

**Mechano- and thermosensitivity of
muscle afferents 4 hours to 7 days after nerve injury
and their responsiveness to TRP agonists**

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Der Dekan

“... The progress of science is tremendously disorderly, and the motivations that lead to this progress are tremendously varied, and the reasons why scientists go into science, the personal motivations, are tremendously varied. I have said ... that science is a haven for freaks, that people go into science because they are misfits, and that it is a sheltered place where they can spin their own yarn and have recognition, be tolerated and happy, and have approval for it...”

Interview with *Max Delbrück* (1978), p. 87. Oral History Project, California Institute of Technology Archives, Pasadena, California.

“... If you're too sloppy, then you never get reproducible results, then you never get reproducible results, and then you never can draw any conclusions; but if you are just a little sloppy, then when you see something startling, (...) you nail it down (...). So I called it the "Principle of Limited Sloppiness"...”

Interview with *Max Delbrück* (1978), p. 76-77. Oral History Project, California Institute of Technology Archives, Pasadena, California.

Summary

Injury of limb nerves leading to neuropathic pain mostly affects also deep somatic nerves including muscle nerves. The role of the afferent and efferent innervation of deep somatic tissues in triggering and maintaining neuropathic pain remains unclear and has not been studied so far. Ectopic impulses in the injured myelinated and unmyelinated afferents occurring spontaneously or being evoked by mechanical or thermal stimulation are considered to be crucial for the development of neuropathic pain. Functional discharge characteristics and molecular transduction mechanisms underlying the mechano- and thermosensitivity of injured muscle afferents have to date not been systematically studied and are practically unknown.

This study on rats concentrates on the discharge characteristics of myelinated (A-fibers) and unmyelinated (C-fibers) muscle afferent neurons using neurophysiological techniques *in vivo*, 4-13 hours or 4-7 days following crush lesion of the lateral *gastrocnemius-soleus* nerve. The injured muscle afferent neurons were studied for: **(a)** their responsiveness to mechanical and thermal stimulation; the rate and pattern of spontaneous activity; the conduction velocity of the injured axons with respect to their response properties to the physiological stimuli and to the spontaneous activity; the response pattern (combinations of responses) to physiological stimuli; **(b)** their responsiveness to local application of the transient receptor potential (TRP) agonists capsaicin (TRPV1), menthol (TRPM8) and mustard oil (TRPA1); **(c)** their responsiveness to activation of postganglionic axons by electrical stimulation of the sympathetic chain; and **(d)** their afterdischarge to electrical stimulation of the muscle nerve.

(1) Four to 13 hours after muscle nerve injury ectopic activity is almost absent in A-fibers but already present in many C-fibers. Four to 7 days after lesion of the muscle nerve almost all injured afferent A- and C-fibers develop ongoing and/or evoked activity originating at the injury site. Mechanosensitivity is prominent in the majority of A- and C-fibers. Heat and cold sensitivity in injured A-fibers and cold sensitivity in injured C-fibers appear to be novel functional properties. In the first two weeks after a nerve injury the proportion of A- and C-fibers exhibiting ectopic activity are double as high in a muscle nerve than in a skin nerve.

(2) Capsaicin dose-dependently activates most heat-sensitive C- and some heat-sensitive A-fibers and desensitizes almost all heat-sensitive muscle afferents for heat stimuli without affecting cold- or mechanosensitivity. Thus heat-sensitivity of most C- and some A-muscle afferents may be dependent on the TRPV1 channel. Heat-sensitivity of the capsaicin-insensitive A-fibers might be mediated by other channels (e.g., the TRPV2 channel, which is reported to be present mainly in medium- to large-diameter primary afferent neurons). Menthol dose-dependently activates all cold-sensitive C-fibers and sensitizes their cold response. Most cold-sensitive A-fibers are depressed in their activity and desensitized in their responses to cold stimuli by menthol. Cold-sensitivity of injured unmyelinated muscle afferents is probably mediated by the TRPM8 channel. However, other channels (e.g., the TRPA1 channel) may be involved in the novel cold- sensitivity of injured muscle A- afferents.

(3) Sympathetic-afferent coupling is probably absent 4-7 days after the lesion of the muscle nerve. Longer regenerating time periods are probably required to accomplish this afferent-efferent cross-talk.

(4) About 20% of the myelinated muscle afferents, but no afferent C-fibers, show afterdischarges in response to suprathreshold electrical stimulation of the muscle nerve with a few impulses at 1 Hz. Lidocaine without causing conduction block or affecting the rate of spontaneous activity, dose-dependently decreases the afterdischarge arguing that the afterdischarge is probably mediated by specific sodium channels.

Whether and to what degree the discharge properties of injured muscle afferents contribute to deep neuropathic pain, dysesthesias and paresthesias as well as other functional changes in patients with nerve injury has to be explored.

Zusammenfassung

Schädigung von Extremitätennerven, welche zu neuropathischen Schmerzen führen, betreffen meistens tiefe somatische Nerven einschließlich Muskelnerven. Die Rolle der afferenten und efferenten Innervation tiefer somatischer Gewebe in der Erzeugung und Aufrechterhaltung neuropathischer Schmerzen ist unklar und wurde bisher experimentell mit neurophysiologischen Methoden wenig untersucht. Die spontane oder evozierte ektope erzeugte Impulsaktivität in geschädigten afferenten Neuronen mit myelinisierten oder unmyelinisierten Axonen ist wichtig in der Erzeugung und Aufrechterhaltung neuropathischer Schmerzen. Das funktionelle Entladungsverhalten und die molekularen Transduktionsmechanismen, welche der Mechano- und Thermosensibilität geschädigter Muskelafferenzen zugrunde liegen, sind bisher nicht systematisch untersucht worden und praktisch unbekannt.

Die vorliegende experimentelle Arbeit an der Ratte beschreibt das Entladungsverhalten myelinisierter (A-Fasern) und unmyelinisierter (C-Fasern) Muskelafferenzen, welches mit neurophysiologischen Methoden *in vivo* 4-13 Stunden und 4-7 Tage nach Quetschläsion des Nervus gastrocnemius-soleus zu lateralen Wadenmuskulatur der Rattenhinterextremität herausgearbeitet worden ist. Folgende Merkmale der geschädigten afferenten Muskelneurone wurden untersucht: **(a)** Die Entladungen auf mechanische und thermische Reizung; die Höhe und das Muster der Spontanaktivität; die Leitungsgeschwindigkeiten der Afferenzen aufgeteilt nach ihrem Entladungsverhalten. **(b)** Die Erregbarkeit der Afferenzen auf die lokale Applikation der transient receptor potential (TRP) Agonisten Capsaicin (TRPV1), Menthol (TRPM8) und Senföl (TRPA1). **(c)** Die Erregbarkeit der Afferenzen bei Aktivierung der postganglionären Neurone, die in den geschädigten Muskelnerven projizieren, durch elektrische Reizung des (sympathischen) lumbalen Grenzstranges. **(d)** Die Nachentladung der Muskelafferenzen auf elektrische Reizung des Muskelnerven.

(1) Vier bis 13 Stunden nach Nervenläsion sind A-Fasern fast nicht ektope aktiv, während viele C-Fasern schon ektope Aktivität zeigen. Vier bis 7 Tage nach Nervenläsion zeigen praktische alle afferenten A- und C-Fasern Spontanaktivität und/oder mechanisch oder thermisch erzeugte Aktivität, die am Läsionsort entsteht. Die meisten A- und C-Fasern sind mechanosensibel. Hitze- und Kaltsensibilität sind neue funktionelle Eigenschaften der geschädigten afferenten A- und C-Fasern. Die Zahl von afferenten A- und C-Fasern mit ektope erzeugter Aktivität ist doppelt so hoch in den geschädigten Muskelnerven als im geschädigten Hautnerven.

(2) Capsaicin aktiviert dosisabhängig die meisten hitzesensiblen C-Fasern und einige hitzesensiblen A-Fasern und desensibilisiert praktisch alle hitzesensiblen Afferenzen für Hitzereize ohne die Antworten auf Kaltreize oder mechanische Reize zu beeinflussen. Die Hitzesensibilität der geschädigten Afferenzen scheint deshalb durch den TRPV1-Kanal vermittelt zu werden. Bei Capsaicin-insensiblen aber hitzesensiblen A-Fasern kann der TRPV2-Kanal, der in afferenten Neuronen mit großen Zellkörpern nachgewiesen worden ist, eine Rolle spielen. Menthol aktiviert dosisabhängig alle kaltsensiblen C-Fasern und sensibilisiert sie für Kaltreize. Menthol hemmt die ektope Aktivität in den meisten kaltsensiblen A-Fasern und desensibilisiert diese Fasern für Kaltreize. Die Kaltsensibilität geschädigter C-Fasern wird wahrscheinlich durch den TRPM8-Kanal vermittelt. Andere TRP-Kanäle (z.B. der TRPA1-Kanal) können die Kaltsensibilität in geschädigten A-Fasern vermitteln.

(3) Eine funktionelle Kopplung von sympathischen efferenten Fasern zu geschädigten Muskelafferenzen ist 4 bis 7 Tage nach Nervenläsion praktisch nicht nachweisbar. Längere Zeitintervalle nach Nervenläsion sind vermutlich nötig für die Entwicklung dieser pathologischen Kopplung nach Nervenläsion.

(4) Überschwellige elektrische Reizung des Muskelnerven mit wenigen Impulsen erzeugt Nachentladungen in etwa 20% der myelinisierten Afferenten, jedoch nicht in unmyelinisierten Afferenzen. Diese Nachentladung ist abhängig von der Reizstärke und kann vollständig blockiert werden durch Applikation von Lidokain an den Reizelektroden in Konzentrationen, die weder die Fortleitung noch die evozierten Aktivitäten blockieren. Die Nachentladungen werden vermutlich durch spezielle Natriumkanäle vermittelt.

Ob und in welchem Ausmaß das ektope Entladungsverhalten mechanisch geschädigter Muskelafferenzen zu neuropathischen Schmerzen, Dysästhesien, Parästhesien und sonstigen funktionellen Störungen bei Patienten mit Nervenläsionen beiträgt, werden zukünftige Untersuchungen zeigen müssen.

List of Abbreviations

AC	Adenylate cyclase
AD	Afterdischarge
ADC	Analog-to Digital Convertor
APs	Action potentials
ASIC	Acid Sensing Ion Channel
ATP	Adenosine Triphosphate
B2	Bradykinin receptor
BDNF	Brain-derived neurotrophic factor
BK	Bradykinin
CAP	Capsaicin
CCI	Chronic constriction injury
CFA	Complete Freund's Adjuvant
CL	Crush lesion
CNS	Central nervous system
CRPS	Complex regional pain syndrome
CU	Cross-union
CV	Conduction Velocity
DAG	Diacyl glycerol
DMSO	Dimethyl sulfoxide
DRG	Dorsal Root Ganglia
E-C	Excitation-contraction
ECG	Electrocardiography
EI	Electrical identification
EP2 or EP4	Prostaglandin E receptor
EPI	Epinephrine
ER	Endoplasmic reticulum
ES	Electrical stimulation
G	Protein G
GS	Gastrocnemius soleus
HTM	High threshold mechanoreceptor
Il6	Interleukin 6
imp/s	Impulses per second
i.m.	intramuscular
i.p.	intraperitoneal

IP3	Inositol tri-phosphate
i.t.	intrathecal
i.v.	intravenous
LTM	Low threshold mechanoreceptor
LST	Lumbar sympathetic trunk
MO	Mustard oil
mRNA	messenger Ribonucleic acid
NAd	Noradrenaline
NE	Norepinephrine
NGF	Nerve Growth Factor
PGE2	Prostaglandin E2
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
P2X	Purino (ATP) receptor
R	Receptor
RA	Rapidly Adapting (mechanoreceptors)
RF	Receptor field
SA	Spontaneous activity
SC	Sympathetic chain
SCS	Sympathetic chain stimulation
SEM	Standard Error of the Mean
SMP	Sympathetically maintained pain
SNL	Spinal nerve ligation
SP	Substance P
TG	Trigeminal Ganglia
TNF α	Tumor necrosis factor α
TP I	Testing period I
TP II	Testing period II
TRAAK	TWIK-related arachidonic acid-stimulated K ⁺ channel
TREK-1	TWIK-related K ⁺ channel (K _{2P} 2.1)
TrkA	Neurotrophic tyrosine kinase receptor type 1
TRP	Transient Receptor Potential
TRPA1	Ankyrin Receptor type 1
TRPM3	Melastatin Receptor type 3
TRPM8	Melastatin Receptor type 8
TRPV1	Vanilloid Receptor type 1
TRPV2	Vanilloid Receptor type 2
TRPV3	Vanilloid Receptor type 3
TRPV4	Vanilloid Receptor type 4
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin-Resistant (Na ⁺ channels)
TTX-S	Tetrodotoxin-Sensitive (Na ⁺ channels)

Content

1 Introduction	1
1.1 Overall description.....	1
1.2 Muscle afferents under physiological conditions.....	4
1.2.1 Fiber composition of muscle nerves.....	4
1.2.2 Physiological properties of muscle afferent fibers.....	4
a. Conduction velocity and spontaneous activity:.....	6
b. Mechanosensitivity.....	7
c. Thermosensitivity.....	8
d. Chemical excitability.....	9
1.3 Injured muscle afferents.....	11
1.3.1 Inflammatory injuries.....	12
1.3.2 Muscle injuries.....	13
1.3.3 Muscle nerve injuries.....	13
1.4 Cutaneous afferents: behavior after nerve injury.....	13
1.5 Sympathetic-afferent coupling.....	15
1.6 Aims of the study.....	19
2 Methods	21
2.1 Nerve lesions and groups of animals.....	21
2.2 Anesthesia and animal maintenance during the experiments.....	22
2.3 Surgery, recording and electrical stimulation.....	23
2.3.1 Surgery and recording.....	23
Fig. 2.3.....	25
2.3.2 Lumbar sympathetic trunk stimulation.....	27
2.3.3 Suprathreshold electrical stimulation.....	29
2.4 Physiological stimulation.....	29
2.4.1 Mechanical stimulation of the muscle nerve.....	29

2.4.2 Thermal stimulation of the muscle nerve	30
2.5 Experimental protocols	31
2.5.1 Group A1 and A2 experiments: functional identification of the injured muscle fibers	31
2.5.2 Group B experiments: testing of TRP agonists	31
2.5.3 Group C experiments: testing of sympathetic stimulation	33
2.5.4 Group D1 experiments: testing of the afterdischarge	34
2.5.5 Group D2 experiments: testing of the effect of lidocaine on the afterdischarge	35
2.6 Data analysis.....	35

3 Results

37

3.1 Ectopic sensitivity of injured muscle afferents (groups A1 and A2).....	37
3.1.1 Proportions of injured muscle A- and C-fibers with ectopic activity	38
3.1.2 Response characteristics	42
a. Mechanosensitivity.....	42
b. Cold sensitivity	45
c. Heat sensitivity.....	50
3.1.3 Spontaneous activity	55
a. Rate and pattern of spontaneous activity	55
b. Spontaneous activity and responsiveness to physiological stimulation.....	57
c. Change of spontaneous activity during experimental interventions. Introduction into the phenomenon of afterdischarge.....	59
3.1.4 Conduction velocity and ectopic response properties	61
a. Recording from sciatic nerve	61
b. Recording from dorsal root	62
3.1.5 Response patterns to physiological stimulation.....	66
a. Comparison of TP I and TP II:	66
b. Comparison of A-fibers and C-fibers in TP II:.....	68
3.2 Responses of the injured muscle afferents to the TRP channels agonists	69
3.2.1 TRPV1 channel agonist capsaicin	72
C-FIBERS:	73
A-FIBERS:	77
3.2.2 TRPM8 channel agonist menthol.....	82
C-FIBERS:	82

A-FIBERS:	87
3.2.3 TRPA1 channel agonist mustard oil	97
C-FIBERS:	99
A-FIBERS:	101
3.3. Responses of injured muscle afferents to electrical stimulation of the sympathetic supply (group C).....	101
3.4. Afterdischarge to electrical stimulation of the muscle nerve (groups D1 and D2)	104
3.4.1. Proportions of fibers with afterdischarge before and after nerve injury	104
3.4.2. Blocking of the afterdischarge by local application of lidocaine	107
4 Discussion	113
4.1. Ectopic sensitivity of the injured muscle afferents (group A1 and A2)	113
4.1.1. Proportions of injured fibers with ectopic activity	114
4.1.2. Functional response characteristics of injured and intact muscle afferents	114
a. Mechanosensitivity.....	114
b. Thermosensitivity	118
c. Spontaneous activity.....	118
4.1.3. Comparison of injured muscle A- and C-fibers.....	119
4.1.4. Importance of A-fibers in the nociception.....	120
4.1.5. Comparison of functional characteristics of injured muscle afferents and injured cutaneous afferents	121
a. Time period I:	122
b. Time period II.....	123
c. Long-term changes.....	126
4.1.6. Axonal mechano- and thermosensitivity	128
4.1.7. Mechanisms underlying ectopic spontaneous activity and mechanosensitivity...	129
4.2. Responses of injured muscle afferent fibers to TRP channels agonists (group B).....	133
4.2.1. Possible transduction mechanisms of heat-sensitivity of injured muscle afferents	133
4.2.2. Capsaicin sensitivity of injured muscle afferents	135
C-FIBERS	135
A-FIBERS	137
4.2.3. Possible transduction mechanisms of cold-sensitivity of injured muscle afferents	138

4.2.4. Menthol and mustard oil sensitivity of injured muscle afferents.....	140
C-FIBERS	140
A-FIBERS	140
4.2.5. Possible mechanisms of the thermo-sensation disorders	142
4.3 Possible explanations of the lack of the responses of injured muscle afferents to electrical stimulation of the sympathetic supply (group C)	144
4.4. Afterdischarge of the muscle A-fibers	146
5 Conclusions	148
Bibliography	151

Chapter 1

Introduction

1.1 Overall description

Nerve injury is followed by multiple changes in the peripheral and central nervous system resulting in neuropathic pain and other somatosensory abnormalities (Jänig 2009, Jänig and Kirillova 2012, Fig. 1.1). Lesioned primary afferent neurons undergo biochemical, morphological, and physiological changes expressed in up- or down-regulation of voltage-gated ionic channels, transduction channels for physiological stimuli, membrane receptors for various chemical (in particular inflammatory) mediators, and the intracellular pathways coupled to them (McMahon et al 2006). Overall the lesioned primary afferent neurons switch to a state of regeneration, sprouting at as well as proximal to the lesion site in the nerve. The neurons may shrink. Neurons with unmyelinated (C-) fibers may die with time if no regeneration to the target tissue occurs (Degn et al 1999; Hu and McLachlan 2003; Jänig and McLachlan 1984; Lekan et al 1997; Tandrup et al 2000). Physiologically lesioned afferent neurons develop spontaneous and evoked ectopic excitability (see *a* in Fig. 1.1; Devor 2006). Starting within 4–6 h after the lesion, this ectopic activity can persist for weeks and months (Blenk et al 1996; Blumberg and Jänig 1984; Gorodetskaya et al 2003; Grossmann et al 2009; Jänig et al 2009; Michaelis et al 1995, 1999).

Also intact (non-lesioned) afferent neurons with C-fibers and possibly A-fibers may change functionally as a consequence of a peripheral nerve lesion. Signalling to intact afferent neurons may occur at three sites: in the nerve, in the target tissue, and in the dorsal root ganglion (see *b*, *c* and *d* in Fig.1.1). After partial nerve lesion intact nerve fibers are surrounded by degenerating nerve fibers, activated Schwann cells, macrophages, and other inflammatory cells distal as well as proximal to the nerve lesion (Scholz and Woolf 2007). This inflammatory milieu may influence the excitability of the afferent neurons, enhance sensory transmission in the dorsal horn of the spinal cord, and/or induce spontaneous activity

CONSEQUENCES OF PERIPHERAL NERVE INJURY

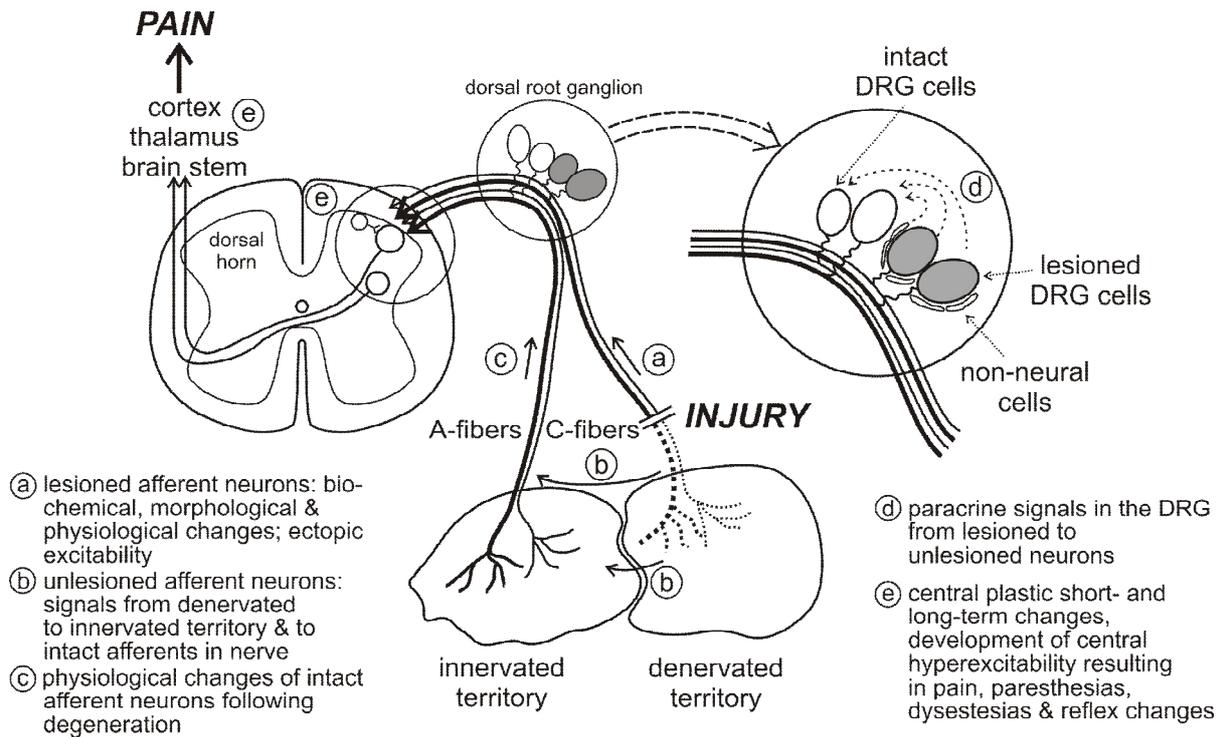


Fig. 1.2. *Consequences of peripheral nerve lesion.* For details see overview. Modified from Jänig et al (2009).

in unlesioned afferent nerve fibers (see *b* and *d* in Fig. 1.1; Ali et al 1999; Wu et al 2001; Djouhri et al 2006).

In the dorsal root ganglion (DRG), signalling from the axotomized DRG cells may influence the intact (non-lesioned) DRG cells, either directly or via non-neural cells (e.g., satellite and other cells) involving neurotrophic factors and their receptors in neurons and satellite cells (McMahon et al 2006; Mendell 2009), followed by changes of excitability and generation of ectopic activity in the cells bodies (see *d* in Fig. 1.1; Michaelis et al 2000).

Changes of lesioned afferent neurons entail short- and long-term plastic alterations in the central representations of the somatosensory system, including the spinal or trigeminal dorsal horn (see *e* in Fig. 1.1) as well as higher brain structures. These central molecular, morphological, and physiological changes may become with time irreversible, depending on the size and type of nerve lesion (Hökfelt et al 2006; Ossipov and Porreca 2008).

Clinical observations and quantitative measurements on patients show, that the efferent sympathetic nervous system can generate or enhance pain, leading to sympathetically maintained pain (SMP) (Baron 2002, 2009; Jänig 2009). Experiments on animals have shown that after complete or partial nerve injury, both damaged and undamaged primary afferent

neurons may express adrenoceptors, develop chemical sensitivity to adrenoceptor agonists and can be activated by electrical stimulation of the sympathetic trunk (Michaelis et al 1996, Chen et al 1996, Baron 1999, Jänig 2009). The mechanisms underlying this sympathetic-afferent coupling are little understood and remain to be explored. The investigation of the coupling of sympathetic fibers to injured muscle afferent fibers is therefore of special interest since almost 50% of the unmyelinated muscle nerve fibers are efferent postganglionic (Mense 1993).

Thus, neuropathic pain and other somatosensory abnormalities following nerve lesion are dependent on multiple changes in the peripheral and central nervous system. Ectopic activity in the lesioned afferent neurons is an important peripheral component triggering and maintaining central hyperexcitability. Thus central hyperexcitability and the plastic changes in the central sensory representations are the mechanistic basis of neuropathic pain. Now discharges in injured and uninjured afferent nociceptive and non-nociceptive neurons may trigger various signs of neuropathic pain (e.g., spontaneous pain, mechanical, or cold allodynia), paresthesias, or dysesthesias (Baron 2009). Up to the present most experimental investigations in the field of neuropathic pain focussed on cutaneous pain following nerve injury. Peripheral and central mechanisms following injury of deep somatic nerves including muscle nerves are largely unexplored. Up to now no quantitative studies of the mechanosensitivity, thermosensitivity, and spontaneous activity of afferent muscle fibers following muscle nerve injury have been conducted.

