated area. In the past decade

different animal (rat) models employing distinct peripheral nerve lesion were developed. Following the lesion, the animals exhibit abnormal reactions to normally innocuous mechanical and/or thermal stimuli which are considered to be equivalent to different components of symptoms of neuropathic pain in human patients such as mechanical or thermal allodynia. These animal models are used to study the mechanisms involved in development and maintenance of neuropathic pain. While almost all the animals that underwent a distinct nerve lesion, such as spinal nerve lesion or spared nerve injury, developed signs of pain-like behaviour, in humans only 5% or less of the subjects with lesion of large peripheral nerves develop signs of neuropathic pain. At present we have no explanation for this discrepancy between the animal behavioural data and the data obtained on humans. One essential factor for development of neuropathic pain is the size of the lesioned nerve(s). Another important factor may turn out to be the genetic predisposition to develop neuropathic pain which has not been systematically investigated up to now. Therefore, great care must be taken in the interpretation of the results obtained from the behavioural and reduced animal models. However, the use of animal models provides insight into the possible cellular mechanisms which lead to the generation of neuropathic pain. Understanding these mechanisms is important for the development of novel therapeutic treatment strategies of neuropathic pain and the use of animal models can predict drug efficacy and responsiveness to different treatment modalities.

5. SUMMARY

The pathophysiological mechanisms of the neuropathic pain following nerve lesion are related to changes in injured afferent neurones, in uninjured neighbouring afferent neurones and in central neurones. In the present study the neurophysiological changes in lesioned and regenerating fibres and intact afferent nerve fibres, innervating a territory adjacent to a denervated area, were investigated. Two models of nerve lesion in the rat were used: 1. The sural nerve was either *crushed* or *sectioned and resutured* allowing the nerve fibres to regenerate to the target tissue (*Regeneration Model*). 2. The tibial and common peroneal nerves were ligated and sectioned, leaving the sural nerve intact (*Spared Nerve Injury [SNI] Model*). In anaesthetised rats spontaneous activity (SA), mechanosensitivity (MS) and thermosensitivity (TS) of the sural afferent myelinated (A-) and unmyelinated (C-) fibres were investigated neurophysiologically *in vivo* 4 days to 4 months in the *Regeneration Model* or 1 day to 10 weeks post *SNI*. In the *SNI* model pain-like behaviour of the rats to mechanical and thermal stimulation of the skin was tested.

A. *Regeneration Model*. Within 3 weeks post nerve lesion the injured nerve fibres were still regenerating in the nerve, some being trapped at the lesion site. One to 4 months post nerve lesion 80% of the A-fibres but only 29% of the C-fibres with functional properties had regenerated and were activated by physiological stimuli from the target tissues of the hind paw. The remaining A- and C-fibres had their receptive fields in the lesioned nerve and had one to three of the following properties: SA, MS, TS. The MS and TS fibres which had regenerated to the target tissues had activation thresholds similar to those in the control animals. The MS and TS fibres with receptive fields in the lesioned nerve had lower activation thresholds than those in the control animals. The incidence of SA was significantly higher in regenerating nerve fibres than in intact nerve fibres in control rats.

B. *SNI Model*. Following *SNI* rats developed an increased behavioural sensitivity of the ipsilateral sural nerve territory to innocuous mechanical and cold stimuli appearing in 24 hours and persisting during 8 weeks of testing. The incidence of SA C-fibres was significantly higher, but the proportion and the activation threshold of the MS A- and C-fibres were not significantly different following *SNI* compared to control animals. Thus the behavioural changes to mechanical stimulation in *SNI* rats might be due to abnormal sensory processing of sensitised dorsal horn neurones in the spinal cord. The proportion of cold sensitive C-fibres was significantly higher and their activation threshold was significantly lower following *SNI* compared to control animals. These alterations might be the basis for abnormal reactions to innocuous cold stimuli of *SNI* rats.

C. *Conclusions*: Neuropathic pain in patients with nerve injury may be related to increased sensitivity of lesioned and unlesioned primary afferent neurones. Unravelling the underlying cellular mechanisms of this increased sensitivity to innocuous stimuli could lead to new therapeutic tools in treatment of neuropathic pain.