So far the most important investigations on small diameter muscle afferents and mechanisms governing muscle pain have been done in experiments on animals, mainly cat and rat (Paintal 1960; Iggo 1961; Kumazawa and Mizumura 1977; Kaufman et al 1983; Mense and Meyer 1985; Diehl et al 1993; Ge and Khalsa 2003) and humans (Simone et al 1994; Marchettini et al 1996) as described in the reviews of Mense (1993, 2012), Graven-Nielsen (2004) and Kaufman (2002). Preliminary results show that the response pattern of muscle nociceptors in humans is indistinguishable from that of muscle nociceptors in animals (cat, dog or rat). The receptors respond to strong local pressure and algescic substances with a discharge frequency and time course similar to that observed in animal experiments. Repetitive electrical microstimulation of the bundles of unmyelinated muscle afferents using tungsten wire electrodes inserted into a muscle nerve generates cramp-like pain sensations that are characteristic of muscle pain (Simone et al 1994). These data support the notion that muscle receptors characterized as nociceptive in animal experiments are the ones that trigger muscle pain in humans when excited.

1.2 Muscle afferents under physiological conditions

1.2.1 Fiber composition of muscle nerves

Afferent fibers innervating skeletal muscle are large-diameter myelinated ($A\alpha$ - or group I fibers; $A\beta$ - or group II fibers), small-diameter myelinated ($A\delta$ - or group III fibers) or unmyelinated fibers (C- or group IV fibers). The nomenclature used to classify these afferent fibers was created by Lloyd (1943) (roman numbers) and by Gasser and Grundfest (1939) (Mense 1993). The most detailed experimental investigation of the properties of small-diameter muscle afferents including muscle nociceptive ones have been conducted by Mense and co-workers in cat and rat (Mense 1993) and by Kumazawa and Mizumura in dog and rat (Kumazawa and Mizumura 1977) (Table 1.1).

In the cat the nerve to the lateral gastrocnemius-soleus muscle is composed approximately of one-third myelinated and two-thirds unmyelinated fibers (Mitchell and Schmidt 1983). Approximately one-half of all fibers are afferent and the other half efferent (myelinated motoaxons, sympathetic postganglionic axons) (Langford 1983, Mitchell and Schmidt 1983) (Fig. 1.2).

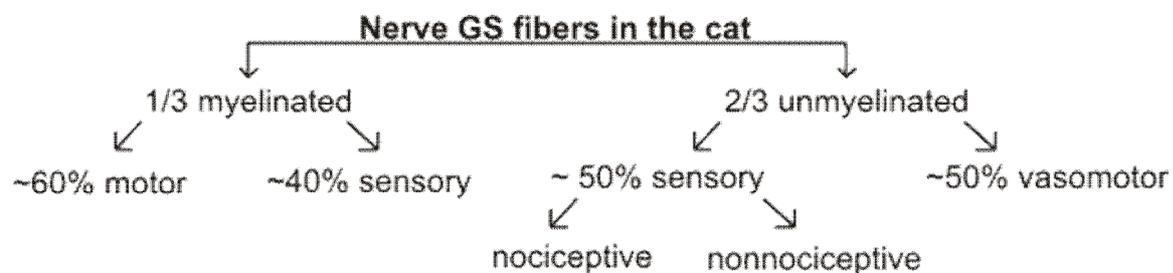


Fig. 1.2. Composition of axons in the nerve to the lateral gastrocnemius-soleus (GS) muscle in the cat (according to Mitchell & Schmidt 1983).

1.2.2 Physiological properties of muscle afferent fibers

Morphological and neurophysiological investigations have shown that the large-diameter myelinated fibers innervate muscle spindles or Golgi-tendon organs. They are mechanosensitive and involved in regulation of length and tension of skeletal musculature. Small-diameter afferent ($A\delta$ -, C-) fibers innervating skeletal muscle or its tendon consist functionally of various types as far as their responses to physiological stimuli are concerned.

	Aα/β-fibers	Aδ-fibers	C-fibers
SA	Many show high rate regular “ongoing” activity originating in muscle spindles	Up to 90% C-fibers exhibit spontaneous activity consisting of single or grouped impulses at irregular intervals and ≤ 0.1 imp/s	
Mechano-sensitivity	pressure or force sensitive, muscle stretch sensitive	1) pressure sensitive: 44% low threshold mechanosensitive (LTM) 33% high threshold mechanosensitive (HTM) tissue-threatening pressure R answering to chemical agents – “pressure pain R” 2) 23% static contraction and stretch-sensitive	1) pressure sensitive: 49% HTM; responsive to chemical agents (“pressure pain R”) 32% LTM; responsive to innocuous muscle stretch, physiological contractions and weak pressure stimuli 2) 19% static contraction sensitive
Thermo-sensitivity	not described	not described	50-60% are heat-sensitive and/or very few cold-sensitive 1) low threshold (20°C-40°C), most of them do not respond to BKN, HTM, – thermoregulation R 2) high threshold (>43°C, <20°C), polymodal nociceptors
Function	Stretch and contraction sensitive; tactile pressure or force sensation	Static contraction sensitive; metaboreception in innocuous and noxious range (sympathetic pressor responses evoked by active muscle contraction; pressure sensation, pain sensation (fast responses)	Static contraction sensitive; metaboreception in innocuous and noxious range; thermoregulation; pressure sensation; pressure pain sensation (slow responses); ischemic pain sensation

Table 1.1. *Physiological properties of muscle afferent fibers* (adapted from Adreani et al 1997, Adreani and Kaufman 1998, Hayes et al 2005, Hoheisel et al 2005, Kaufman et al 1983, Light et al 2008, Mense and Meyer 1985, 1988; Mense and Stahnke 1983; Taguchi et al 2005; for review see Kumazawa and Mizumura 1977, Mense 1993, Mense 2012). SA – spontaneous activity, BKN - bradykinin, HTM – high threshold mechanoreceptor, LTM – low threshold mechanoreceptor, R - receptor.

Afferent muscle A δ -fibers are either nociceptive, low pressure sensitive or contraction-sensitive. Unmyelinated muscle afferents are either nociceptive, low pressure sensitive, contraction-sensitive or thermosensitive (Table 1.1). The main type of receptive endings connected to small-diameter afferent fibers is the free nerve endings, mostly associated with small-diameter arterial blood vessels. Group IV fibers are assumed to terminate exclusively in free nerve endings while group III fibers supply both free nerve endings and other types of muscle receptors (e.g., Paciniform corpuscles). The Schwann cells ensheathing the small-diameter afferents leave small patches of the axonal membrane uncovered, the so-called exposed axon areas, which are presumably the sites of action for chemical agents that are present in the normal active skeletal muscle as well as in pathologically altered skeletal muscle.

In vitro investigations of muscle afferent neurons using calcium imaging of dorsal root ganglion (DRG) cells show that small-diameter muscle afferent neurons may be divided, according to their chemo-sensitivity, into low and high metabolite-responsive afferent neurons (Light et al 2008). The small-diameter muscle afferent neurons are involved in activation of cardiovascular and respiratory systems during muscular exercise (Kaufman and Foster 1996; Mitchell et al 1983), in sensations during exercise, as well as muscle fatigue, deep pressure sensation (Graven-Nielsen et al 2004; Rivers and Head 1908), and pain sensations (Marchettini et al 1996, Simone et al 1994; Torebjörk et al 1984).

The separation of the small-diameter afferent neurons innervating skeletal muscle into distinct types that are associated with several functions as propagated by Mense and others (Mense 1986, 1993) is still a matter of discussion. After all there is considerable overlap in the functional characteristics of the different groups of small-diameter muscle afferents. Therefore, the different functions mediated by the central nervous system (sensations including pain; adaptation of cardiovascular system and respiration) could also be triggered and supported by one population of small-diameter muscle afferents being mechanosensitive as well as chemosensitive in which the mechanical thresholds range from low-threshold to high-threshold.

a. Conduction velocity and spontaneous activity:

The conduction velocities (CV) of the large-diameter myelinated afferent fibers innervating skeletal muscle in the rat are: A α - > 50 m/sec, A β - 50-20 m/sec; small-diameter myelinated A δ - 20-2 m/sec, and unmyelinated C-fibers - < 2 m/sec (Lawson & Waddell 1991, Waddell et al 1989, Kirillova et al 2011a). The conduction velocity of muscle afferents may vary according to species, age, body size, nerve type and the temperature of the preparation (Djoughri and Lawson 2004). In the GS muscle of the cat, the conduction velocity of nociceptive afferent units spans the entire spectrum of 0.3-30 m/sec (Paintal 1960; Iggo 1961; Mense and Meyer 1985; Mense 1986). Also fibers of the same neurons may conduct more slowly in the dorsal root than in the peripheral nerve (Waddell et al 1989). Microneurographic data have shown that muscle nociceptors in humans likewise possess afferent fibers conducting in the range 0.6-13.5 m/sec (Simone et al 1994; Marchettini 1996). However other groups report that the proportion of nociceptors among A β -fibers (>20m/sec) is also relatively high (rating from 18% to 30%) in various nerves in different species (Djoughri and Lawson 2004).

Whether non-insured muscle afferents have spontaneous activity is very much debated. Many of the large-diameter myelinated afferents show high rate regular spontaneous activity originating in muscle spindles. It has been shown by some groups that intact muscle A δ -afferents (Kumazawa and Mizumura 1977) and up to 90% of the intact muscle C-fibers exhibit a low rate of spontaneous activity (less than 0.1 imp/s) (Berberich et al 1988; Diehl et al 1993, Kumazawa and Mizumura 1977; Taguchi et al 2005). The functional significance of the spontaneous discharge is unknown. It will probably not elicit subjective sensations because single impulses in (cutaneous) nociceptive fibers have been reported not to reach consciousness (Torebjörk and Hallin 1974). Other studies reported no spontaneous activity in intact muscle afferents in humans (Marchettini 1996).

b. Mechanosensitivity:

Direct probing of muscle tissue may excite group II muscle afferent fibres (Lewin and McMahon 1991). Thus A β -fibers are the main candidates for mediating the pressure or force sensation. Approximately 40% of the group IV units have a low mechanical threshold and respond to everyday stimuli such as weak deformation of muscle tissue, and some of them respond to stretch and active contractions over a wide range of force (Paintal 1960, Kaufman et al 1983; Mense and Meyer 1985; Abrahams 1986). Furthermore, non-painful pressure sensations can be evoked mechanically from muscle tissue in humans (Graven-Nielsen et al. 2004). These low-threshold mechanosensitive (LTM) units likely mediate innocuous pressure sensations from muscle, i.e. they are mechanoreceptors (Graven-Nielsen et al 2004; Hoheisel et al 2005). Another possible function the LTM receptors is that of “ergoreceptors”, i.e. they help adjust respiration and circulation during muscular exercise (Leshnower et al 2001).

Most group IV and many group III muscle afferent fibers cannot be excited by small degrees of muscle stretch (Iggo 1961; Kumazawa and Mizumura 1977; Kaufman et al 1982). These afferents have a high mechanical threshold and also respond to noxious chemical stimuli. Paintal (1960) denoted these receptors “pressure-pain receptors” and suggested that one of their functions was to elicit pain sensations from muscle. In a recent study about 60% of the group IV muscle receptors did not respond to innocuous muscle stretch, physiological contractions, and weak pressure stimuli but required noxious (tissue-threatening) pressure for activation (Hoheisel et al 2005). Their discharge rate still increased at stimulus strengths within the noxious range. They thus fulfilled the major requirements for a nociceptor. Often, muscle nociceptors exhibiting these response properties are called high-threshold

mechanosensitive (HTM) receptors. Among group III muscle receptors, the proportion of HTM endings is smaller than among group IV units. The membrane of HTM receptors may be equipped with as yet unidentified mechanosensitive transient receptor potential (TRP) channels (e.g., TRPV4 channel) that have a high mechanical threshold (Liedtke 2005; for a review see Hu et al 2006).

c. Thermosensitivity:

There is little evidence in the literature suggesting that subjective sensations of warmth or cold can be elicited from deep tissues. The group of Graven-Nielsen (2002) showed that i.m. injections of hot 48°C isotonic saline in humans induced muscle pain in contrast to isotonic saline at lower temperatures (8-38°C). In contrast to thermal skin stimuli, the i.m. injections of warm or cold isotonic saline did not cause any thermosensation. The only function of the thermosensitive units which could be speculated upon is a participation in thermoregulation, meaning that thermosensitive receptors in deep tissues might form an additional input channel to thermoregulatory centers.

Heat sensitivity has been reported in about 40 to 60% of intact muscle mechanosensitive C-fibers (Kumazawa and Mizumura 1977; Taguchi et al 2005). 50% of intact muscle mechanosensitive C-fibers are capsaicin-sensitive (Hoheisel et al 2004). Cold sensitivity was observed only in a few intact muscle C-fibers (Kumazawa and Mizumura 1977; Mense 1986, 1993; Taguchi et al 2005). Activation of intact muscle A-fibers by heating or cooling the muscle has not been reported in the literature.

All studies conducted so far on muscle group-IV receptors agree that some of these units (19%, in the study of Mense and Meyer 1985) respond to small temperature changes in the innocuous range (20-40°C). Many thermosensitive endings exhibit a discharge behavior similar to that of cutaneous thermoreceptors and may fulfil thermoreceptive functions. They differ from nociceptors in that most of them do not respond to BKN (Mense 1986). Possibly, they form an input channel for thermoregulation and do not trigger a conscious sensation when activated (Jessen et al 1983, Graven-Nielsen et al 2002).

Other group-IV muscle receptors have been reported to have a thermal threshold in the noxious range (> 43°C or < 20°C) (Iggo 1961; Kumazawa and Mizumura 1977). Most of these units also respond to endogenous algogenic agents and noxious local pressure and thus behave like polymodal nociceptors in the skin. It has to be kept in mind that many cutaneous mechanoreceptors exhibit a certain dependence of their discharges on temperature; these

receptors may behave like “spurious thermoreceptors”. Therefore, a susceptibility of a receptive ending to temperature changes does not necessarily reflect a thermoreceptive or thermonociceptive function (Mense 1993).

Many deep tissues including skeletal muscle, dura, testis and colon are reported to be innervated by polymodal mechano-heat-sensitive primary afferents. The heat sensitivity of deep tissues and the transduction mechanism involved in it are believed to subserve a nociceptive function sensing inflammatory changes after tissue injuries (Reeh and Pethö 2000). This hypothesis is supported by growing evidence that several inflammatory mediators, including bradykinin, PGE₂, histamine, low pH act on nociceptors, at least partly, by lowering the threshold of their heat and cold transduction mechanisms so profoundly that normal body temperatures become a driving force of excitation resulting in deep pain sensation. The specificity of muscle afferent’s thermosensitivity at the molecular level has almost not been studied so far. Only Mense’s group has shown that half of the unmyelinated muscle afferent fibers exhibit capsaicin sensitivity (Hoheisel et al 2004).

d. Chemical excitability:

In addition to the involvement in pressure sensation, static contraction, thermoregulation, muscle pain, muscle primary afferent neurons are very important as the afferent arm of sympathetic reflexes evoked by muscle contraction (see review by Kaufman and Hayes 2002). Thus, primary afferent endings in muscle could detect and encode muscle work using both mechanical and metabolic information. These “work receptors” were defined as “ergoreceptors”, a subset of which could be “metaboreceptors”, i.e. sensory endings that encode the metabolic state in skeletal muscle during muscle work (Kniffki et al 1981).

Many reports have suggested the existence of at least two populations of metaboreceptive group III and group IV muscle afferents. According to this classification, one population is suggested to detect innocuous levels of metabolites contributing to sympathetic pressor responses evoked by active muscle contraction, but not to muscle pain. The other population is suggested to detect noxious levels of metabolites contributing to muscle pain (Kniffki et al 1981; Mense 1993). Recent *in vitro* investigations of muscle afferent neurons using calcium imaging of dorsal root ganglion cells confirmed this idea and showed that small-diameter muscle afferent neurons may be divided, according to their chemosensitivity, into low metabolite responsive (46,5%) and high metabolite (53,5%) responsive afferent neurons (Light et al 2008).

Most high metabolite-responding DRG neurons innervating muscle were found among the smallest neurons innervating muscle and therefore have most likely unmyelinated (group IV) or thinly myelinated (group III) axons. It is likely that at least some of these afferent neurons have high thresholds to mechanical or thermal stimuli; thus these muscle afferents are polymodal nociceptive afferents (Adreani and Kaufman 1998; Berberich et al 1988; Haouzi et al 1999; Kaufman et al 1984; Mense and Meyer 1985).

Molecular receptors found on cardiac and skeletal muscle afferent neurons do not respond to a single metabolite in the physiological range, but rather to a combination of two or more factors produced by muscle contraction (Naves and McCleskey 2005). Studies of Light and co-workers also showed that combinations of high concentrations of protons, lactate, and ATP activate more DRG neurons than individual or pairs of metabolites (Light et al 2008).

In general, according to the literature, the following types of molecular receptors are likely to be present in small diameter muscle afferent neurons (Caterina and Julius 1999, Mense and Meyer 1985, McCleskey and Gold 1999, Light et al 2008, Mense 2009, Mense 2012, in press):

- receptors for inflammatory substances, like bradykinin (BKN, B1 and B2 receptor), 5-hydroxytryptamine (5-HT, serotonin, 5-HT₃ receptor), and prostaglandins (PGE₂, EP₂ receptor).
- receptors for protons, like acid-sensing ion channels (ASIC1) and the transient receptor potential vanilloid receptor (TRPV1). ASIC receptors are likely to be major mediators of responses to increased concentrations of metabolites produced in contracting muscles (Light, 2008). TRPV1 is sensitized by and responds to an increase in protons concentration and to heat and is important under exhaustive muscle work, ischemia, inflammation or tonic contraction. A specific agonist for this receptor molecule is capsaicin. In inflamed tissue, TRPV1 receptors have been reported to be upregulated (Carlton and Coggeshall 2001). Many investigators have proposed that TRPV1 receptors could play a role in muscle pain (Hoheisel et al 2004; Kaufman et al 1983; Marchettini, 1996; Rau et al 2007). They showed that capsaicin injected into a muscle immediately excite group III and IV afferents with moderate or high mechanical thresholds followed by a sustained discharge of these fibers. Under these conditions subjects reported cramping muscle pain.
- purinergic receptors, which bind adenosine triphosphate (ATP) and the products of ATP degradation such as adenosine (P₂X receptors). ATP is released from damaged muscle

cells. When injected i.m. in humans, ATP causes pain (Mörk et al 2003). ATP is a potent modulator of ASIC3, dramatically enhancing the responsiveness to pH in the normal physiological range (Naves and McCleskey 2005). It is thus possible that both ASIC and P2X receptors are necessary to detect muscle metabolites in the physiological range.

- receptors for growth factors, such as nerve growth factor (NGF) (TrkA receptor) or brain-derived neurotrophic factor (BDNF) (TrkB receptor). NGF excites exclusively high threshold mechanoreceptors (HTM) (Hoheisel et al 2005). NGF is present in free nerve endings of the muscle of rats (Reinert et al 1998), and is synthesized in muscle tissue. In inflamed muscle, its synthesis is increased (Petzet and McMahon 2006).
- receptors for excitatory amino acids, like glutamate receptors. In humans, injection of glutamate into skeletal muscle elicits pain, which was stronger in women than in men (Svensson et al 2005, Ge et al 2005).
- opioid receptors, which are upregulated during tissue inflammation.

Other algescic agents may activate muscle nociceptors without binding to specific receptor molecules, for example, high intracellular concentrations of potassium ions, high sodium concentrations or hypertonicity of the solutions. ATP, protons, capsaicin, and hypertonic saline excite both HTM and LTM endings, and therefore are not specific stimulants for nociceptors (Mense 2012, in press). Receptors for bradykinin, potassium, and arachidonic acid metabolites such as prostaglandin E2 and various cytokines are likely to sensitize muscle afferent endings under a variety of conditions and may be responsible for enhanced perceptions of fatigue and pain (Hayes 2006, Hoheisel 2005; Kaufman 1983, Kaufman and Rybicki 1987, Mense 1993; Rotto and Kaufman 1988, Rotto 1990). Muscle nociceptive afferent neurons are equipped with tetrodotoxin-resistant (TTX-r) Na⁺ channels as are nociceptive cutaneous afferent neurons (Steffens et al 2003). Muscle low threshold pressure sensitive A α / β -fibers are blocked by TTX, thus being TTX-sensitive (TTX-s).

1.3 Injured muscle afferents

Various techniques can be applied to produce experimental muscle pain. Among them are: 1) intramuscular injection of inflammatory agents, such as hypertonic saline or capsaicin; 2) mechanical stimuli, such as eccentric exercise, compression by a tourniquet or electrical stimulation of muscles; or 3) injury of the muscle nerves.

1.3.1 Inflammatory injuries

Carrageenan injected into the cat hindlimb muscles generates within 1 to 2 hours a powerful inflammatory response in the muscle associated with the release of 5-hydroxytryptamine, histamine, bradykinin, and prostaglandins. These substances stimulate or sensitize muscle nociceptors (Berberich et al 1988). The main effect of inflammation on muscle group III nociceptors is that they develop spontaneous activity. Interestingly, their mechanical thresholds do not change. This finding led the investigators to conclude that this group of receptors might trigger spontaneous pain and dysesthesias associated with myositis, an inflammatory disease of skeletal muscle; assuming that “carrageenan-evoked inflammation” is a realistic model of this disease (Gautam et al 2010).

Another technique to induce muscle pain is injection of an algogenic substance, like capsaicin. It produces a cramp like pain, and microneurographic recordings in humans show a continuous activation of group III and IV afferents (Marchettini et al 1996, Mense 2009).

In animal experiments, a long-lasting inflammation (exceeding 2 weeks) can be induced by injecting complete Freund’s adjuvant (CFA) into a muscle or other substances. One of the inflammation-induced changes in muscle group III/IV units is spontaneous activity in normally silent units. However, even in chronic inflammation the mean discharge rate in group IV units did not exceed 1 Hz (Diehl et al 1993). Another effect is sensitization of HTM receptors for mechanical stimuli. In inflamed tissue they respond to weak stimuli, i.e. former HTM receptors behave like LTM units. The inflammation-induced sensitization of muscle nociceptors is at least partly due to the release of PGE₂. Cyclooxygenase blockers abolish the resting discharge and restored the high mechanical threshold of group IV units in inflamed muscle (Diehl et al 1993). Chronic CFA myositis is associated with an increased “innervation density” with substance P (SP)-containing nerve endings in rats (Reinert et al 1998). SP is predominantly present in nociceptive afferent neurons. Therefore, these changes may contribute to an enhanced pain sensation of an inflamed muscle.

Activation of microglia appears to be a key factor in the generation of nociceptive behavior of myositis rats. After prevention of the microglial activation by intrathecal administration of the glial blocker minocycline, the myositis-induced allodynic behavior and the reduction of the exploratory motor activity was largely attenuated (Chacur et al 2009).

1.3.2 Muscle injuries

Some painful cases of muscle damage can be produced by *eccentric exercise*. There are two prominent signs of damage in a muscle immediately after it has been subjected to a series of eccentric contractions: Sarcomeres in myofibrils are disrupted and the excitation–contraction (E–C) coupling system is damaged. During repeated eccentric contractions the number of disrupted sarcomeres increases, until a point is reached where membrane damage occurs. Finally the damage to elements of the E–C coupling machinery becomes apparent and the muscle fibers may die (Proske, 2001).

The question of whether eccentric exercise leads to damage of afferent muscle receptors remains open. Proinflammatory cytokines (especially IL-6, but not TNF α) are likely to be involved in maintaining muscle hyperalgesia, by sensitizing high threshold mechanosensitive muscle afferents (HTM) (Sluka et al 2007). The sensory neuron is suggested to be the functionally important site in the processing of this type of hyperalgesia.

1.3.3 Muscle nerve injuries

The discharge properties developing in large-diameter and small-diameter muscle afferents after injury of a muscle nerve were not systematically investigated up to now. Up to the present most experimental investigations in the field of neuropathic pain focussed on cutaneous pain following nerve injury. The knowledge about the ectopic discharge pattern of injured somatic afferent neurons innervating skeletal muscle could make a major contribution to unravel the peripheral mechanisms of neuropathic pain. It is not far-fetched suggesting that the discharge pattern of injured muscle afferent neurons and those of injured cutaneous afferent neurons are different.

1.4 *Cutaneous afferents: behavior after nerve injury*

Injured cutaneous afferent fibers were quantitatively studied for their responses to mechanical, cold and heat stimuli applied to the nerve injury site and to the nerve distal to the injury site 7 to 14 days after crush lesion of the sural nerve when the fibers could not have regenerated to the target tissues and 1 – 15 months after crush lesion of the sural nerve when the afferent nerve fibers could have regenerated to the target tissue assuming regeneration rates of about 1 mm/day for unmyelinated fibers and 2-4 mm/day for myelinated fibers

(Sunderland 1978, 1991, Gorodetskaya et al 2003, 2009, Grossmann et al 2009a). In total, in the first two weeks after *sural* nerve injury 43% myelinated and 49% unmyelinated cutaneous lesioned afferent fibers show distinct responses to mechanical and thermal stimuli applied to their regenerating terminals in the injured nerve (Gorodetskaya et al 2003, Jänig et al 2009). That could mean that many neurons innervating skin shrink and could die after nerve lesion (Hu and McLachlan 2003). The quantitative distribution of these response characteristics to physiological stimuli are statistically not significantly different from those observed in intact cutaneous afferent fibers. Thus the molecular transductions mechanisms for innocuous and noxious cold stimuli, for mechanical stimuli and for heat stimuli are fully preserved in the injured cutaneous afferent neurons, when compared to the control, whether they regenerate to the target tissue or stop regenerating and terminate in the injured nerve months (and probably years) after injury. (Gorodetskaya et al 2003, 2009; Grossmann et al 2009a,b; Jänig et al 2009).

C-FIBERS:

Afferent cutaneous C-fibers were divided functionally into three groups based on their responses to the physiological stimuli:

- *Cold-sensitive non-nociceptive C-fibers (type 1 cold-sensitive)*. These fibers exhibit graded responses to cooling of skin or nerve, have a low threshold to cold stimuli, are depressed in their activity by warming and have rates of spontaneous activity of about 3-5 imp/s at 30°C. They are practically mechanoinsensitive; some are excited by heat stimuli. These fibers can be activated by the TRP (transient receptor potential) M8 agonist menthol (Teliban, 2012).
- *Nociceptive type 2 cold-sensitive C-fibers*. These afferent fibers have a high threshold to cold stimuli (most below 10°C). Most of them are mechano- and/or heat-sensitive. These fibers are menthol-insensitive (Teliban, 2012).
- *Cold-insensitive C-fibers* which are heat- or mechanosensitive or both. Most of these fibers are nociceptive. A few are non-nociceptive.

A-FIBERS:

Injured A-fibers and control A-fibers are predominantly mechanosensitive, very few are weakly cold-/heat-sensitive; only 7% have spontaneous activity. The thresholds of mechanosensitive A-fibers range from 0.7 to >70 mN (median 7.5 mN), some being low-

threshold (threshold ≤ 10 mN, median 2 mN) and some high-threshold (threshold ≥ 20 mN, median 45 mN).

1.5 Sympathetic-afferent coupling

For almost a century it has been suggested that in certain clinical syndromes that are characterized by severe pain, activity in the sympathetic nervous system may be involved in the generation of pain. This assumption is mainly based upon two observations: (1) the pain is spatially correlated with signs of autonomic dysfunction; and (2) blocking the efferent sympathetic supply to the affected extremity relieves the pain in some patients (Bonica 1990, Price et al 1998, Baron and Jänig 2010). The pain component that is relieved by specific sympatholytic procedures is called “sympathetically maintained pain” (SMP) (Raja et al 1991).

There is no obvious indication for direct or indirect coupling between the efferent sympathetic systems and the afferent systems in the peripheral tissues leading to pain, discomfort or other sensations maintained by the sympathetic outflow. This situation may change after trauma with or without a nerve lesion. Activity in sympathetic neurons may now lead to SMP (Baron and Jänig 2010). During inflammation or following nerve lesion, the efferent (noradrenergic) innervation of the affected tissue may feed back to the primary afferent neurons and activate them, enhancing their spontaneous activity or increasing their responsiveness to mechanical or thermal stimuli. This in turn would amplify the physiological impulse transmission in the spinal or trigeminal dorsal horn (sensitization of dorsal horn neurons) or would enhance hyperexcitability of these neurons after a nerve lesion (Jänig 2009).

Several animal models for neuropathic pain involving controlled nerve lesions, have been used to determine the contribution of the sympathetic nervous system to neuropathic pain (for review see Jänig 2009, Baron and Jänig 2010). The interventions performed were surgical or chemical sympathectomy, systemic or local application of adrenoceptor blockers such as phentolamine (α_1 , α_2), prazosin (α_1), or yohimbine (α_2), or of adrenoceptor agonists such as epinephrine, norepinephrine or clonidine (α_2), or intraperitoneal application of guanethidine (which is taken up by the noradrenergic terminals and depletes norepinephrine).

After *complete or partial nerve injury* in experimental animals, surviving afferents may develop noradrenergic sensitivity (Fig. 1.3). Some myelinated and unmyelinated afferents innervating a stump neuroma can be excited by epinephrine or by stimulation of sympathetic

efferents that project into the neuroma (Blumberg and Jänig 1984, Burchiel 1984, Devor and Jänig 1981, Scadding 1981, Sato 1991, O'Halloran 1997). However, in the early stages after the nerve injury the activation of afferent fibers requires rather high (nonphysiological) frequencies of stimulation of the sympathetic supply. In a chronic neuroma model or in a chronic nerve cross-union model (≥ 1 year after injury), electrical stimulation of the sympathetic trunk at physiological frequencies (0.5–4 Hz) were capable of activating regenerated C-fibers, probably with nociceptive functions (Häbler et al 1987); this activation was mediated by an adrenergic or by a nonadrenergic mechanism (Jänig 1990).

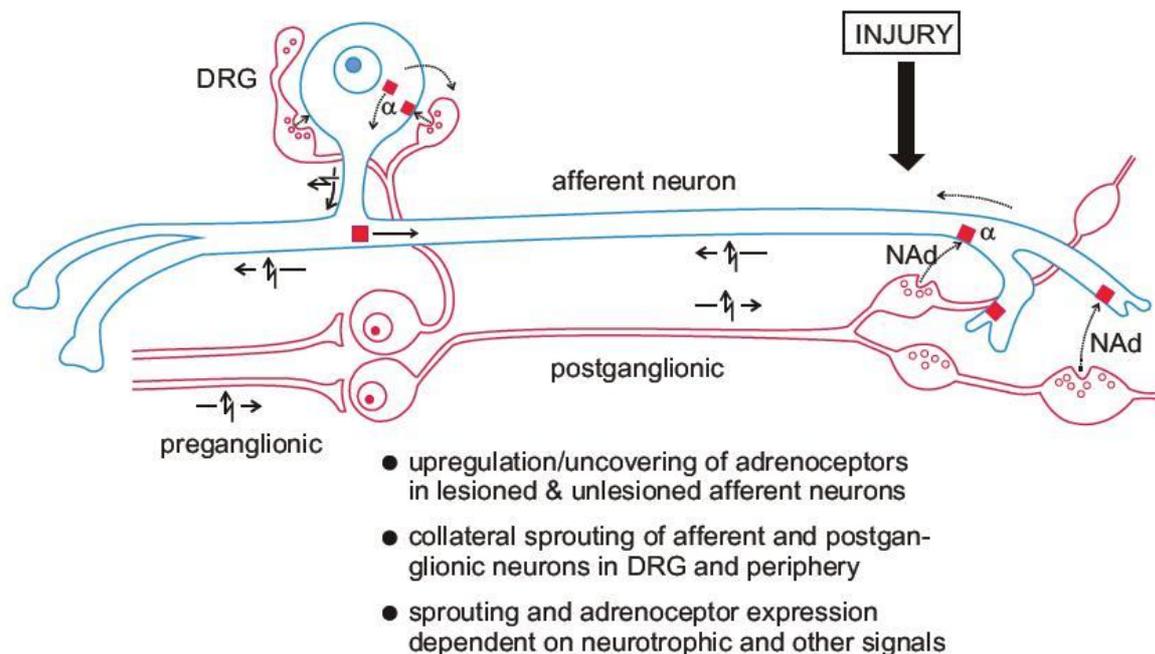


Fig. 1.3. Influence of sympathetic activity and catecholamines on primary afferent neurons following nerve injury. Sympathetic and afferent neurons start to sprout in the periphery and dorsal root ganglion (DRG). Afferent neurons express adrenoceptors in their membranes. The sympathetic-afferent coupling is located in the periphery and in the DRG. It occurs in lesioned and possibly unlesioned afferent neurons. It is mediated by norepinephrine (noradrenaline; NAd) released from sympathetic postganglionic neurons and by α -adrenoceptors expressed at the plasma membrane of afferent neurons. The type of adrenoceptor is mainly α_2 in rats and possibly α_1 in humans (taken from Baron and Jänig 2010).

In addition to these peripheral interactions, coupling of sympathetic and afferent neurons may also occur within the dorsal root ganglion (DRG; Fig. 1.3). After a complete nerve lesion, some DRG somata with A β -fibers and a few with C-fibers develop ectopic activity. Electrical stimulation of sympathetic efferents innervating the DRG may lead to an α_2 -adrenoceptor mediated increase of this spontaneous activity (Chen et al 1996, Devor et al 1994, Michaelis et al 1996). This increased adrenergic sensitivity is paralleled by

morphological changes within the DRG (Fig. 1.3). After the nerve lesion, sympathetic postganglionic fibers that normally innervate blood vessels within the DRG sprout to form basket-like terminals around large primary afferent somata that project into the injured nerve (Chung et al 1996, Chung et al 1997, McLachlan et al 1993). The study of Chung (1997) also suggests that not only axo-somatic interactions between sympathetic and sensory neurons but also axo-axonal interactions are possible.

The relevant findings are summarized as follows (Baron and Jänig 2010): (1) In the first 2–3 weeks after nerve lesion, most A-fiber neurons with spontaneous activity and very few silent A-fiber neurons are excited. At later time periods, when the catecholaminergic sprouting in the DRG is more prominent, most spontaneously active A-fiber neurons are inhibited during stimulation of the sympathetic supply and are rarely excited. (2) Very few afferent neurons with unmyelinated axons respond to electrical stimulation of the sympathetic trunk; most of them are inhibited in their activity (Devor et al 1994, McLachlan et al 1993, Michaelis et al 1996). (3) The coupling from sympathetic postganglionic fibers to the afferent cell bodies in the DRG probably occurs only with spontaneously active muscle afferent neurons with A-fibers (Michaelis et al 2000). (4) Activation of the afferent neurons, via the DRG, requires high-frequency stimulation of the sympathetic neurons at 5 to 20 Hz. Sympathetic pre- and postganglionic neurons rarely discharge at these high frequencies under physiological conditions. (5) Mechanical allodynic and hyperalgesic behavior shown by rats with L5 spinal nerve injury (transection and ligation) is not dependent on the innervation of the DRG by sympathetic neurons (Ringkamp et al 1999). The rate of ectopic activity originating in the DRG, 15 to 45 days after spinal nerve injury, is not dependent on activity in sympathetic neurons (Liu et al 2000). (6) Afferent neurons in the DRG may be activated indirectly by ischemia generated by decrease of blood flow during vasoconstriction in the DRG following electrical stimulation of the sympathetic trunk and not directly via adrenoceptors expressed in the DRG cells (Häbler et al 2000). (7) Axotomized medium- to large-diameter DRG neurons express α_{2A} -adrenoceptors after sciatic nerve injury (Birder and Perl 1999). However, these upregulated adrenoceptors disappear after several weeks as perineural catecholaminergic sprouting develops, and only small depolarizations can be generated by high concentrations of norepinephrine in axotomized DRG somata (de Armentia et al 2003).

Any hypothesis about chemical norepinephrine mediated coupling between sympathetic and afferent neurons would have to explain how functional adrenoceptors appear in the membrane of nociceptive and other afferent neurons as a consequence of a nerve lesion or

inflammation. The cellular mechanisms underlying the increased sensitivity are unknown. An obvious explanation is either novel expression or upregulation of existing adrenoceptors. Alternatively, the receptors may normally be present on primary nociceptive afferents but not be functional; they may become uncovered and effective during the response to damage or inflammation. This possibility is consistent with the finding that α_{2A} - and α_{2B} -adrenoceptor mRNA is normally constitutively present in DRG cells (Pieribone 1994). After nerve lesion, mRNA for α_{2A} -adrenoceptors is clearly upregulated mainly in large-diameter afferent DRG neurons (Shi et al 2000). Importantly, injured as well as uninjured neurons showed this phenotypic switch. However, these changes only lasted for up to 72 days, arguing that this noradrenergic sensitivity is very unlikely to be relevant for the chronic changes seen in patients.

Although this situation appears to be clear, the mechanisms underlying this coupling are still unclear. Indeed, although we have relatively good animal models about the sympathetic-afferent coupling after the nerve lesion (Jänig and Koltzenburg 1992, Jänig and Häbler 2000; Jänig and Baron 2001; Jänig 2012; Jänig and Levine 2006), (a) in some animal nerve lesion models sympathetic-afferent coupling plays practically no role (or this role can be shown only under unphysiological stimulation of sympathetic neurons (Häbler et. al. 2000)); (b) some findings suggest that adrenergic sensitivity of afferents and sprouting of sympathetic fibers in dorsal root ganglia are not sufficient for the development of sympathetically maintained pain (McLachlan et al 1993; Michaelis et al 1996); (c) so far, it is unknown which efferent sympathetic outflow channel (e.g., to the skin or to deep somatic tissues, such as skeletal muscle) is involved in the pathologic sympathetic afferent coupling (Schattschneider et al 2006); (d) most studies were done on cutaneous afferents or afferents in mixed nerves and so there is no data about the sympathetic-afferent coupling in deep somatic tissues; and (e) there is no data about the mechanisms of this coupling in the case of pain without nerve lesion (e.g., type CRPS I patients with SMP (Price et al 1998; Baron et al 2002, Jänig & Baron 2002, 2003, Jänig 2009)).

Baron and co-workers (Baron et al 2002) have clearly shown that in patients with CRPS I (no nerve lesion present) and sympathetically maintained pain (SMP), the sympathetic-afferent coupling must take place in the deep somatic tissues of the affected extremity. In addition to a coupling in the skin, a sympathetic-afferent interaction is likely to happen in other tissues, particularly in deep somatic domains such as bone, muscle, or joints, which are richly innervated by sympathetic fibers. These structures are rather painful in patients with complex regional pain syndromes (Wasner et al 1998, Baron 1999, 2001, Veldman 1993),

which lends support to this notion. Furthermore, patients might exist who are characterised by a selective or predominant sympathetic-afferent interaction in deep somatic tissues sparing the skin. Later the same group (Schattschneider et al 2006) showed that SMP in deep somatic tissues is prominent in first 6 months after trauma. The investigation of the coupling of sympathetic fibers to injured muscle afferent fibers is therefore of special interest.

1.6 Aims of the study

Small and large diameter muscle afferents are involved in regulation of motor activity, adaptation of cardiovascular system and respiration during muscle effort, thermoregulation, non-painful sensations and painful sensations. According to these global functions are the deep somatic afferent neurons innervating skeletal muscle differentiated into several types characterized by their responses to physiological mechanical, thermal or chemical stimuli. The mechanisms underlying these responses are based on several types of transduction channels, membrane channels for chemical signals, and ionic channels related to excitability and conduction. Nerve injuries followed by neuropathic pain, dysesthesias and paresthesias mostly involve (in addition to skin nerves) deep somatic nerves including those to skeletal muscle. Functional discharge characteristics and molecular transduction mechanisms underlying thermosensitivity of injured muscle afferents have to date not been systematically studied and are practically unknown when compared to the functional discharge characteristics of injured cutaneous afferent neurons. Therefore, this study concentrates on the discharge characteristics of myelinated and unmyelinated muscle afferent neurons using neurophysiological techniques, 4-13 hours or 4-7 days following crush lesion of a muscle nerve. The injured muscle afferent neurons were studied for:

- their responsiveness to mechanical and thermal stimulation; the rate and pattern of spontaneous activity; the conduction velocity of the injured axons with respect to their response properties to the physiological stimuli and to the spontaneous activity; the response pattern (combinations of responses) to physiological stimuli;
- their responsiveness to local application of the transient receptor (TRP) agonists capsaicin (TRPV1), menthol (TRPM8) or mustard oil (TRPA1);
- their responsiveness to activation of postganglionic axons by electrical stimulation of the sympathetic chain; and
- their afterdischarge to electrical stimulation of the muscle nerve.

Chapter 2

Methods

The neurophysiological experiments were performed on 99 male Wistar rats weighting between 300-570 grams. The animals were housed singularly in plastic cages with sawdust as bedding material and were provided with water and food ad libitum. The experimental protocols were approved by the local Animal Care Committee.

2.1 Nerve lesions and groups of animals

The animals were assigned to the following experimental groups:

- Group A1 experiments: Rats with an acute gastrocnemius-soleus nerve lesion.

In 22 rats the lateral gastrocnemius-soleus nerve was crushed 4-13 hours (7.2 ± 0.3 hours) before the recording from afferent fibers started (time period I [TP I]). The responsiveness to mechanical and thermal stimulation and the rate and pattern of spontaneous activity of the injured afferent nerve fibers were studied.

- Groups A2, B and C experiments: Rats with a chronic gastrocnemius-soleus nerve lesion.

In 67 rats the lateral gastrocnemius-soleus nerve was crushed under general anesthesia (pentobarbital sodium [Narcoren, Merial GmbH, Halbergmoos, Germany], 60 mg/kg, i.p.) and aseptic conditions 4-7 days before the neurophysiological experiments (called chronic nerve lesion; time period II [TP II]). The nerve was exposed in the left hindlimb. Under visual control through a stereomicroscope, a length of about 0.5 mm of the nerve was crushed 5-10 mm proximal to its entry into the skeletal muscle, using fine watchmaker's forceps. The crush was repeated three times each for a few seconds resulting in a translucent nerve. The wound was closed and healing was uneventful. In the time period of 4-7 days after nerve injury nerve fibers do not regenerate to the skeletal muscle assuming a regeneration speed of 1-3 mm/day (Sunderland 1978, 1991). In the rats with chronic GS nerve lesion the injured afferent fibers were investigated for

their responses: 1) to mechanical and thermal stimulation and for their rate and pattern of spontaneous activity (15 rats; group A2); 2) to local application of the transient receptor (TRP) agonists capsaicin (TRPV1), menthol (TRPM8) and mustard oil (TRPA1) (25 rats; group B); 3) to activation of postganglionic axons by electrical stimulation of the sympathetic chain (17 rats; group C).

- Group D1 and D2 experiments: Rats without or with acute GS nerve lesion.

10 rats without nerve lesion or with crush injury of the GS nerve, performed acutely during recording from intact afferent fibers, were used to investigate three questions: 1) Testing for afterdischarge (before and after the crush injury). 2) Physiological characterisation of the muscle nerve fibers in the first minutes after nerve lesion. 3) Testing of the afterdischarge after local application of the sodium channel blocker lidocaine.

2.2 Anesthesia and animal maintenance during the experiments

On the day of the final experiments the rats were re-anesthetised with Pentobarbital-Sodium (Narcoren, 40-50 mg/Kg, i.p.). A catheter was inserted into the jugular vein for the administration of drugs and Tyrode's solution (Fig. 2.1). Tyrode's solution is a solution that is roughly isotonic with interstitial fluid and used in physiological experiments and tissue culture. It contains NaCl (0.8%), KCl (0.02%), CaCl₂ (0.02%), MgCl₂ (0.01%), NaHCO₃ (0.1%), NaH₂PO₄ (0.025%) and glucose (0.1%). Additional doses of Pentobarbital were given regularly (10 mg/kg/h, i.v.) in order to maintain a sufficient level of anesthesia as judged from the absence of spontaneous blood pressure fluctuations. Arterial blood pressure was measured continuously via a catheter inserted into the tail artery. For this purpose a transducer was used (LM-22 provided by List, Darmstadt). A controlled administration of drugs and fluid was maintained during the experiment. The mean arterial blood pressure measured via a catheter in the tail artery was always ≥ 80 mmHg.

The acid-base status regularly determined was in the range of pH = 7.4, P_{CO2} = 35-40 mmHg, P_{O2} ≥ 100 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. The ventilation was performed with 20% O₂-enriched room air under positive pressure (ventilation pump RUS 1300, FMI, Egelsbach, Germany) at a frequency of 70 strokes/minute. Rectal temperature was monitored and kept close to 37.0°C by means of a

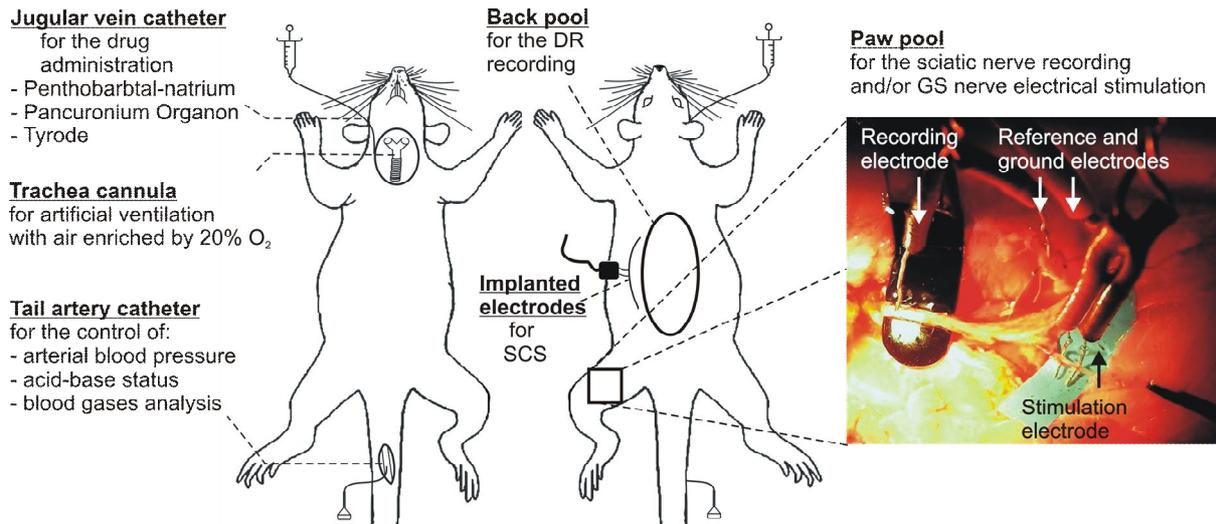


Fig. 2.1. *Preparation of the animal.* The catheter inserted into the jugular vein was used for regular administration of the anesthetic (Pentobarbital-Sodium), the relaxant (Pancuronium Organon) and Tyrode's solution. Over a catheter inserted into the tail artery the arterial blood pressure was measured as well as blood samples were taken at regular intervals (every hour) for determination of the acid-base status (pH, base excess, P_{O_2} , P_{CO_2}). A cannula was inserted into the trachea for artificial ventilation of the rat. Recording was done from fine filaments pulled out either from sciatic nerve (sciatic nerve pool) or from the dorsal root (DR) L4 or L5. The zoom-out photo of the left hind paw shows the original set-up of the experiment, when recording was performed from the sciatic nerve. The pools were made from the surrounding skin flaps, the sciatic nerve or dorsal roots were placed on a black perspex platform and electrodes for recording were positioned above. Gastrocnemius-soleus (GS) nerve fibers were identified electrically using stimulation electrodes positioned under the lateral GS nerve. In the group C rats a specially designed pair of electrodes was implanted around the sympathetic chain (SC) for activation of postganglionic axons by electrical stimulation (SCS).

servo-controlled heating blanket under the rat. At the end of the experiments, the animals were killed under deep anesthesia by an intravenous injection of a saturated potassium chloride solution. All experiments had been approved by the local animal care committee of the state administration and were conducted in accordance with German Federal Law.

2.3 Surgery, recording and electrical stimulation

2.3.1 Surgery and recording

In 77 rats the left lateral gastrocnemius-soleus nerve and the sciatic nerve up to the sciatic notch were exposed. In these experiments the sciatic nerve was put on a rigidly fixed small black perspex platform approximately 10 mm proximal to the separation of the gastrocnemius-soleus nerves from it. A pool was formed from the skin flaps and filled with warm (30°C) paraffin oil (Fig. 2.1; 2.2).

Dorsal roots and spinal cord were covered by paraffin oil in a pool made of the surrounding skin. The dorsal root L4 or L5 was put on a rigidly fixed small Perspex

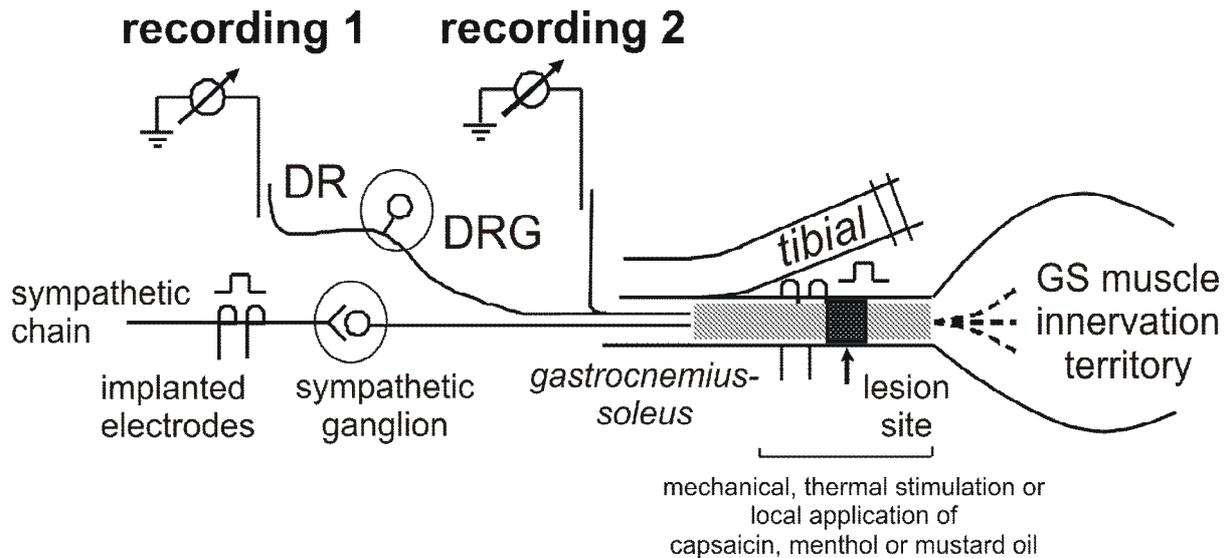


Fig. 2.2. Schematic representation of the gastrocnemius soleus (GS) nerve fiber preparation. Fine filaments teased either from dorsal root L4 or L5 (recording 1) or from sciatic nerve (recording 2) were put on a platinum recording electrode to measure the activity in the afferent nerve fibers; the reference electrode was connected to the nearby tissue. The GS nerve was isolated from the surrounding tissue and put on a pair of electrodes for electrical stimulation and identification of the nerve fibres as C- or A-fibres. In the group A1 experiments the GS nerve was crushed 4-13 hours before the recording from afferent fibers started. In the group A2, B and C experiments the nerve was crushed 4-7 days before the neurophysiological experiments. Afferent A- and C-fibers were physiologically characterized by their responses to mechanical and thermal (cold, heat) stimuli applied directly to the GS nerve from 3mm proximal to 3mm distal to the injury site of the nerve. In the group B experiments, after the physiological characterization of the nerve fibres, menthol, capsaicin, mustard oil or tyrode was applied to the nerve injury site. Group C animals underwent additional implantation of a pair of stimulation electrodes at the sympathetic chain. In these rats the fiber responses to activation of postganglionic axons by electrical stimulation of the sympathetic chain were examined. In the group D1 and D2 experiments the nerve was crushed during recording of the afferent fibers (D1) or was left intact (D2).

platform. Before isolating filaments from the sciatic nerve or from the dorsal root L4 or L5 the common peroneal nerve (after carefully separating it from the sciatic nerve), the sural nerve and the tibial nerve were transected.