ZUSAMMENFASSUNG

Die pathophysiologischen Mechanismen neuropathischer Schmerzen nach Nervenläsionen hängen von den Veränderungen in den geschädigten afferenten Neuronen, intakten benachbarten afferenten Neuronen und in zentralen Neuronen ab. In dieser Arbeit wurden in der Ratte die neurophysiologischen Veränderungen in geschädigten und regenerierenden Afferenzen und in intakten Afferenzen zu Territorien, die dem denervierten Arealen benachbart sind, untersucht. Zwei Nervenläsionsmodelle wurden benutzt: 1. Der N. suralis wurde entweder *gequetscht* oder *durchtrennt und wieder angenäht*, so dass die Nervenfasern zum Zielgewebe regenerieren konnten (*Regenerationsmodel*). 2. Der N. tibialis und der N. peroneus communis wurden ligiert und durchtrennt, wobei der N. suralis intakt gelassen wurde („*Spared Nerve Injury*“ [*SNI*] Model). Spontanaktivität (SA), Mechanosensibilität (MS) and Thermosensibilität (TS) der afferent myelinisierten (A) und nichtmyelinisierten (C) Nervenfasern im N. suralis wurden neurophysiologisch *in vivo* 4 Tage bis 4 Monate nach Läsion im Regenerationsmodel und 1 Tag bis 10 Wochen nach Läsion im SNI Model untersucht. Im SNI Model wurde das schmerzhaft Verhalten der Ratten auf mechanische und thermische Reizung der Haut untersucht.

A. *Regenerationsmodel*. Innerhalb von 3 Wochen nach Nervenläsion waren die durchtrennten Nervenfasern im Prozess der Regeneration. Ein bis 4 Monate nach der Nervenläsion konnten 80% der A-Fasern aber nur 29% der C-Fasern mit physiologischen Entladungsverhalten vom Zielgewebe der Hinterpfote aktiviert werden. The restliche A- und C-Fasern hatten ihre rezeptiven Felder im Nerven. Afferenzen mit rezeptiven Feldern im Nerven hatte eine bis drei der folgenden funktionellen Eigenschaften: SA, MS, TS. Die Aktivierungsschwellen der MS und TS Afferenzen, die zum Zielgewebe regeneriert waren, waren quantitativ von denen in Kontrolltieren nicht zu unterscheiden. Die MS und TS Afferenzen mit rezeptiven Feldern im geschädigten Nerven hatten niedrigere Aktivierungsschwellen als die Hautafferenzen in Kontrolltieren. Außerdem war die Häufigkeit von Spontanaktivität in den geschädigten Afferenzen höher als in intakten Hautafferenzen in Kontrolltieren.

B. *SNI Model*. Nach SNI zeigten Ratten in ihrem Verhalten quantitativ im ipsilateralen Territorium des N. suralis eine erhöhte Empfindlichkeit auf nichtnoxische Reizung von Mechano- und Kaltrezeptoren. Diese erhöhte Empfindlichkeit trat nach 24 Stunden auf und blieb über den Testzeitraum von 8 Wochen bestehen. Die Häufigkeit von SA C-Fasern war signifikant höher, aber die die Häufigkeit und Aktivierungsschwellen der MS A- and C-Fasern waren nicht verschiedenen von denen in Kontrolltieren. Hieraus folgt, dass die Verhaltensveränderungen auf mechanische Hautreizung in Ratten mit SNI vermutlich eine Folge der veränderten Informationsverarbeitung in sensibilisierten spinalen Hinterhornneuronen ist. Die Häufigkeit von Kälte-sensiblen C-Fasern war signifikant höher und ihre Aktivierungsschwellen waren signifikant niedriger in Ratten mit SNI als in Kontrollratten. Diese Veränderungen können das veränderte Verhalten der SNI Ratten auf Kaltreize erklären.

C. *Schluss*: Neuropathische Schmerzen in Patienten mit Nervenläsionen können teilweise erklärt werden durch veränderte Sensibilität geschädigter und ungeschädigter primär

afferenter Neurone. Aufklärung der zellulären Mechanismen, die diesen Veränderungen zugrunde liegen, kann zu neuen Ansätzen

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