On the black perspex platforms fine strands were teased out of the dorsal site of the sciatic nerve or from the dorsal root L4 or L5 and put on a platinum electrode for recording; the indifferent electrode being connected to the nearby tissue. The registration usually started with the recording of spontaneous activity followed by electrical identification of the nerve fibers in this filament (Fig. 2.3). After the electrical identification was done the stimulation electrodes were removed from the nerve for further recording of spontaneous activity and responses to mechanical, thermal stimulation or local application of capsaicin, menthol or mustard oil (Fig. 2.2).

The signals recorded from the afferent nerve fibers in the nerve strands were differentially amplified 1000 times using a differential amplifier (10 M Ω input impedance) (Fig. 2.4). The signals were filtered by a bandpass filter between 120 Hz to 1-1.2 KHz for

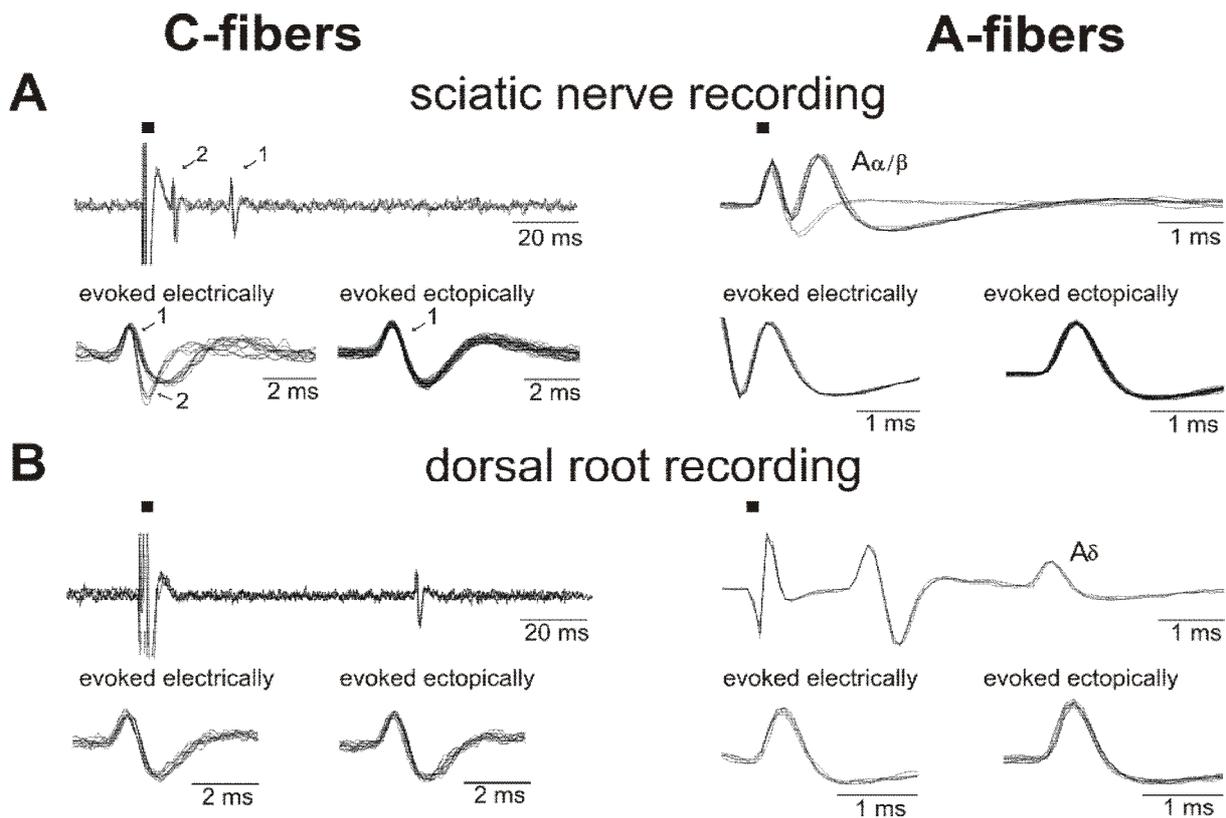


Fig. 2.3. Identification of A- and C-fibers in the lateral gastrocnemius-soleus nerve. The conduction velocity was determined from the latency of the responses in filaments isolated from the sciatic nerve (A) or isolated from the dorsal root L4 or L5 (B) to single pulse stimulation of the gastrocnemius-soleus nerve (upper records, several times superimposed; stimulus indicated by the square). The shapes of these electrically evoked responses were compared with those of the action potentials occurring spontaneously or being evoked by mechanical or thermal stimulation (evoked ectopically) of the nerve injury site (lower records, several times superimposed). The A-fiber in A was an A α/β -fiber and in B probably an A δ -fiber (conduction velocity 12 m/s).

unmyelinated fibers and between 120 Hz to 12 KHz for identifying myelinated fibers. The network interferences were filtered by the noise eliminator (Quest Scientific, Hum Bug, Canada). Action potentials (APs) generated by different axons could also be discriminated using a window discriminator. The output of the window discriminator was connected to a loud-speaker for the audio control of the recording. The arterial blood pressure, electrocardiogram (ECG), intratracheal pressure, thermal stimuli, marker signals and neural activity were recorded simultaneously and were analog-digital converted using the System Spike II (Cambridge Instruments) and fed into a computer. During the experiments a preliminary template analysis of the neural activity was done using the Spike II System to discriminate the APs generated by different fibers. In this way we were able to discriminate spikes of the same size but with different shapes. Files were stored on hard disc for the off-line analysis. APs recorded from the axons were additionally monitored in real time using a digital storage oscilloscope (Classic 5000, Gould Instrument System) (Fig. 2.4).

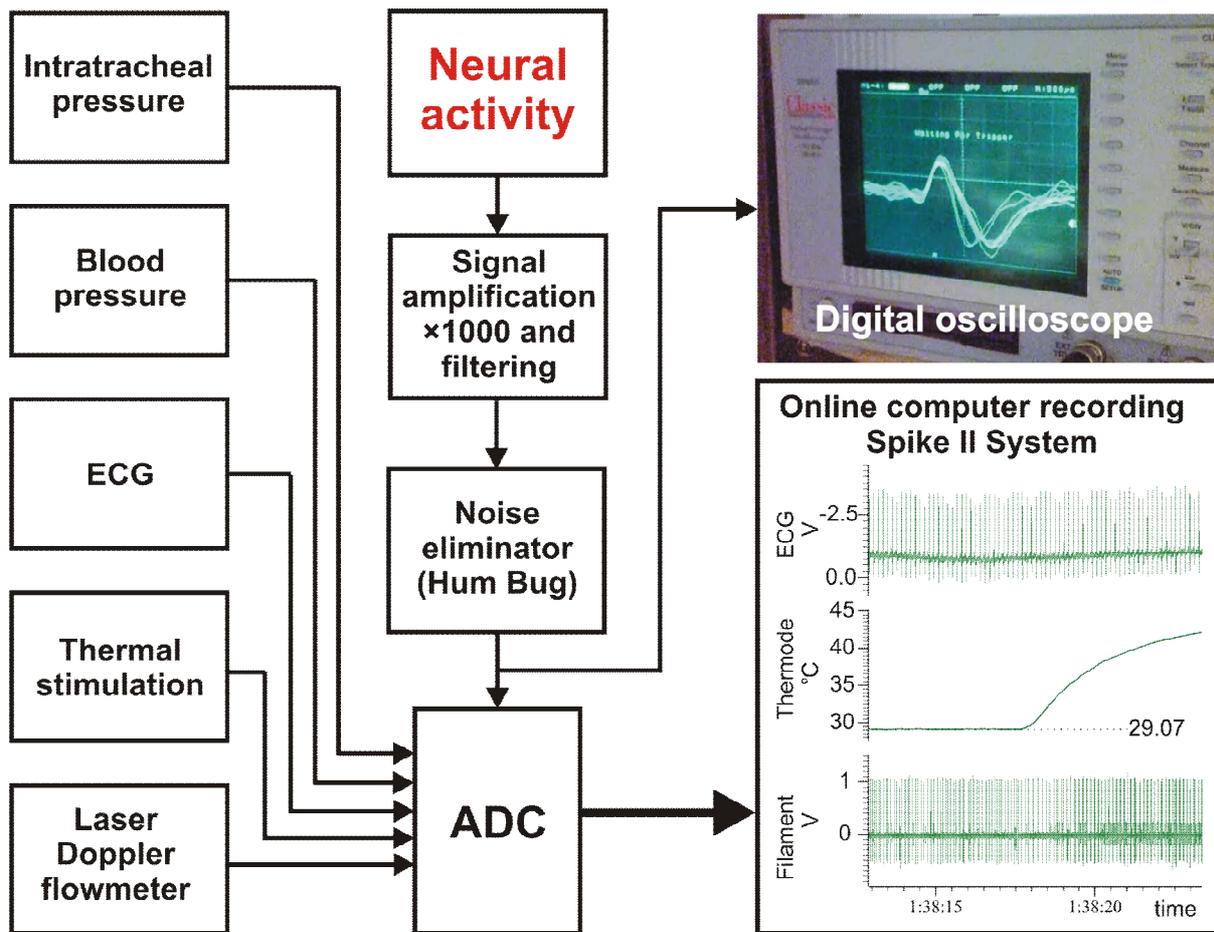


Fig. 2.4. *Set-up of the recording situation.* Nerve fiber action potentials were amplified and filtered (120 Hz to 1-1.2 KHz for unmyelinated fibers; 120 Hz to 12 KHz for identifying myelinated fibers). Additionally an active frequency filter was used (Hum Bug). Neuron action potentials, intratracheal pressure, arterial blood pressure, electrocardiogram (ECG), thermal stimuli, data from laser Doppler flowmeter and the marker signal for stimuli were fed into the computer program Spike II (for the online computer recording) and digital oscilloscope (for the signal monitoring) via an analog-to-digital convertor (ADC).

The lateral gastrocnemius-soleus nerve was isolated from the surrounding tissue by a semiflexible plastic sheet on a length of 5-6 mm between the site where it was connected to the skeletal muscle and the site where it joined the sciatic nerve, the injury site being in the middle (Fig. 2.1). The nerve was positioned on a pair of platinum electrodes for electrical stimulation with square wave pulses of 0.1 ms (A-fibers) or 0.5 ms (C-fibers) duration at strengths of up to 30 Volts (A-fibers 0.3 – 2 Volts; C-fibers 3-30 Volts). The distance between the proximal stimulation electrode and the recording electrode at the sciatic nerve or the recording electrode at the dorsal root was 13.9 ± 0.3 mm (range 9-20 mm) and 74.6 ± 1.1 mm (range 61-83 mm), respectively. In TP I 88% of the A-fibers and 62% of the C-fibers and in TP II almost all A-and C-fibers were identified electrically on the basis of their conduction velocities (by measuring the latency of the responses to single pulse stimulation of the

gastrocnemius-soleus nerve; see upper records in Fig. 2.3). The distinction between afferent A- and C-fibers in the rat based on the conduction velocity of their axons was made according to Lawson and co-workers (Lawson and Waddell 1991, Waddell et al 1989; see also Lawson 2005). Fibers conducting at >2 m/s were considered to be myelinated and fibers conducting at ≤ 2 m/s as unmyelinated. Waddell and Lawson (1991) show that afferent neurons with axons conducting at 1.3 – 2 m/s belong either to C-fiber or A δ -fiber afferent neurons. Neurons with these fibers were rare in our sample (see Fig. 3.1).

In the remaining A- or C-fibers (most of them in TP I) the exact latency of response to electrical stimulation of the muscle nerve was not measured because of the overlap of the electrically evoked action potentials. However, in these cases it was always possible to classify the fibers as A- or C-fibers on the basis of the size and shape of the action potentials in relation to the electrically or ectopically evoked action potentials (Blenk et al 1996). Under our filter conditions (see above) action potentials recorded from A-fibers were usually two-phasic and larger than action potential recorded from C-fibers (see superimposed action potentials in Fig. 2.3, right part). Action potentials recorded from C-fibers were usually tri-phasic (see superimposed action potentials in Fig. 2.3, left part).

2.3.2 Lumbar sympathetic trunk stimulation

In 17 rats (group C), before the dorsal root surgery, the left lumbar sympathetic trunk (LST) was exposed between paravertebral ganglia L2 and L4 (Baron et al 1988) using a retroperitoneal approach and carefully freed from the surrounding connective tissue. The isolated sympathetic chain between ganglion L2 and L3 was placed on a self-designed pair of silver cuff electrodes (Fig. 2.5) (Fenik et al 2001).

The teflon insulated silver wires (wire \varnothing 0,125mm, \varnothing with Teflon cover 0,15mm, A-M-System INC) were freed from their cover and stretched through the soft flexible tube (\varnothing 0.5 mm) opened from one side. After positioning the tube with the electrode wires around the sympathetic chain, it was embedded by silicone elastomer without harming the tissue (“Kwik-Cast”, World Precision Instruments). Then the LST was crushed rostral to the cuff holder and caudal to ganglion L2. The auxiliary plastic electrode holders were glued to the tissue with super glue (cyanoacrylate). The lateral opening of the body was closed by suturing deep tissues and skin and the silver wires were attached via mini-connectors with the electrical

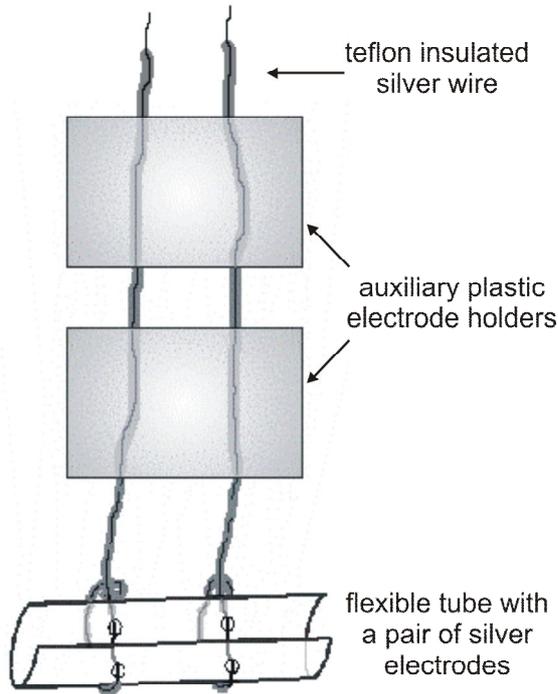


Fig. 2.5. Schematic representation of the electrodes at the lumbar sympathetic chain

stimulator. As a control for the correct electrode's implantation the blood flow changes within the cutaneous innervation territory of the segment L5 were measured on the plantar skin of the left hindpaw using a laser Doppler flowmeter device (MBF3D, Moor Instruments, Axminster, Devon, UK). The output of this device does not provide absolute values of blood flow, but arbitrary units which are called "flux". Repetitive electrical stimulation of the LST (e.g. at 7V and 5 Hz as shown in Fig. 2.6) leads to a decrease in blood flow in skin. This functional test, monitoring the correct positioning of the pair of stimulation electrodes at the LST, was performed at the beginning of every group C experiment. The threshold stimulus strength to elicit a blood flow change and the stimulus strength to elicit the maximal blood flow decrease were determined.

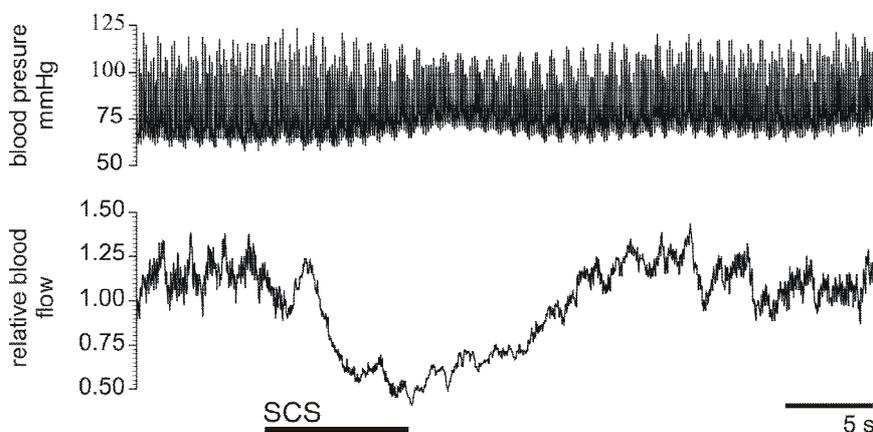


Fig 2.6. Simultaneous measurement of the blood pressure (mmHg) and relative blood flow within the L5 innervation territory on the plantar skin of the left hindpaw before, during and after repetitive electrical stimulation of the sympathetic chain stimulation (SCS) (7V, 5Hz).

2.3.3 Suprathreshold electrical stimulation

Since the event of afterdischarge (AD) was not fully investigated earlier, in this project we included suprathreshold electrical stimulation (ES) in the testing protocol of noninjured and injured muscle afferent fibers (group D1 and D2 experiments). Standard ES used were 5 stimuli of 30Volt (0.5ms, 1Hz). In some afferent fibers the strength-dependent response was studied, by increasing successively the strength of the ES (2V, 4V, 6V, 8V, 10V, 12V, 14V, 16V, 18V, 20V, 30V; 5 stimuli, 0,5ms, 1Hz).

2.4 Physiological stimulation

In the animals with GS nerve injury mechanical and thermal stimuli were applied from 3 mm proximal to 3 mm distal to the injury site of the nerve. In the control animals without nerve injury physiological stimuli were applied to the hind paw area (bending) and to the GS muscle (mechanical probing with a fine-tipped blunt glass rod).

2.4.1 Mechanical stimulation of the muscle nerve

Mechanosensitivity of injured muscle afferents was tested first qualitatively with a fine-tipped blunt glass rod (tip diameter 0.5 mm). Activation thresholds were determined with calibrated von Frey fiberglass filaments with circular plain tips of 0.5 mm diameter and forces of 0.3 to 100 mN (Table 2.1., Marstock^{nervtest}, Fruhstorfer, Marburg, Germany).

von Frey Filament	1	2	3	4	5	6	7	8	9	10	11	12
mN	0,3	0,45	0,7	1	2	7,5	10	20	30	45	70	100

Table 2.1. Conversion table of the von Frey filament in mN.

The mechanical stimuli were applied repetitively at 70 stim/min to the nerve which was attached to and held in position by the semiflexible plastic sheet that isolated the nerve from the surrounding tissue. This way of mechanical stimulation was reproducible for the weak von Frey filaments but less so for von Frey filaments >10 mN. Therefore, we could reliably determine the mechanical thresholds of the injured afferent fibers, but not the stimulus-response functions for mechanical stimuli using von Frey filaments >10 mN. An afferent fiber

was considered to be mechanosensitive if it generated ≥ 3 action potentials in 10 s during the repetitive mechanical stimulation of the nerve in silent fibers or in fibers with spontaneous activity ≤ 0.5 imp/s. In fibers with spontaneous activity of >0.5 imp/s mechanosensitivity was defined as an increase of activity by about 50% during the repetitive mechanical stimulation.

2.4.2 Thermal stimulation of the muscle nerve

Thermosensitivity of the injured nerve fibers was tested qualitatively using a fine-tipped metal rod (tip diameter 1.5 mm) at temperatures of 5°C or about 50°. After identification of the exact “receptive field”, the thermosensitivity was tested quantitatively using a U-shaped water-perfused thermode (Fig. 2.7). The length of the contact site with the nerve was 2 mm. The stimulus covered a distance of 4 mm along the nerve. The temperature was measured at this contact site (see Gorodetskaya et al 2003).

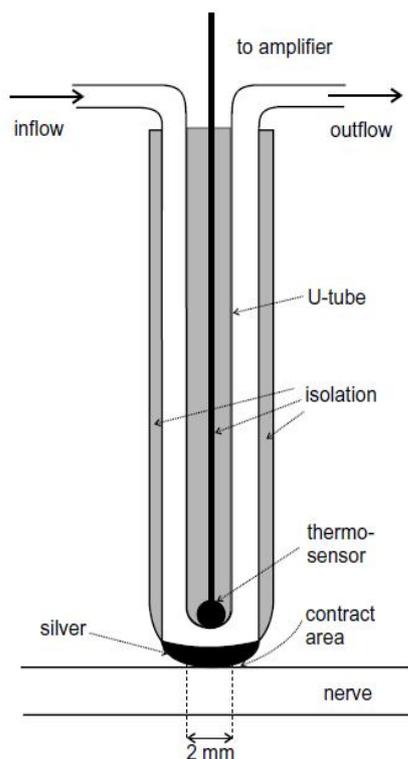


Fig. 2.7. Device used for thermal stimulation of the nerve. The thermode consisted of a U-shaped tube which is inserted in a brass tube of approx. 75 mm in length and 5 mm in diameter. The outer walls of the U-tube are thermally isolated. At its convexity (the contact areas with the nerve) the thermal isolation of the U-tube was interrupted at 1 mm x 2 mm to allow an optimal thermal conduction to the nerve. To achieve different temperatures at the contact site, water at temperatures ranging from about 3 to 52°C was perfused through the tube. A small thermo-sensor about 1mm in diameter (KTY 21-7, Siemens, Germany; time constant 1.5 s) 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 (see Gorodetskaya et al 2003).

The nerve fibers were stimulated either with a series of cold stimuli of 5-28°C (5, 10, 15, 20, 25°C) or with a series of heat stimuli of 35-50°C (48-50, 45, 40, 35°C) both in ascending or descending order. All thermal stimuli lasted for approximately 20–30 seconds starting from a baseline temperature of about 30°C. A fiber was considered to be cold or heat

activated either if at least 3-5 action potentials were evoked during a stimulus in a silent fiber or in fibers with spontaneous activity at rates of ≤ 0.5 imp/s or if the rate of spontaneous activity increased by 50% in fibers with spontaneous activity of >0.5 imp/s.

2.5 Experimental protocols

Initially when the project started the 3 experimental protocols A1, A2 and C were planned (Fig. 2.8) to functionally characterize the muscle afferents 4-13 hours after nerve injury (time period I [TP I], group A1 experiments) and 4-7 days after nerve injury (time period II [TP II], group A2 experiments), and to study the modulation of the ectopic discharges by the electrical stimulation of the sympathetic chain (time period II [TP II], group C experiments). The group B and most group D experiments were conducted after the group A and group C experiments.

2.5.1 Group A1 and A2 experiments: functional identification of the injured muscle fibers

The lateral GS nerve was crushed either 4-13 hours (time period I [TP I], group A1, 22 rats) or 4-7 days (time period II [TP II], group A2, 15 rats) before recording from the afferent fibers started. Every filament isolated from the sciatic nerve or dorsal root was tested for nerve fibers by electrical stimulation of the GS nerve (Fig. 2.8). If the filament contained a small number of GS nerve fibres (≤ 10 fibers electrically identified or $\leq 3-4$ fibres activated ectopically) with sufficient signal-to-noise ratio the fibers were further analysed for their spontaneous activity (SA) and responses to physiological or chemical stimuli. In case of a filament which contained a fiber with SA, first the SA was recorded for 3-5 minutes in order to determine its rate and pattern. This was followed by a series of thermal stimuli using the thermode (Fig. 2.7) and of mechanical stimuli using von Frey filaments applied from 3mm proximal to 3 mm distal to the injury site of the nerve. In this way, all individual nerve fibres were characterized within a filament in relation to their ectopic SA and evoked activity.

2.5.2 Group B experiments: testing of TRP agonists

In the group B (35 rats) the lateral gastrocnemius-soleus nerve was crushed 4-7 days before the neurophysiological experiments (TP II) and filaments with afferent fibers were

EXPERIMENTAL PROTOCOLS

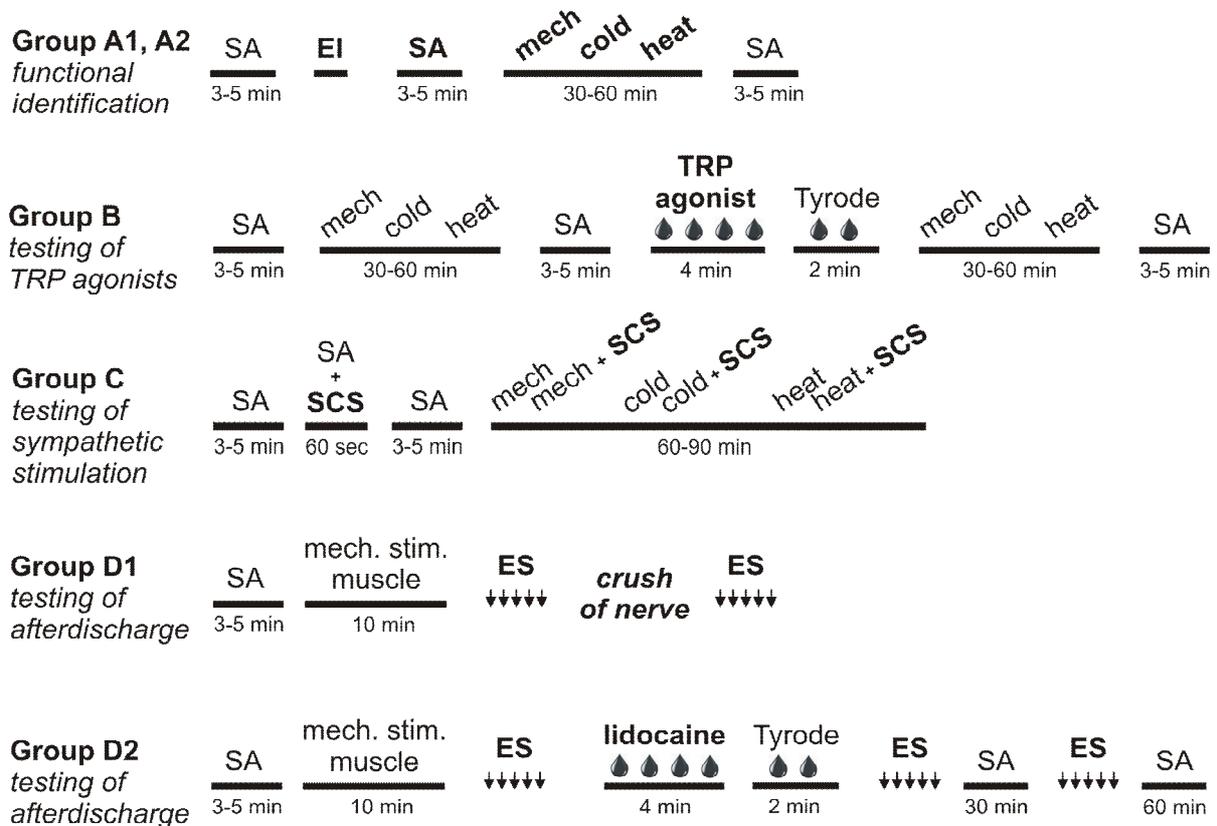


Fig. 2.8. Experimental procedures. Functional identification: studying the rate and pattern of spontaneous activity (SA), electrical identification (EI), and qualitative measurement of the responsiveness to mechanical (mech) and thermal stimulation (cold, heat) were done in the group A1 (time period I [TP I], 22 rats) and group A2 (time period II [TP II], 15 rats) experiments. The responses to the local application of the transient receptor (TRP) agonists capsaicin (TRPV1), menthol (TRPM8) or mustard oil (TRPA1) and the qualitative comparison of the thermal and mechanical responses before and after application of one of the agonists were tested in the group B experiments (TP II, 35 rats). TRP agonist substances were washed out using Tyrode's solution. The responses to activation of postganglionic axons by electrical stimulation of the sympathetic chain (SCS) were examined in the group C experiments (TP II, 17 rats). Afterdischarge (AD) in afferent fibers was studied before and after crush injury of the nerve in the group D1 experiments (7 rats). Five electrical 0.5 ms stimuli (ES) at 1 Hz and 30 Volt were applied to the GS nerve. In some neurons the stimulus-response relation was studied by applying the ES in ascending order (2V, 4V, 6V, 8V, 10V, 12V, 14V, 16V, 18V, 20V; 5 stimuli, 0.5ms, 1Hz). The afferent nerve fibers investigated for AD were characterized by their responses to physiological stimulation of the nerve injury site or of the GS muscle. In the group D2 experiments (3 rats) after an afferent fiber with AD was found lidocaine (1mg/mL) was applied locally to the electrical stimulation site. After washing out the lidocaine with Tyrode, the afferent fiber was repeatedly tested for AD by suprathreshold ES for ≥ 30 min.

teased from the sciatic nerve. First it was tested whether a filament contained fibres with at least one of the following functional properties: cold sensitivity or/and heat sensitivity or/and mechanosensitivity. After the nerve fibers in the filament were fully characterized as in the group A protocol (see testing procedure in 2.5.1), the transient receptor potential (TRP) V1 agonist *capsaicin* (50 μ M, 100 μ M, 1mM and 10mM), the TRPM8 agonist *menthol* (10 μ M, 50 μ M, 100 μ M, 1mM, 4mM) or the TRPA1 agonist *mustard oil* (100 μ M, 1mM) in ascending

concentration were applied to the receptive fields of the afferent nerve fibers in the nerve, followed by a washout with Tyrode's solution. For the topical application small pieces of filter paper (4 x 4 mm) soaked in the respective capsaicin, menthol or mustard oil solution were placed on the nerve covering about 6-8 mm of the nerve that included the lesion site and the nerve up to 3 mm distally from which the fiber could be excited by mechanical or thermal stimulation. The volume of these solutions was $\geq 5\mu\text{L}$; this volume was about 5 to 10 times larger than the volume of the lateral gastrocnemius-soleus nerve covered by this volume. Thus, the concentration of menthol, capsaicin or mustard oil at the nerve surface was the concentration of menthol or capsaicin in the solution. Every dose was applied for 3-4 min and followed by a wash-out period with Tyrode's solution for at least 2 min.

The spontaneous activity was recorded before, during and for 3-5 minutes after application of the TRP agonists. To measure the degree of sensitization or desensitization of the afferent fibers by the TRP agonists for thermal or mechanical stimulation, after the application of TRP agonists, the responsiveness to physiological stimulation of the afferent fibres was tested again (Fig. 2.8). When more than one concentration of TRP agonists was applied, the responses of the nerve fibres to the physiological stimuli were tested after every application.

Capsaicin, menthol and mustard oil are water-soluble in picomolar concentrations only. To solve menthol or mustard oil we used pure ethanol and then reached our final testing concentrations by further diluting it with Tyrode's solution, so that 4mM, 1mM, 100 μM menthol solutions contained 0.2%, 0.05% and 0.005% ethanol respectively; and 10mM, 1mM, 100 μM mustard oil solutions contained 1%, 0.1%, 0.01% ethanol. Before most applications of menthol or mustard oil we tested the effect of 0.1, 0.2, 0.5, 1% solution of ethanol (vehicle) on the nerve fibers. Ethanol is a TRPV1 agonist at concentrations $\geq 0.5\%$ (Trevisani 2002). According to pharmacological investigation of Turgut et al (2004) 10mM, 1mM and 100 μM capsaicin solutions could be reached only in 20%, 2% and 0.2% of ethanol, respectively. Therefore we solved capsaicin in Tyrode's solution heated to 65-67°C and obtained in this way seemingly clear suspensions of 50 μM , 100 μM , 1mM and 10mM capsaicin which had concentration-dependent effects on the afferent nerve fibers.

2.5.3 Group C experiments: testing of sympathetic stimulation

In group C (17 rats) fine filaments were recorded from the dorsal root L5 or L4 (recording 1, Fig. 2.2). At the beginning of every experiment the strength of sympathetic

chain stimulation (SCS) at 2-10 Hz to obtain maximal decrease of blood flow through the plantar skin, using laser Doppler flowmetry, was determined (Fig. 2.6). This strength was used throughout the rest of the experiments.

The recording started with the identification of the nerve fibers (Fig. 2.3). In case of a filament which contained a spontaneously active (SA) fiber, first the SA was recorded for 3-5 minutes. This was followed by the SCS for 60 sec and then again recording of SA for 3-5 minutes (Fig. 2.8). Then the testing series of mechanical stimuli using von Frey filaments and of thermal stimuli using the thermode were applied with and without SCS (Fig. 2.9). The duration of SCS alone or in combination with the physiological stimuli was 60 sec. In this way, all individual nerve fibres were characterized by their ectopic excitability and the activation thresholds in relation to the SCS.

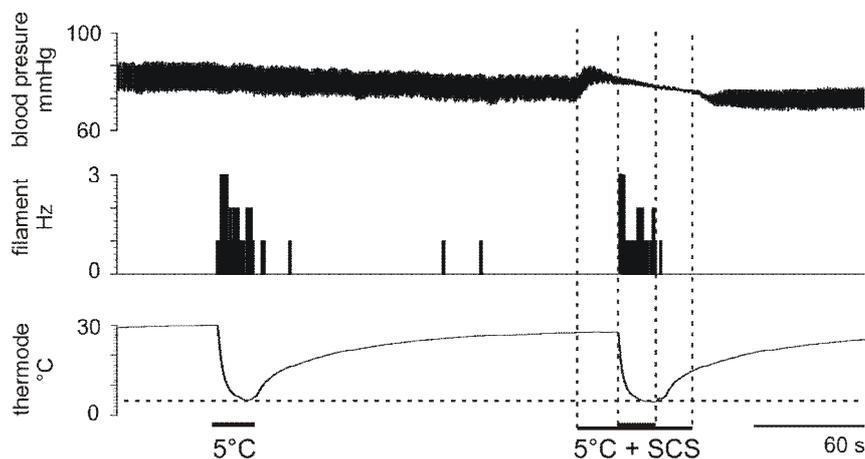


Fig. 2.9. Activation of an injured afferent nerve fiber by cold (5°C, 20s) stimulation applied to the nerve injury site with and without repetitive electrical stimulation of the sympathetic chain (SCS, 5V, 5Hz).

2.5.4 Group D1 experiments: testing of the afterdischarge

In group D1 (7 rats) afterdischarge (AD) in afferent fibers to electrical nerve stimulation was studied before and after crush injury of the nerve (Fig. 2.8). A train of 5 suprathreshold electrical stimuli (ES) at 30 Volt (0.5 ms, 1 Hz) was applied to the GS nerve. In some afferent fibers the stimulus-response relation of the AD was studied by varying the strength of the ES in an ascending order (2V, 4V, 6V, 8V, 10V, 12V, 14V, 16V, 18V, 20V; 5 stimuli, 0.5ms, 1Hz). Before the nerve injury the nerve fibers were tested for their responses to mechanical

probing of the GS muscle and after nerve injury to mechanical and thermal stimuli applied to the injury site according to protocol A1.

2.5.5 Group D2 experiments: testing of the effect of lidocaine on the afterdischarge

In 10 experiments the hypothesis was tested whether the AD in muscle afferent fibers generated by electrical stimulation of the muscle nerve is dependent on the presence of sodium channels in the nerve fibers. Lidocaine (sodium channel blocker) in a concentration of 0.01, 0.1 and 1mg/mL (corresponding to solutions of approximately 0.04, 0.4 and 4mM/L) was applied locally to the stimulation electrode site of the GS nerve for 4 min in the same way as described for the application of the TRP agonists (see 2.5.2). After the application lidocaine was washed out with Tyrode's solution and the response of the afferent fiber to ES of the nerve (5 stimuli, 30V, 0,5ms, 1Hz) was tested repeatedly every 30 min.

2.6 Data analysis

Neural activity, temperature of the thermode for stimulation of the injured nerve, arterial blood pressure, electrocardiogram and intratracheal pressure were simultaneously fed into a computer using the Spike II System (Cambridge Electronic Design Ltd, 4 Science Park, Milton Road, Cambridge, UK) (Fig. 2.4). All parameters were continuously monitored to guarantee that we were recording from the same fiber since the action potentials sometimes changed in size and shape with time. Data analysis was performed offline using the general purpose capture and analysis package Spike II. In this analysis size and shape of the action potentials recorded from the nerve fibers were used to discriminate the signals recorded from the afferent fibers (see lower records in Fig. 2.3 and Fig. 2.10).

For multi-unit recordings, the Spike II System contains tools for sorting spikes based on the spike waveform shape. For this purpose several similar action potentials were added together to a template, so that all action potentials whose image points were at least 70-90% similar to the template and whose amplitude differed by not more than 5-20% from the amplitude of the template, were assigned as one nerve fiber (Fig. 2.10).

The neural activity during the various stimuli was evaluated quantitatively. The initial rate (impulses per second) of spontaneous activity (SA), which was recorded before the

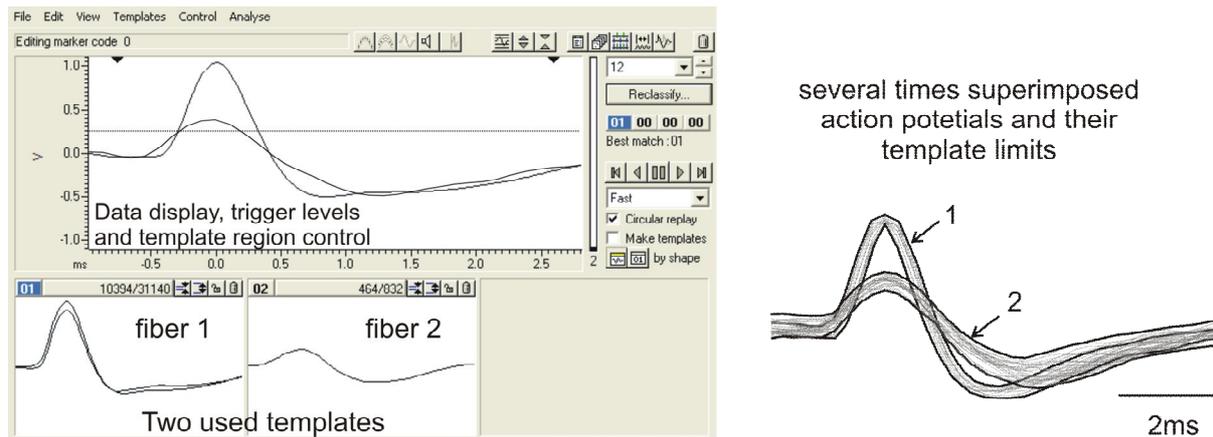


Fig. 2.10. Off-line template analysis window and allocation of action potentials to single individual nerve fibers. The template analysis window contains four main regions: a status and tool bar at the top, a data display area with the trigger levels, a control area on the right and a region with up to 20 templates at the bottom. The program scans the data in the channel set, displays triggered action potentials and creates their templates. The criteria for the new template creation are user-defined by setting the percentage of the width change in the amplitude (maximum per cent amplitude change for the match, 3-20%) and the shape (minimum percentage of points in template, e.g. 70-90%). The analysis of the action potentials of the two-fiber data recording is shown in this specific example. The thick black lines mark the borders of the templates. The thin grey lines show several times superimposed individual action potentials corresponding in their shape to the respective template. Thus they are assigned to two separate units (fiber 1 or fiber 2).

testing period was taken as baseline (Fig. 2.8). In most figures the baseline rate of SA is deduced from the activity evoked by thermal or mechanical stimuli or application of TRP agonists or vehicle.

Quantitative measurements are either expressed as mean \pm standard error of the mean (SEM) or median \pm interquartile range (Fig. 2.11). For statistical analysis the χ^2 -test, the Student's t-test, ANOVA or the Wilcoxon-Mann-Whitney U-test were used. The normality of the distribution was measured by means of the Shapiro-Wilk tests with random checks ($n < 50$) or the Kolmogorow-Smirnow test ($n > 50$). The distributions of the discharge frequency were modified as a Velleman-Hoaglin (1981) boxplot with median (2nd quartile), 1st and 3d quartile, upper and lower whisker ($\pm 1.5 \times$ interquartile range) (Fig. 2.11).

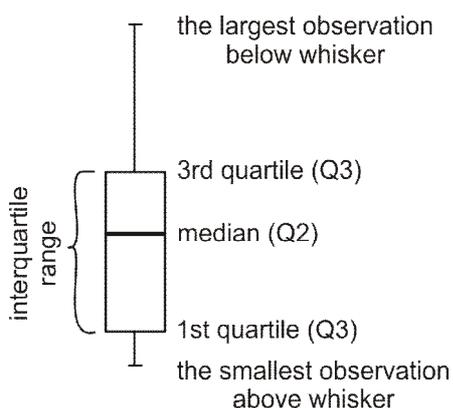


Fig. 2.11. Boxplot modified from Velleman and Hoaglin (1981). Upper and lower ends correspond to the closest observations to the whisker value ($\pm 1.5 \times$ interquartile range). The box (box between 3rd and 1st quartile) encloses the middle 50% of the distribution. The line in the box marks the median. The boxplot is a quick way of examining one or more sets of data graphically. Boxplots do have some advantages taking up less space and are therefore particularly useful for comparing distributions between several groups or sets of data.

Chapter 3

Results

3.1 Ectopic sensitivity of injured muscle afferents (groups A1 and A2)

Muscle afferent fibers are usually referred to as group I to group IV afferent fibers, groups I and II afferents being large diameter myelinated ($A\alpha/\beta$ -) fibers, group III afferents small diameter myelinated ($A\delta$ -) fibers and group IV afferents unmyelinated (C-) fibers. In our study we had no criteria to discriminate between groups I, II or III fibers. Therefore we will use throughout the text the terms A-fibers (or myelinated fibers) and C-fibers (or unmyelinated fibers) (see Fig. 3.1).

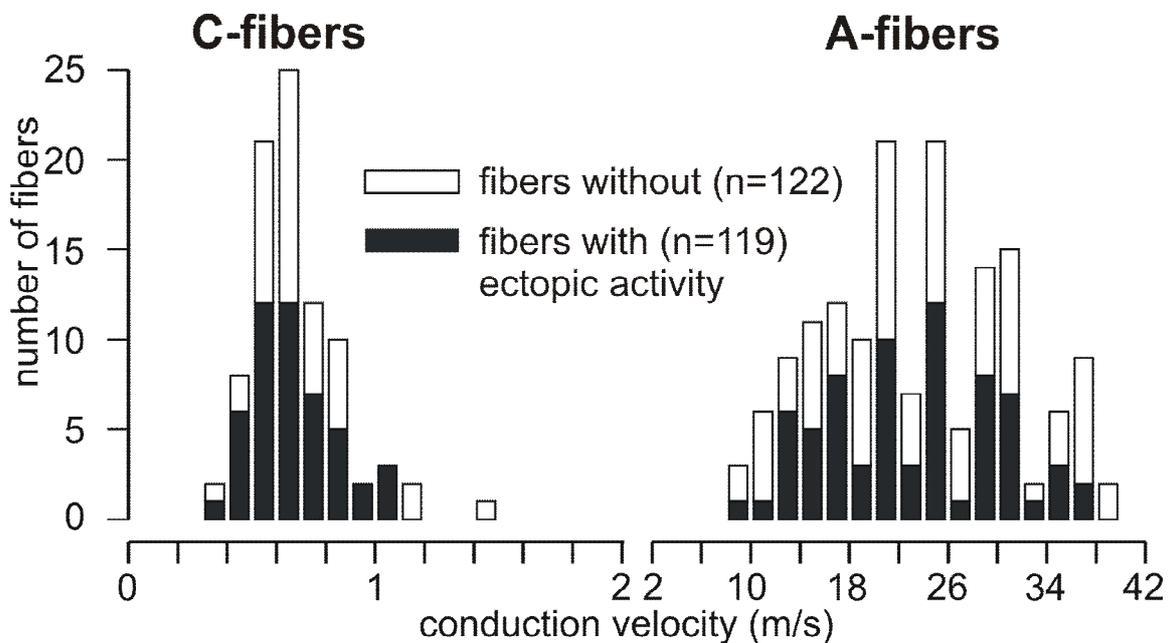


Fig. 3.1. Conduction velocity of A- and C-fibers in the lateral gastrocnemius-soleus nerve. Distribution of conduction velocities in time period II (TP II) (4-7 days after nerve crush). Data from 23 experiments in which all electrically identified fibers were tested. Black parts of the columns: Fibers showing spontaneous activity and/or responses to mechanical and/or thermal stimuli applied to the nerve injury site. Open parts of the columns: Fibers which did not show spontaneous activity, mechanosensitivity or thermosensitivity.

In 22 rats with acute lateral gastrocnemius-soleus nerve lesion (4-13 hours after nerve injury, time period I (TP I)) and in the 67 rats with a chronic gastrocnemius-soleus nerve lesion (4-7 days after nerve injury, TP II) we studied systematically all nerve fibers in the filaments, which were isolated either from the sciatic nerve or from dorsal root L4 or L5 and activated by electrical stimulation of the lateral gastrocnemius-soleus nerve (Fig. 2.2, 2.8), for their ectopic spontaneous and evoked activity and for their conduction velocity.

The study of the functional characterization of the injured muscle fibers is based on the recording from 208 injured afferent A-fibers and 165 injured afferent C-fibers which could be activated by electrical stimulation of the lateral gastrocnemius-soleus nerve and exhibited at least one form of discharge property (spontaneous activity, mechanosensitivity, cold sensitivity and/or heat sensitivity).

3.1.1 Proportions of injured muscle A- and C-fibers with ectopic activity

The term “ectopic activity” of an afferent fiber denotes activity originating spontaneously at the injury site or in dorsal root ganglion cells of afferent neurons and activity evoked by mechanical or thermal stimulation of afferent neurons at the injury site. The first signs of the developed ectopic activity in the injured muscle nerve appeared 4 hours after nerve lesion. This is in accordance with previous studies on an acutely damaged skin nerve (Michaelis et al 1995, Blenk et al 1996).

In time period I (TP I) (4-13 hours after nerve injury) 5.4% of the electrically identified A-fibers and 37.9% of the electrically identified C-fibers isolated from the sciatic nerve exhibited ectopic activity (Table 3.1).

	4-13 hours rec. sciatic n.	χ^2 -test	4-7 days rec. sciatic n.	χ^2 -test	4-7 days rec. dorsal r.
A-fibers	12/224 (5.4%)	p<0.000	153/278 (55.0%)	p<0.05	41/61 (67.2%)
C-fibers	39/103 (37.9%)	p<0.02	98/160 (61.3%)	p<0.05	20/24 (83.3%)

Table 3.1. Percentages of electrically identified fibers with spontaneous and/or evoked ectopic activity. Values are percentages of electrically identified fibers with spontaneous activity (SA) and/or activity evoked by mechanical and/or thermal stimulation of the nerve injury site. The fibers recorded from the sciatic nerve (rec. sciatic n.) were obtained in 19 experiments (4-13 hours) and 36 experiments (4-7 days). The fibers recorded from the dorsal root (rec. dorsal r.) L4 or L5 were obtained in 19 experiments.

In 36 experiments (all group A2 (13 rats) and part of group B (23 rats)) of TP II (4-7 days after nerve injury) we studied fibers in the filaments which were isolated from the sciatic nerve. In these experiments 55% of the electrically identified A-fibers and 61.3% of the electrically identified C-fibers were ectopically active (Table 3.1). Thus, the proportion of ectopically active afferent A- and C-fibers significantly increased in TP II compared to TP I ($p < 0.0001$ for A-fibers and $p < 0.02$ for C-fibers, χ^2 -test).

The distribution of the conduction velocity of these fibers shows a clear separation between C- and A-fibers, only one fiber conducting in the range of 1.3 to 2 m/s. The distribution of the conduction velocities of A-fibers did not show a separation between A δ - (group III) and A α/β - (group I/II) fibers (Fig. 3.1).

About 50% of the myelinated axons in the gastrocnemius-soleus nerve are somatomotor axons (Mitchell and Schmidt 1983; Peyronnard et al 1986; Sittiracha and McLachlan 1986) and 45% of the unmyelinated axons are sympathetic postganglionic axons (Baron et al 1988). Assuming that injured somatomotor and postganglionic axons do not exhibit spontaneous or evoked ectopic activity originating at the injury site (Schmelz et al 1998; Schmidt et al 1995) we conclude that all or most myelinated and unmyelinated fibers showing no ectopic activity are efferent fibers (Fig. 3.1) and that therefore all or most injured muscle afferents show ectopic activity in TP II. This conclusion is supported by the finding that the proportions of electrically identified A- and C-fibers which were isolated from the dorsal root L4 or L5 and exhibited ectopic activity were significantly higher than the proportions of electrically identified A- and C-fibers with ectopic activity which were isolated from the sciatic nerve since motoneurons and postganglionic neurons do not project through the dorsal roots to the periphery (Table 3.1).

Seventy-nine per cent of the A-fibers and 83% of the C-fibers with ectopic activity were recorded from filaments isolated from the sciatic nerve and the remaining fibers were isolated from the dorsal root L5 (see upper part of Tables 3.2 and 3.3). There were no statistically significant differences in the functional characteristics (incidence of spontaneous activity, mechano- or thermosensitivity or their combinations) between the afferent fibers isolated from the sciatic nerve and the afferent fibers isolated from the dorsal root. Therefore both populations of afferent fibers will be described together.

Numbers and discharge characteristics of A-fibers 4-13 hours (time period I) and 4-7 days (time period II) after crush injury of the lateral gastrocnemius-soleus nerve

	4-13 hours			4-7 days		
	rec sciatic nerve	rec dorsal root	total	rec sciatic nerve	rec dorsal root	total
Number of fibers with ectopic activity	12	2	14	153	41	194
Number of electrically identified fibers	224	6	230	278	61	339

INDIVIDUAL DISCHARGE CHARACTERISTICS

Spontaneous activity (SA)	4	1	5 (35.7%)	79	18	97 (50%)
Mechanosensitivity	8	1	9 (64.3%)	140	37	177 (91.2%)
Cold sensitivity	0	0	0%	103	19	122 (62.9%)
Heat sensitivity	0	0	0%	87	14	101 (52.2%)

COMBINATIONS OF DISCHARGE CHARACTERISTICS

Mech only/SA	8/0	1/0	9/0 (64.3%)	33/11	13/4	46/15 (23.7%)
Cold only/SA	0	0	0%	6/1	0	6/1 (3.1%)
Heat only/SA	0	0	0%	4/2	0	4/2 (2.1%)
Mech and cold/SA	0	0	0%	25/5	10/2	35/7 (18%)
Mech and heat/SA	0	0	0%	11/5	5/2	16/7 (8.2%)
Cold and heat/SA	0	0	0%	1/1	0	1/1 (0.5%)
Mech, heat & cold/SA	0	0	0%	71/52	9/6	80/58 (41.2%)
SA only	4	1	5 (35.7%)	2	4	6 (3.1%)

Table 3.2. Numbers and discharge characteristics of injured afferent A-fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve. The fibers recorded from the sciatic nerve (rec. sciatic nerve) were obtained in 19 experiments (4-13 hours) and 36 experiments (4-7days). The fibers recorded from the dorsal root (rec. dorsal root) L4 or L5 were obtained in 19 experiments. The numbers behind the slash indicate the numbers of fibers with spontaneous activity (SA) (included in the number before the slash). The percentages of individual discharge characteristics are expressed in respect to the total number of fibers showing ectopic discharges.

Numbers and discharge characteristics of C-fibers 4-13 hours (time period I) and 4-7 days (time period II) after crush injury of the lateral gastrocnemius-soleus nerve

	4-13 hours			4-7 days		
	rec sciatic nerve	rec dorsal root	total	rec sciatic nerve	rec dorsal root	total
Number of fibers with ectopic activity	39	8	47	98	20	118
Number of electrically identified fibers	103	10	113	160	24	184

INDIVIDUAL DISCHARGE CHARACTERISTICS

Spontaneous activity (SA)	23	2	25 (53.2%)	54	12	66 (55.9%)
Mechanosensitivity	29	6	35 (74.5%)	48	11	59 (50%)
Cold sensitivity	5	6	11 (23.4%)	33	14	47 (39.8%)
Heat sensitivity	22	3	25 (53.2%)	66	12	78 (66.1%)

COMBINATIONS OF DISCHARGE CHARACTERISTICS

Mech only/SA	13/8	1/0	14/8 (29.8%)	15/7	1/0	16/7 (13.6%)
Cold only/SA	0	2/0	2/0 (4.3%)	6/1	2/1	8/2 (6.8%)
Heat only/SA	9/5	0	9/5 (19.1%)	30/18	2/2	32/20 (27.1%)
Mech&cold/SA	3/1	2/1	5/2 (10.6%)	8/2	4/2	12/4 (10.2%)
Mech&heat/SA	11/7	1/1	12/8 (25.5%)	17/7	2/2	19/9 (16.1%)
Cold&heat/SA	0	0	0	11/9	4/2	15/11 (12.7%)
Mech,heat&cold/SA	2/1	2/0	4/1 (8.5%)	8/7	4/2	12/9 (10.2%)
SA only	1	0	1 (2.1%)	3	1	4 (3.4%)

Table 3.3. Numbers and discharge characteristics of injured afferent C-fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve. For details see legend Tab. 3.2.

3.1.2 Response characteristics

I will first describe the discharge properties of the injured A- and C-fibers evoked by physiological stimulation. Then I will describe the spontaneous activity and the pattern of discharge of the injured afferent fibers. The numbers of afferent neurons exhibiting individual and combinations of discharge characteristics to the physiological stimuli are shown in Tables 3.2 and 3.3. All percentages will be expressed with respect to the total numbers of afferent A- or C-fibers that exhibited at least one type of ectopic activity.

a. Mechanosensitivity

	Mechanosensitive A-fibers		Mechanosensitive C-fibers	
	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity
TP I	9/230 (4%)	9/14 (64%)	35/113 (31%)	35/47 (74.5%)
TP II	177/339 (52%)	177/194 (91%)	59/184 (32%)	59/118 (50%)

Table 3.4. Numbers of mechanosensitive injured afferent fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve. The percentages of mechanosensitive fibers from all electrically identified fibers are given in the first column; the second column shows percentages of mechanosensitive fibers from all ectopically active ones.

A-FIBERS.

In TP I only very few (9/230) A-fibers showed mechanosensitivity (4%) (Table 3.4). In 4 A-fibers the early stages of the development of the mechanosensitivity were observed (Fig. 3.2), but since the level of sensitivity was not strong enough (increase of the activity was less than 50%) these fibers were not characterized as mechanosensitive.

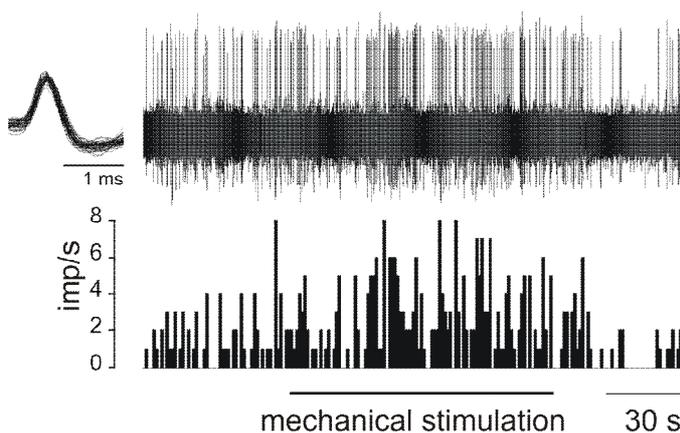


Fig. 3.2. Mechanosensitivity of an A-fiber (TPI). Recording 10 hours after crush injury. Mechanosensitivity tested qualitatively with a fine-tipped blunt glass rod (tip diameter 0.5 mm) applied repetitively at 70 stim/min to the nerve injury site. During the repetitive mechanical stimulation an increase of its activity was less than 50%.

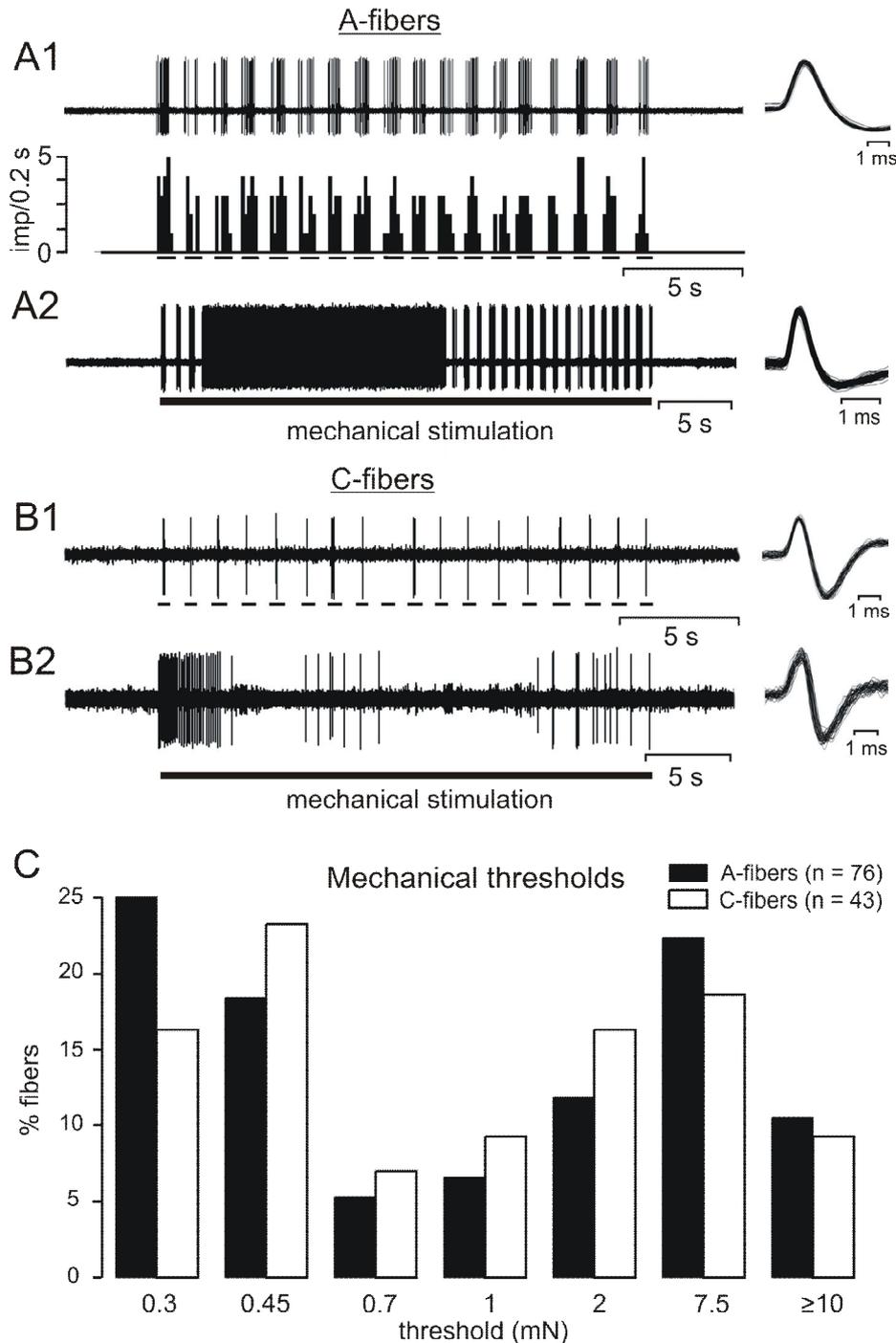


Fig. 3.3. Mechanosensitivity of A- and C-fibers. A-B. Responses of injured A- (A) and C-fibers (B) to mechanical stimulation of the nerve injury site. Mechanical stimuli were applied with glass fiber von Frey filaments at suprathreshold strengths and at a rate of 70 stim/min under visual control through a stereomicroscope. In A1 and B1 the single stimuli are indicated to show the phasic responses of the afferent fibers. The A-fiber in A2 switched from phasic responses to a tonic response during repetitive stimulation. The C-fiber in B2 did not respond phasically to most repetitive stimuli. Insets on the right, action potentials several times superimposed. C. Distribution of activation thresholds of injured A- and C-fibers to mechanical stimulation of the nerve. The strength of the von Frey filament applied to the receptive field in the nerve generating ≥ 3 action potentials in the first 10s of stimulation in fibers being silent or having an spontaneous activity ≤ 0.5 imp/s was defined as activation threshold. In fibers with spontaneous activity > 0.5 imp/s the threshold was an increase of activity by 100%. Thresholds of A-fibers measured in TP II. Distributions of thresholds of C-fibers were not significantly different between TP I and TP II ($p > 0.05$, Wilcoxon-Mann-Whitney U-test, Fig. 3.5). The data from both time periods were therefore pooled together. Activation thresholds of A-fibers and C-fibers were not significantly different in TP II ($p > 0.05$, Wilcoxon-Mann-Whitney U-test).

In TP II 177/339 (52%) of all electrically identified A-fibers could be activated mechanically, thus 91% of the injured A-fibers with ectopic activity were mechanosensitive. Most A-fibers showed brisk responses with high instantaneous frequencies to repetitive mechanical stimulation at about 1 Hz (Fig. 3.3A1). Some of them switched to tonic discharge during this repetitive mechanical stimulation (Fig. 3.3A2). The mechanical thresholds ranged from 0.3 - >10 mN (median 0.7 mN, n=76) and showed a bimodal distribution with modal values of about 0.3 – 0.45 mN and 7.7 mN (Fig. 3.3C). Although not systematically investigated it was obvious that the mechanosensitive A-fibers were also very stretch-sensitive. During the positioning of the thermode on the lateral gastrocnemius-soleus nerve (for thermal stimulation) the nerve was always slightly stretched leading sometimes to strong activation of the A-fibers (Fig. 3.4) followed sometimes by the depression of spontaneous activity.

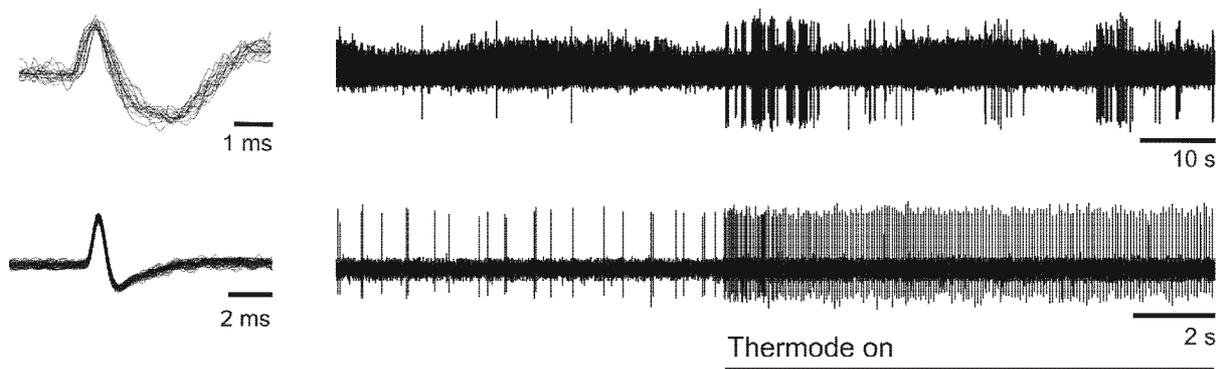


Fig. 3.4. *Stretch-sensitive injured muscle afferents.* Original records from an unmyelinated (upper recording) and an myelinated (lower recording) highly mechanosensitive fiber activated during the positioning of thermode.

C-FIBERS.

35/113 (31%) in TP I and 59/184 (32%) in TP II of the electrically identified C-fibers showed mechanosensitivity (Table 3.4). The incidence of mechanosensitivity in TP I in C-fibers was significantly higher than in A-fibers (35/113 (31%) C-fibers versus 9/230 (4%) A-fibers, $p < 0.000$, χ^2 -test). In TP II it was significantly lower than in A-fibers (59/184 (32%) C-fibers versus 177/339 (52%) A-fibers, $p < 0.000$, χ^2 -test). The magnitude of the responses to mechanical stimulation of the nerve injury site was lower than in A-fibers. Mechanosensitive C-fibers responded usually only with one impulse to a brief stimulus applied with a suprathreshold von Frey filament or a glass rod (Fig. 3.3B1) or they responded rather irregularly to repetitive mechanical stimulation of the nerve injury site (Fig. 3.3B2). Distributions of mechanical thresholds of C-fibers were not significantly different between TP

I and TP II ($p > 0.05$, Wilcoxon-Mann-Whitney U-test, Fig. 3.5). The data from both time periods were therefore pooled together (Fig. 3.3C). The thresholds ranged from 0.3 - >10 mN (median 0.7 mN, $n=43$) and showed a bimodal distribution similar to that of A-fibers with modal values of 0.45 and 7.5 mN. They were statistically not significantly different from those in A-fibers ($p > 0.05$, Wilcoxon-Mann-Whitney U-test).

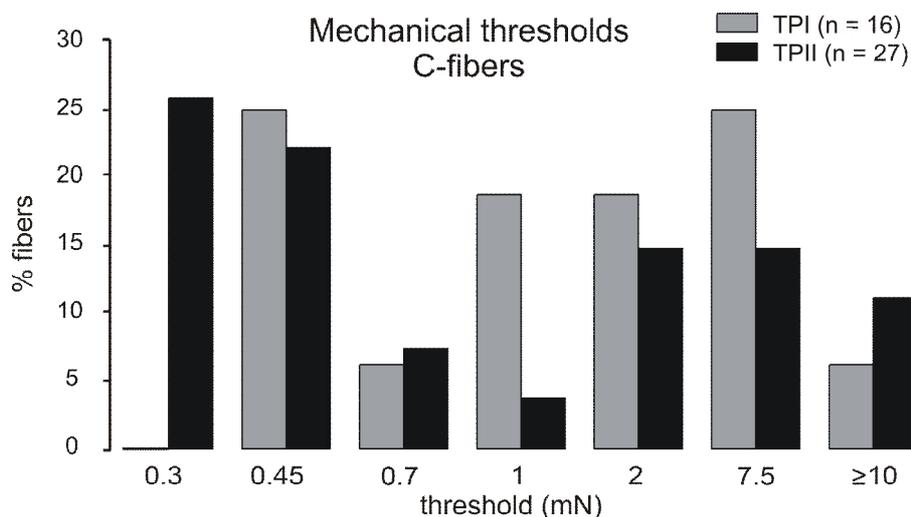


Fig. 3.5. Distribution of activation thresholds of injured C-fibers (TP I and TP II) to mechanical stimulation of the nerve. Distributions of thresholds of C-fibers were not significantly different between TP I and TP II ($p > 0.05$, Wilcoxon-Mann-Whitney U-test).

b. Cold sensitivity

	Cold-sensitive A-fibers		Cold-sensitive C-fibers	
	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity
TP I	0/230 (0%)	0/14 (0%)	11/113 (10%)	11/47 (23.4%)
TP II	122/339 (36%)	122/194 (63%)	47/184 (25.5%)	47/118 (40%)

Table 3.5. Numbers of cold-sensitive injured afferent fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve.

A-FIBERS.

In TP I cold sensitivity was absent in injured A-fibers (Table 3.5). In 2 A-fibers the early stages of the development of the cold sensitivity were observed (Fig. 3.6). The responses of these A-fibers were delayed and/or not reproducible and/or weak (increase of the activity was less than 50%), thus these fibers were not characterized as cold-sensitive.

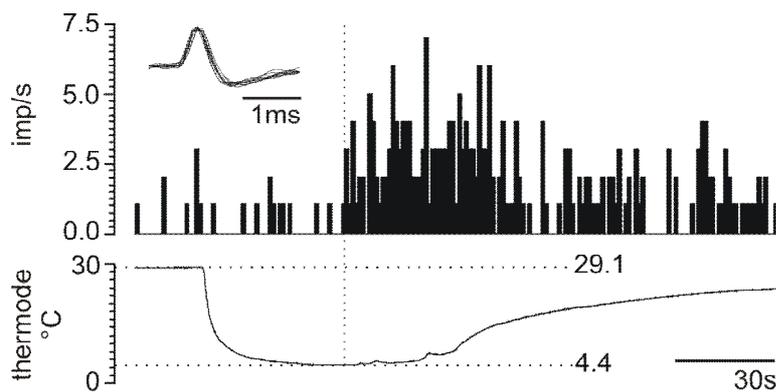


Fig 3.6. Cold-sensitivity of an A-fiber recorded 11 hours after nerve injury (TP I). The cold stimulus of 4-5°C was applied with a thermode to the lesion site of the nerve. The response of the A-fiber was delayed and not reproducible when the same stimulus was applied again 10 minutes and 30 minutes later.

In TP II 63% of the A-fibers with ectopic activity were cold-sensitive showing graded responses to cold stimuli (Fig. 3.8A). The pattern of the response to cold stimuli was always not bursting in contrast to the mostly bursting pattern of the response to heat stimuli (Fig. 3.13). Cold-sensitive A-fibers were separated into two groups. Cold-sensitive A-fibers with spontaneous activity showed larger responses to cold stimuli than silent cold-sensitive A-fibers (Fig. 3.9A and B). The maximal responses in spontaneously active A-fibers occurred mostly at 10 to 15°C (Fig. 3.9B2) and in silent A-fibers at <10°C (Fig. 3.9A2). The thresholds to cold stimuli ranged from 6 to 27°C. They were significantly lower in spontaneously active A-fibers than in silent A-fibers (Fig. 3.7; $p < 0.001$, Wilcoxon-Mann-Whitney U-test).

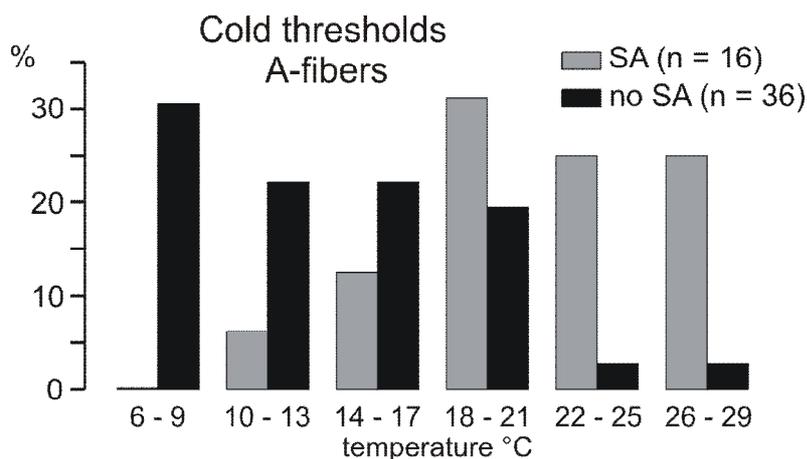


Fig. 3.7. Distribution of cold thresholds of A-fibers in TP II. The afferent fibers were separated into silent fibers (no spontaneous activity [SA]) and fibers with SA.

Cold sensitivity in the injured myelinated afferents in TP II was mostly combined with mechano- and/or heat sensitivity (Table 3.2). The largest population of injured A-fibers was mechano-cold-heat-sensitive (80/194); most of these fibers (58/80) were spontaneously active and had low mechanical and thermal thresholds.

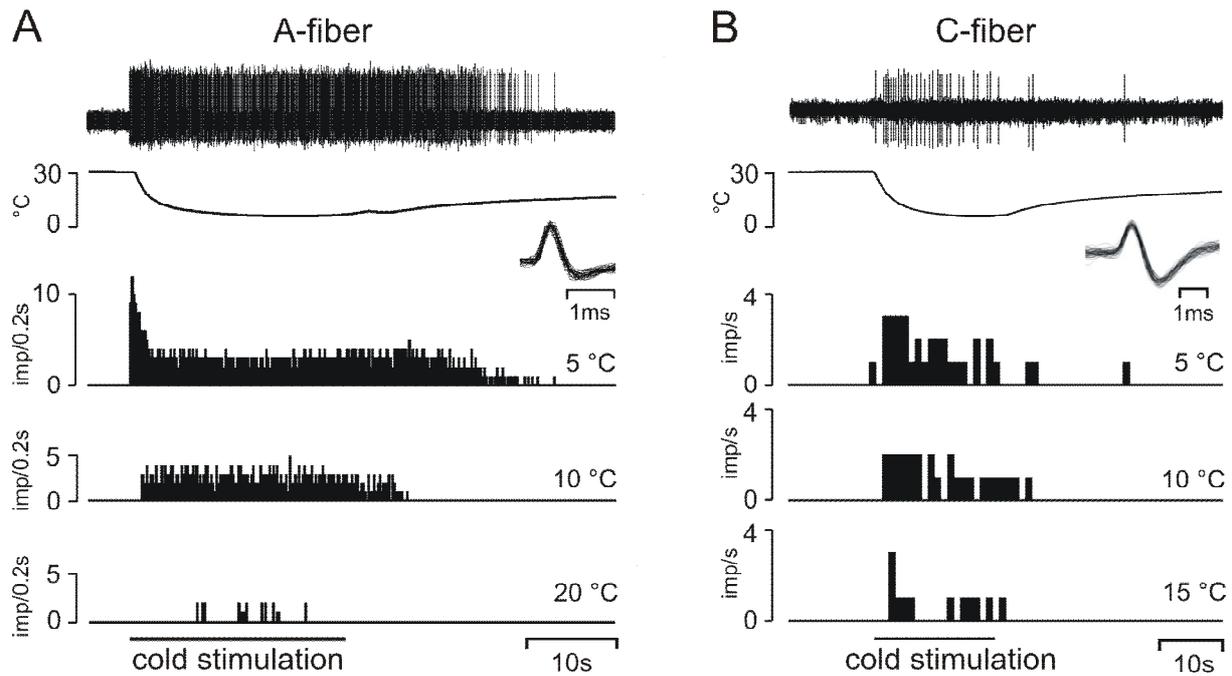


Fig. 3.8. Cold sensitivity of A- and C-fibers. A. Graded responses of an A-fiber to cold stimulation of the nerve. The fiber was silent at 30°C. Inset, action potential several times superimposed. Data from TP II. B. Graded responses of a C-fiber to cold stimulation of the nerve. The fiber was silent at 30°C. Inset, action potentials several times superimposed. Data from TP II.

C-FIBERS.

The frequency of C-fibers with cold sensitivity was higher in TP II (40%) than in TP I (23.4%; $p < 0.05$, χ^2 -test). It was significantly lower than that of injured cold-sensitive A-fibers in TP II (47/118 fibers versus 122/194 fibers; $p < 0.000$, χ^2 -test; Table 3.5). The response pattern to cold stimuli was mostly tonic and not bursting (Fig. 3.8B), but varied considerably between C-fibers (Fig. 3.10B). In most C-fibers these responses were maximal at temperatures of $\leq 10^\circ\text{C}$ (Fig. 3.10B). The thresholds ranged from 5 to 20°C (median 15°C ; inset in Fig. 3.10). The discharge rate at 5°C was significantly lower in C-fibers than in cold-sensitive A-fibers ($p < 0.001$, Wilcoxon-Mann-Whitney U-test; Fig. 3.9 and 3.10).

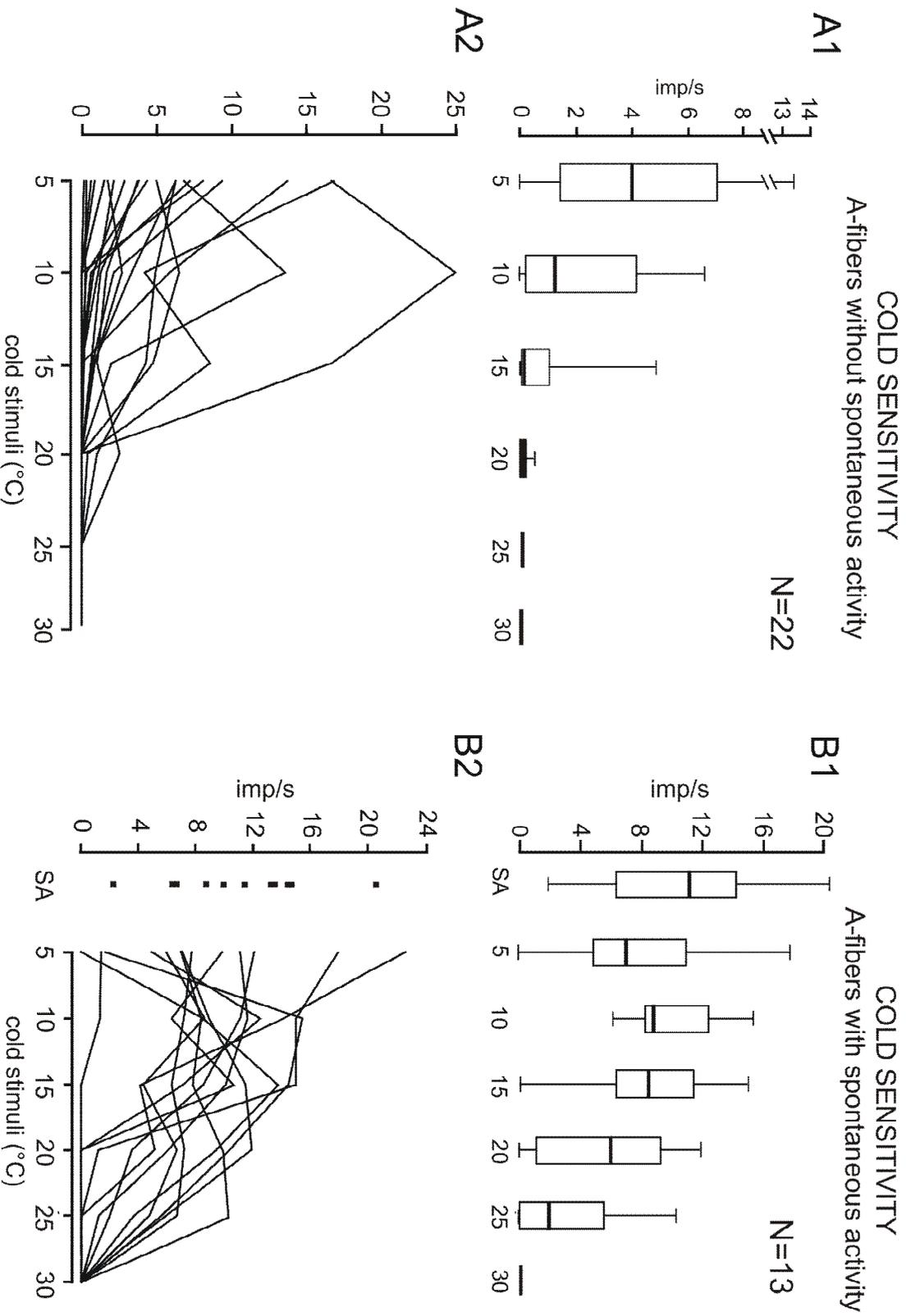


Fig 3.9. Stimulus-response functions of A-fibers to cold stimuli in TP II. The afferent fibers were separated into silent fibers (no spontaneous activity [SA]; A1, A2) and fibers with SA (B1, B2). A2 and B2 show the responses of single afferent fibers and A1, B1 the population responses (median plus interquartile range). The rate of SA was subtracted from the response to cold stimuli. The ordinate scales are the activity in imp/s during stimuli of 20 s duration (see Fig. 3.7, A).

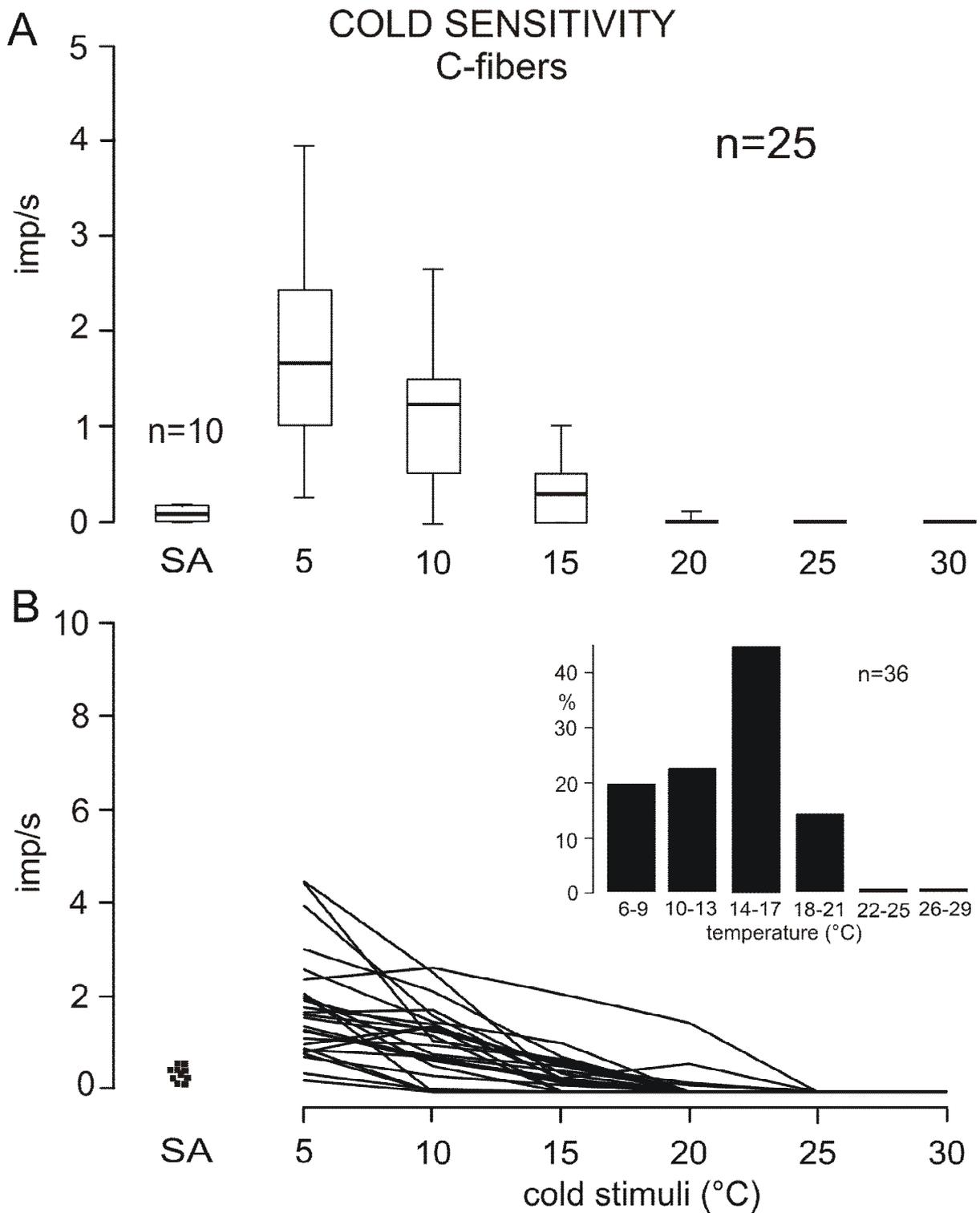


Fig. 3.10. Stimulus-response functions of C-fibers to cold stimuli in TP II. A. Population responses (median plus interquartile range). B. Responses of single afferent fibers. The rate of SA in 12 fibers was subtracted from the response to cold stimuli. The ordinate scales are the activity in imp/s during stimuli of 20 s duration (see Fig. 3B). Inset: Distribution of cold thresholds of C-fibers in TP I and TP II.

c. Heat sensitivity

	Heat-sensitive A-fibers		Heat-sensitive C-fibers	
	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity	Numbers of electrically identified fibers	Numbers of fibers with ectopic activity
TP I	0/230 (0%)	0/14 (0%)	25/113 (22%)	25/47 (53%)
TP II	101/339 (30%)	101/194 (52%)	78/184 (42.4%)	78/118 (66%)

Table 3.6. Numbers of heat-sensitive injured afferent fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve.

A-FIBERS.

Heat sensitivity in injured A-fibers was absent in TP I (Table 3.6). In the late stages of TP I (starting in the 10th hour after nerve injury) few A-fibers started to develop heat sensitivity (Fig. 3.11), but since the level of sensitivity was not strong enough (increase of the activity was less than 50%) these fibers were not characterized as heat-sensitive.

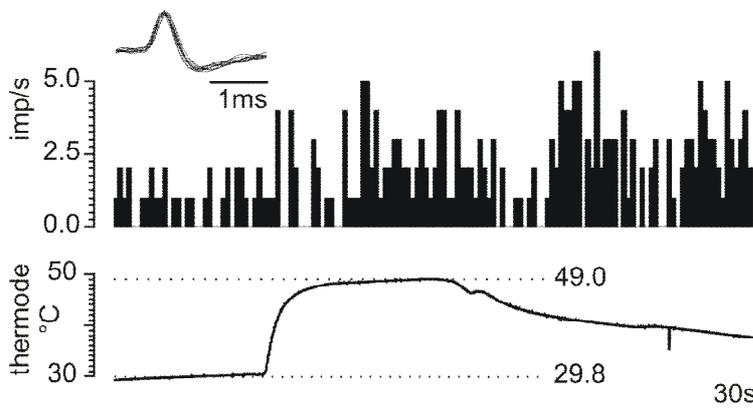


Fig. 3.11. Heat-sensitivity of an A-fiber 12 hours after nerve injury (TP I). A heat stimulus of 49°C was applied with a thermode on the lesion site of the nerve. The increase of the activity during stimulation was less than 50%.

In TP II heat sensitivity is present in 52% of the ectopically active A-fibers. The responses to heat stimuli of 20 to 30s duration applied to the nerve injury site were graded (Fig. 3.14A). In most heat-sensitive A-fibers the activation outlasted the stimulus by 1-4 minutes. The latency of the responses varied, starting either immediately, during the stimulus or even after the stimulus (Fig. 3.12).

In comparison with the tonic discharge pattern during cold stimuli the discharge pattern during heat stimulation was mostly bursting (Fig. 3.13, 3.14A) (bursting in 37.5%, bursting followed by regular discharges in 40.6% and regular in 21.9% of the heat-sensitive A-fibers).

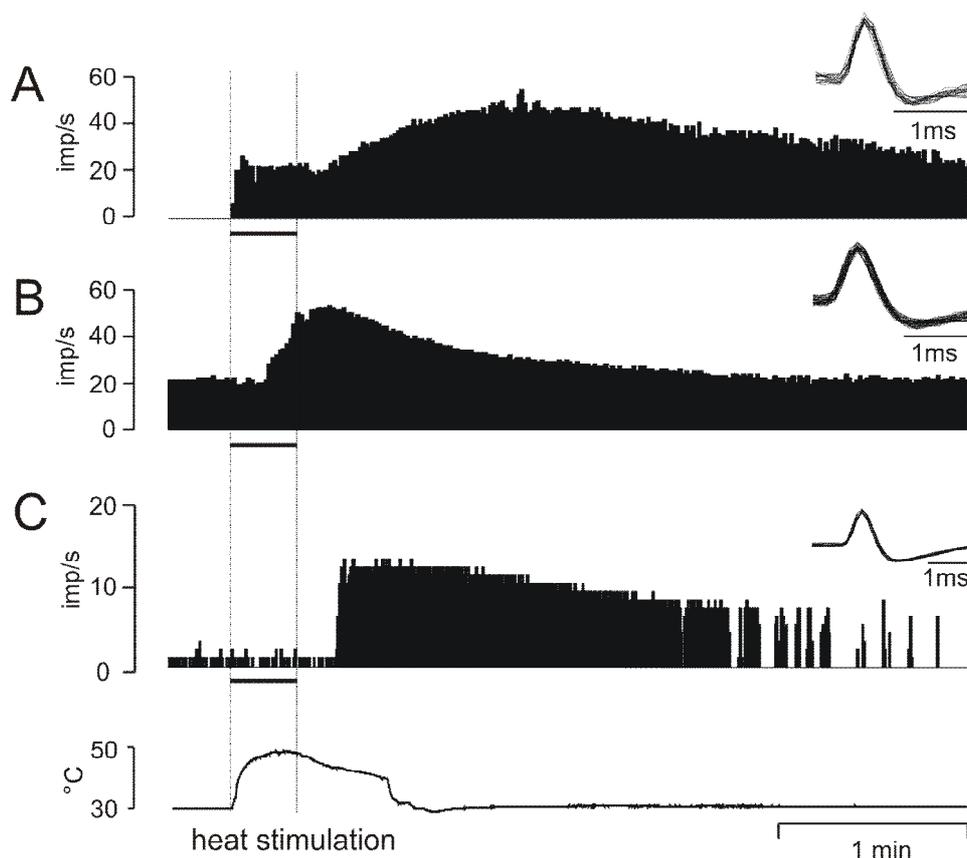


Fig. 3.12. Different latencies of responses of three A-fibers to heating stimuli applied to the nerve injury site. The dotted second vertical line indicates the end of the stimulus. The activation outlasted the stimulus in these fibers. The fiber in A was immediately activated and increased its activity after the stimulus for about 1 min. The fiber in B started to be activated in the middle of the heat stimulus. The fiber in C started to be activated more than 10s after the stimulus when the temperature was still about 43°C.

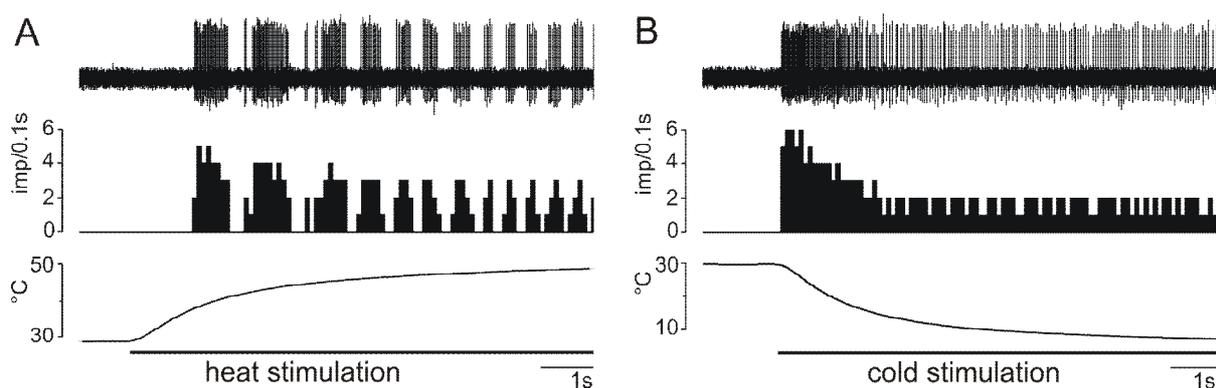


Fig. 3.13. Typical mechano-cold-heat-sensitive A-fiber with a bursting pattern during a heat stimulus (A) and a tonic discharge pattern during a cold stimulus (B).

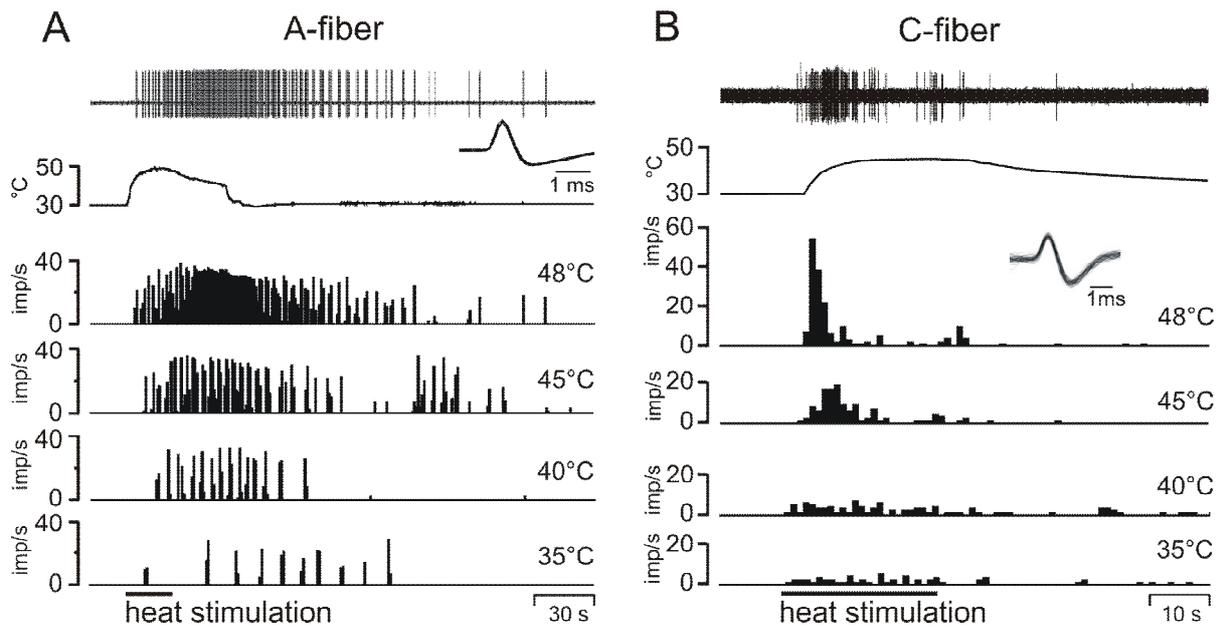


Fig. 3.14. Responses of an A- (A) and a C-fiber (B) to graded heat stimuli. Note that the response of the A-fiber was long-lasting and bursting. Insets, action potentials several times superimposed. Data from TP II.

Two types of heat sensitivity of A-fibers were observed (as it was in case with the cold sensitivity). Heat-sensitive A-fibers with spontaneous activity showed significantly stronger and longer responses to heat stimulation than silent heat-sensitive A-fibers at stimuli of 40 and 45 °C (Fig. 3.15; 40°C $p < 0.0002$, 45°C $p < 0.01$, Wilcoxon-Mann-Whitney U-test). The heat threshold was significantly lower in spontaneously active A-fibers than in silent A-fibers (inset in Fig. 3.15A; $p < 0.01$, Wilcoxon-Mann-Whitney U-test).

C-FIBERS.

Heat sensitivity was present in 53.2% of the injured C-fibers in TP I and in 66.1% in TP II (Table 3.6). In TP II its frequency was significantly higher than in A-fibers (78/118 fibers versus 101/194 fibers, $p < 0.02$, χ^2 -test). As in the heat-sensitive A-fibers the thresholds ranged from 35 to 50°C (median 40°C; inset in Fig. 3.16A2 and B2). The responses to heat stimuli were bursting (63%) or tonic (37%). They did not outlast the stimulus and were not delayed with respect to the stimulus (Fig. 3.14B). The stimulus-response functions varied between individual C-fibers (Fig. 3.16A2 and B2). The discharge rate during heat stimuli of 20-30s duration was significantly lower in C-fibers than in A-fibers (Fig. 3.15, 3.16; $p < 0.002$ for 48°C, Wilcoxon-Mann-Whitney U-test).

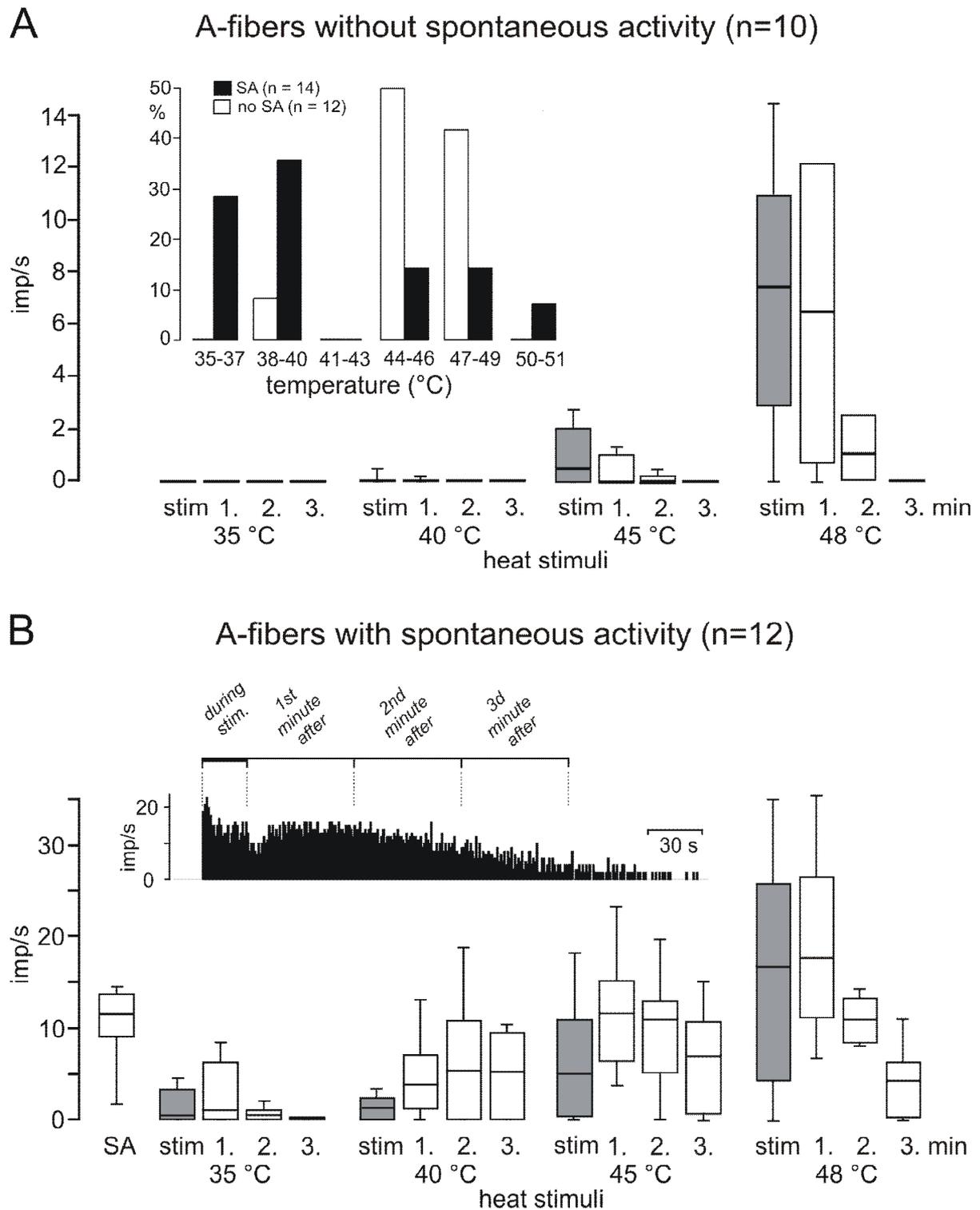


Fig. 3.15. Stimulus-response functions of A-fibers to heat stimuli. The fibers were separated into silent fibers (no spontaneous activity [SA]; A) and fibers with SA (B). The responses were separated into those during stimulation (grey columns) and those in the 1, 2, and 3 minute after stimulation (open columns; see in set in B). The SA was subtracted from the responses to heat stimuli. Medians and interquartile range. Inset in A: Distribution of activation thresholds to heat stimuli. Data from TP II.

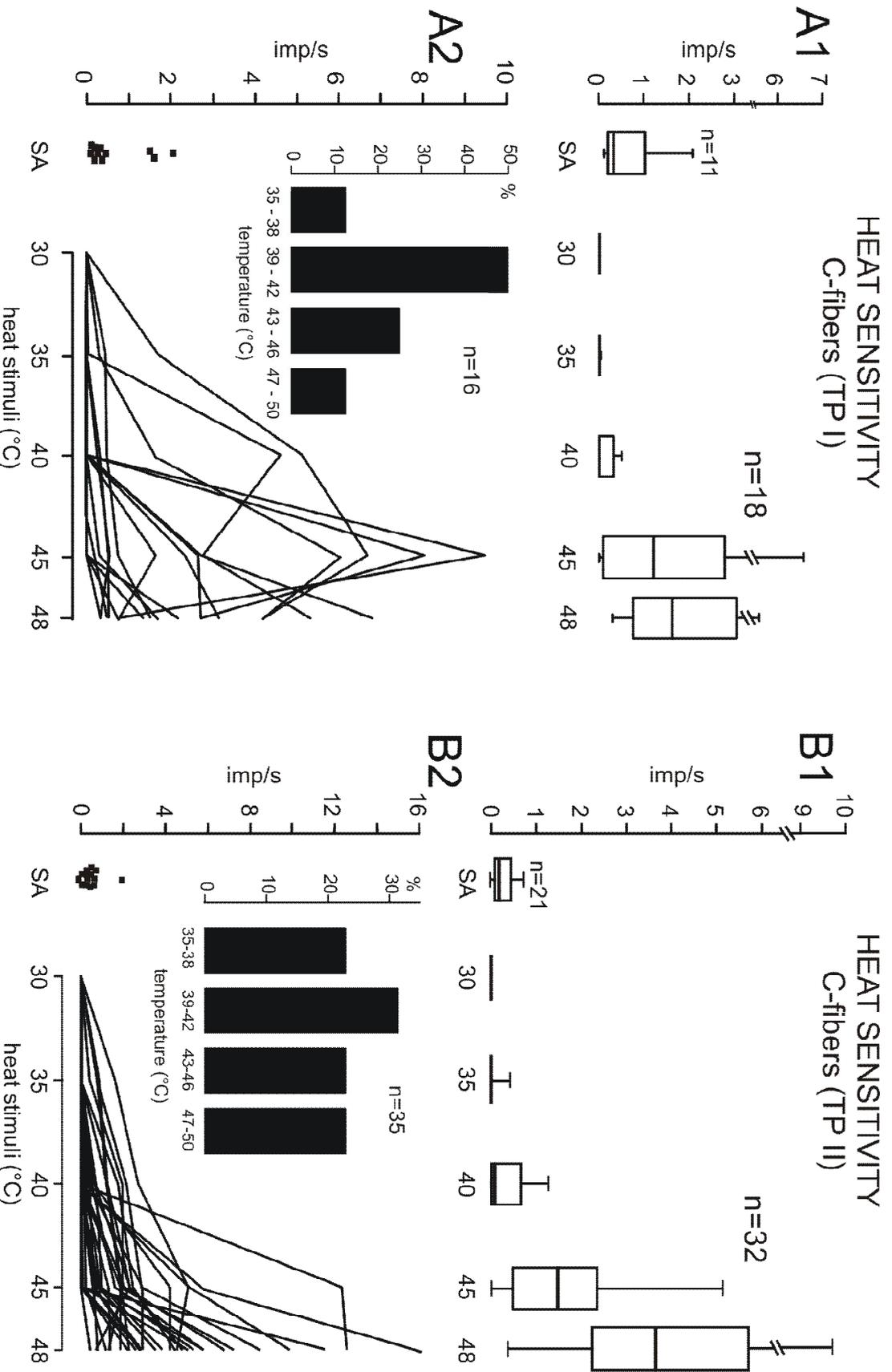


Fig. 3.15. Stimulus-response functions of C-fibers to heat stimuli (TP I and TP II). The spontaneous activity (SA) in 11 fibers (TP I) and 21 fibers (TP II) was subtracted from the responses to heat stimuli. Medians and interquartile range. Inset: Distribution of activation thresholds to heat stimuli.

3.1.3 Spontaneous activity

a. Rate and pattern of spontaneous activity

A-FIBERS.

In TP I spontaneous activity in A-fibers was almost absent. In TP II 50% of the A-fibers with ectopic activity exhibited spontaneous activity. The rate of spontaneous activity in these fibers measured before the fibers were activated by mechanical or thermal stimuli ranged from 0.01 to 38.8 imp/s (mean \pm SEM, 10.9 ± 0.9 imp/s, median 9.5 imp/s, N=96; Fig. 3.17).

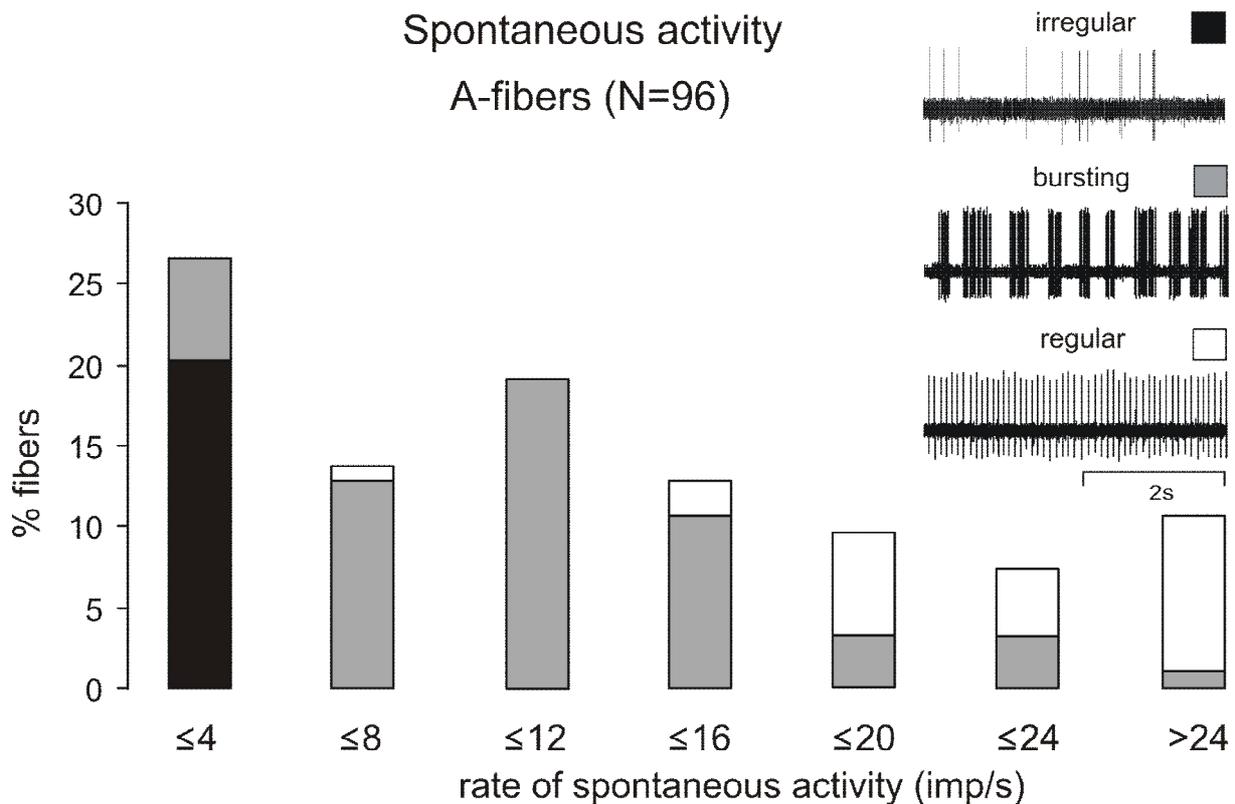


Fig. 3.17. Distribution of rate of spontaneous activity (SA) in A-fibers. SA in A-fibers from TP II. Insets: Examples of irregular, bursting and regular SA.

The pattern of spontaneous activity was irregular (20.2%), regular (23.4%) or bursting (56.4%) showing in the interval histograms of the activity either one peak or two to three peaks (Fig. 3.18A, B and C). Some 9% of the A fibers discharged in doublets and/or triplets (Fig. 3.18, C); these fibers usually had a regular pattern of spontaneous activity. The mean discharge rate was significantly higher in afferent fibers with regular activity than in afferent fibers with bursting or irregular activity (22.8 ± 1.6 imp/s [regular, N= 22] versus 10.4 ± 0.8

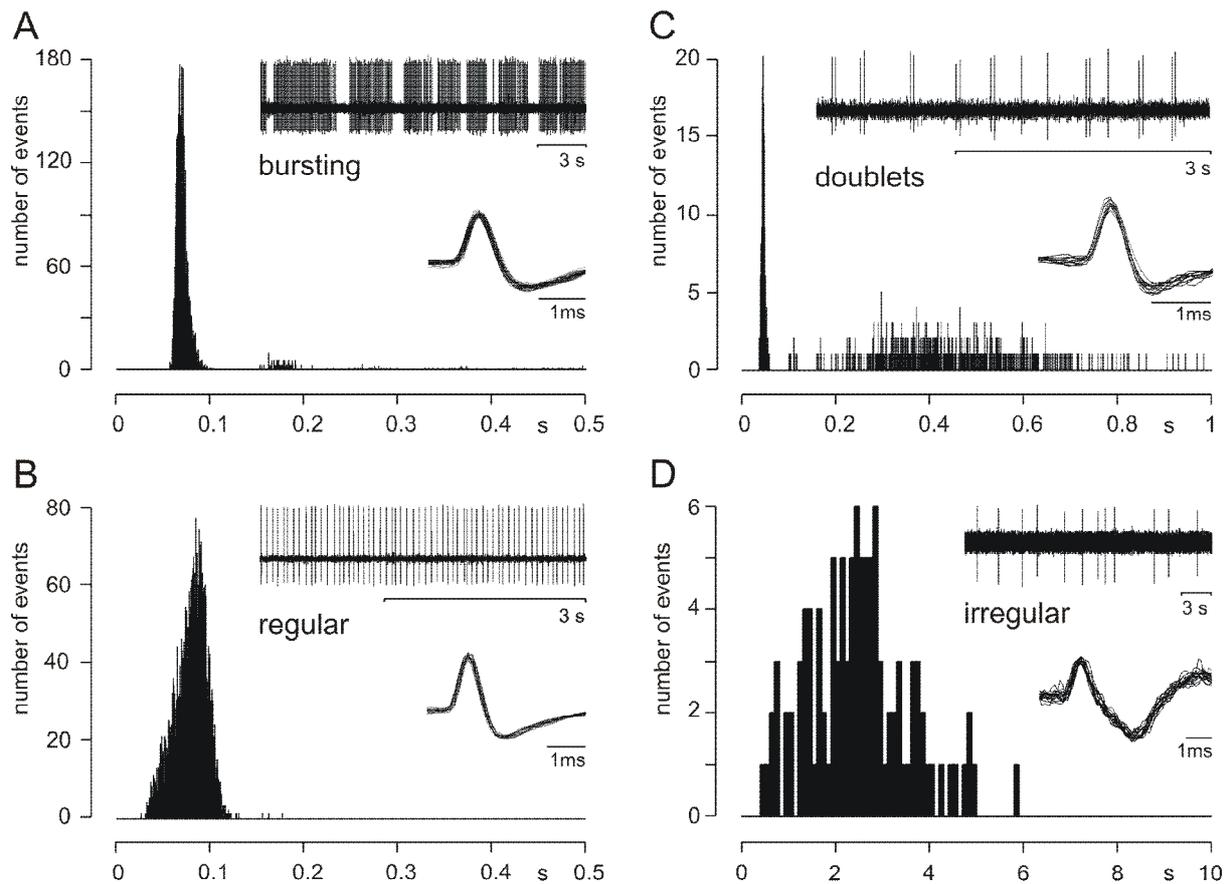


Fig. 3.18. Interspike interval histograms of the bursting (A), regular (B), doublet (C) and irregular (D) pattern of spontaneous activity (SA). A, B, C – myelinated fibers, D – unmyelinated fiber. Insets: Original records of SA and action potentials several times superimposed.

imp/s [bursting, N=53], $p < 0.0002$; 10.4 ± 0.8 imp/s [bursting, N=53] versus 0.95 ± 0.23 [irregular, N=19], $p < 0.0001$, Wilcoxon-Mann-Whitney U-test).

The rate of spontaneous activity was not higher in A-fibers conducting at ≥ 20 m/s (11.7 ± 1.4 imp/s, N=53) than in A-fibers conducting at < 20 m/s (10.9 ± 1.5 imp/s, N=26), most of the latter being possibly A δ - (group III-) fibers. The rates of spontaneous activity in fibers isolated from the sciatic nerve and fibers isolated from the dorsal root were not significantly different (11.7 ± 1.0 imp/s [N=79] vs 9.8 ± 2.4 imp/s [N=17]).

C-FIBERS.

Fifty-three per cent of the C-fibers with ectopic activity had spontaneous activity in TP I and 56% in TP II. The rate of spontaneous activity ranged from 0.02 - 6.95 imp/s (median 0.32 imp/s, N=85; Fig. 3.19). In TP I the rate of spontaneous activity was significantly higher than in TP II (0.13 - 7.0, median 0.5 imp/s [N=25] vs. 0.01 - 6.4, median 0.22 imp/s [N=60], $p < 0.02$, Wilcoxon-Mann-Whitney U-test). The rate of spontaneous activity was significantly

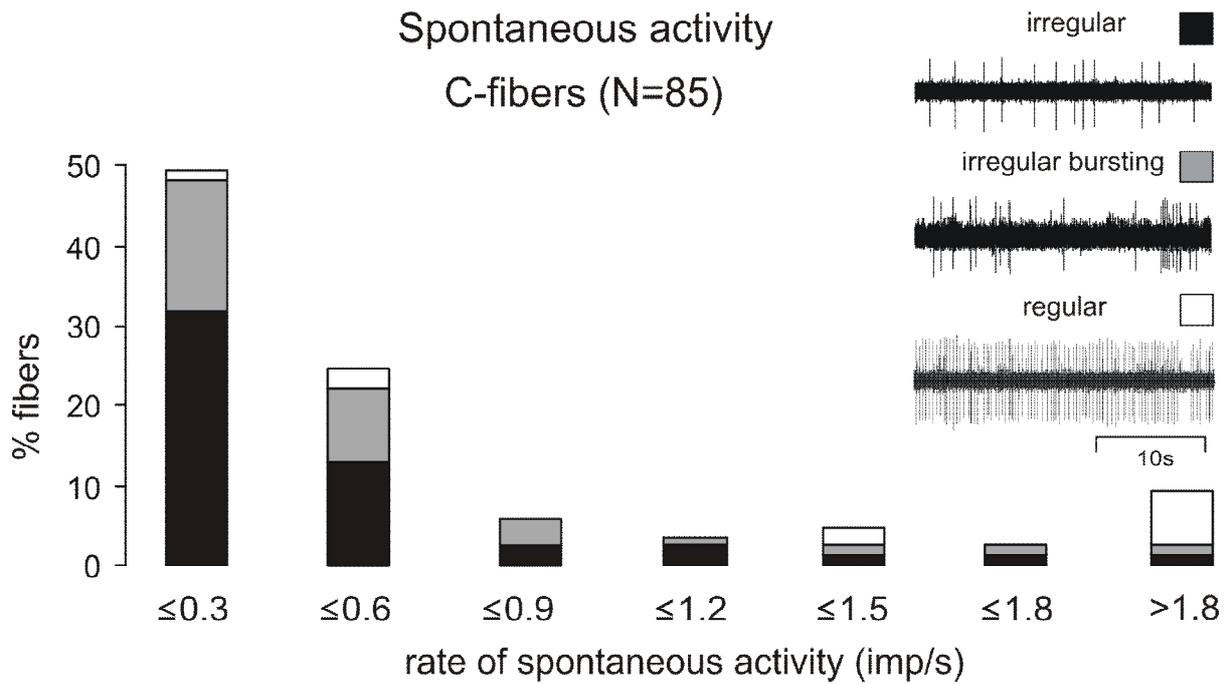


Fig. 3.19. *Distribution of rate of spontaneous activity (SA) in C-fibers.* SA in C-fibers from TP I (N=25) and TP II (N=60). The rate of SA in C-fibers was significantly higher in TP I than in TP II (see text). Insets: Examples of irregular, irregular bursting and regular OA.

lower in C-fibers than in injured A-fibers whether conducting at ≥ 20 m/s or < 20 m/s ($p < 0.0001$, Wilcoxon-Mann-Whitney U-test).

The pattern of spontaneous activity in C-fibers was mostly irregular (Fig. 3.18D). An irregular pattern was observed in 53% of the C-fibers (0.01 – 1.95, median 0.2 imp/s, N=45), irregular bursting in 34.1% (0.06 – 2.1, median 0.33 imp/s, N=29) and regular in 12.9% (0.18 – 6.95, median 2.4 imp/s, N=11) of the whole population of the spontaneously active C-fibers (Fig. 3.19). The rate of spontaneous activity was significantly higher in regularly discharging C-fibers than in C-fibers discharging irregularly or irregularly in bursts ($p < 0.000$). The rates of spontaneous activity in fibers isolated from the sciatic nerve and fibers isolated from the dorsal root in TP II were not significantly different (0.02 - 6.4, median 0.24 imp/s [N=48] vs. 0.01 – 0.94, median 0.21 imp/s [N=12]).

b. Spontaneous activity and responsiveness to physiological stimulation

A-FIBERS.

As described above myelinated fibers with spontaneous activity showed significantly stronger and longer responses to cold and heat stimulation than silent A-fibers (for details see chapter 3.1.2b and c; Figs. 3.9, 3.15). The thresholds to cold and heat stimuli were significantly lower in spontaneously active A-fibers than in silent A-fibers (Fig. 3.7 and inset

in Fig. 3.15; $p < 0.001$, Wilcoxon-Mann-Whitney U-test). However the rate of spontaneous activity was not correlated with their responsiveness to one of the physiological stimuli. Also the incidences and the rate of spontaneous activity were not significantly different between the two populations of low (LTM) and high threshold mechanosensitive (HTM) A-fibers (Fig. 3.3; 42% of the LTM A-fibers [0.3 - 0.7mN] were spontaneously active [mean rate of SA: 14.2 ± 0.3 imp/s], 35% of the HTM A-fibers [≥ 1 mN] were spontaneously active [mean rate of SA: 10.0 ± 0.2 imp/s]).

C-FIBERS.

Separate analysis of the incidence of the spontaneous activity among the heat-sensitive (heat-sensitive only and heat-mechanosensitive) and cold-sensitive (cold-sensitive only and cold-mechanosensitive) unmyelinated muscle fibers showed, that in both time periods (TP I and TP II) the spontaneous activity of the C-fibers was correlated with their heat sensitivity (in TP I 13 out of 21 heat-sensitive C-fibers were spontaneously active [13/21; 61.9%], in TP II – 29/51 [56.9%]). Cold sensitivity was more often observed among C-fibers without spontaneous activity (in TP I – 5 out of 7 cold-sensitive fibers had no spontaneous activity [5/7; 71.4%], in TP II – 14/20 [70%]). The rate of spontaneous activity in C-fibers was not correlated with their responsiveness to thermal stimuli.

Type of C-fiber	TP I	TP II
Heat-sensitive only/SA	9/5	32/20
Heat-mechanosensitive/SA	12/8	19/9
Cold-sensitive only/SA	2/0	8/2
Cold-mechanosensitive/SA	5/2	12/4

Table 3.7. Numbers of heat-sensitive and cold-sensitive injured afferent C-fibers 4-13 hours (TP I) and 4 to 7 days (TP II) after crush injury of the lateral gastrocnemius-soleus nerve. The numbers behind the slash indicate the numbers of fibers with spontaneous activity (SA) (included in the number before the slash).

As shown for the myelinated fibers, the incidences and the rate of spontaneous activity were not significantly different between low (LTM) and high threshold mechanosensitive (HTM) C-fibers (Fig.3.3; 25% of the LTM C-fibers were spontaneously active [mean rate: 0.41 ± 0.2 imp/s], 50% of the HTM C-fibers were spontaneously active [mean rate: 0.47 ± 0.2 imp/s]).

c. Change of spontaneous activity during experimental interventions. Introduction into the phenomenon of afterdischarge.

In general the rate and pattern of the spontaneous activity in all unmyelinated fibers and in most myelinated fibers before and after experimental testing were stable and were not significantly different (Table 3.8).

	<i>A-fibers</i>	<i>C-fibers</i>
<i>1. measurement</i>	15.9 2.4-39.2	0.66 0.12-4.0
<i>2. measurement</i>	13.4 2.2-35.5	0.77 0.1-3.9
<i>N</i>	42	26
<i>Wilcoxon paired test</i>	<i>p</i> =0.1	<i>p</i> =0.56

Table 3.8. Change of the rate of spontaneous activity before and after testing procedure (imp/s; median, range). The rate of spontaneous activity was measured two times at intervals of 30 to 40 min in most afferent fibers. The rates were not significantly different between both measurements (Wilcoxon matched-pairs-signed-rank test).

A special group of A-fibers (about 20%) had unstable rates of spontaneous activity during recording. These fibers had the following characteristics:

- During electrical identification in the unmyelinated fiber range (1-40V, 0.3-1Hz, 0.5ms) these myelinated afferent fibers (for which this strength of the stimulation was suprathreshold) exhibited afterdischarges (AD) at a very high rate (Fig. 3.20A, for details see chapters 3.4 and 4.4);
- During recording these A-fibers showed spontaneous irregular bursts up to 150 Hz (Fig. 3.20B I and II);

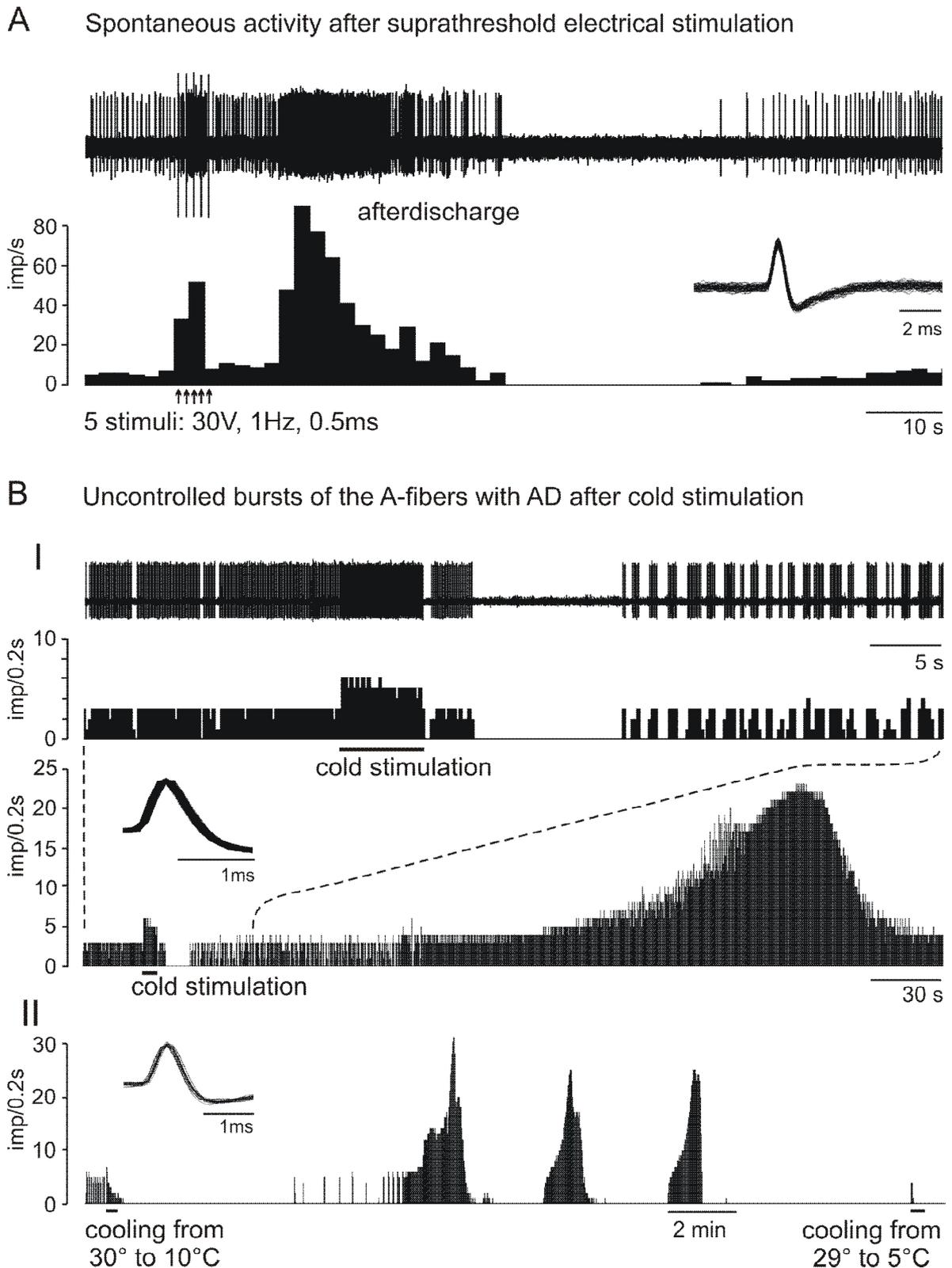


Fig. 3.20. Myelinated fibers showing afterdischarge (AD). A. A-fiber with afterdischarge (AD) following suprathreshold stimulation of the nerve (5 stimuli, 30V, 1Hz, 0.5ms). B. Spontaneous activity, spontaneous irregular bursts and not reproducible responses to thermal stimulation of two myelinated fibers with AD (B I and II). Note the spontaneous waves of activity with continuously increasing rate of discharge.

- The thermosensitivity of these A-fibers was instable in that that the responses to the thermal stimuli were not reproducible and caused irregular bursts (Fig. 3.20B II).

The phenomenon of afterdischarge (AD) was noticed first 13 years ago in Prof. W. Jänig's laboratory by the Dr. Xianguo Liu (unpublished observation). In the present study the phenomenon of AD was reproduced and investigated systematically. It is described in the chapter 3.4. Overall, AD was observed in about 20% of all myelinated muscle A-fibers and only in a few unmyelinated fibers. Interestingly, cutaneous myelinated afferent fibers never exhibited afterdischarges to electrical stimulation of the skin nerve at high strength.

3.1.4 Conduction velocity and ectopic response properties

a. Recording from sciatic nerve

The distribution of the conduction velocities (CV) of the fibers recorded from the sciatic nerve showed no criteria to discriminate between groups I, II or III fibers in both tested time periods. Therefore we used throughout the text the terms A-fibers (or myelinated fibers) and C-fibers (or unmyelinated fibers) (see Figs. 3.1, 3.21, 3.22, 3.23). A significant shift to lower values of the CV were observed in chronically lesioned neurons (4-7 days after gastrocnemius-soleus nerve lesion, time period II (TP II)) in both populations of A- ($p < 0.05$, Wilcoxon signed rank test) and C-fibers ($p < 0.0005$, t-test) in comparison to the acutely lesioned fibers (4-13 hours after nerve injury, time period I (TP I)) (Fig. 3.21; C-fibers: 0.98 ± 0.05 [range 0.5 - 1.6] m/s in TP I vs. 0.77 ± 0.03 [range 0.3 - 1.4] m/s in TP II; A-fibers: 24.84 ± 0.78 m/s in TP I vs. 22.56 ± 0.46 m/s in TP II).

Figures 3.22 and 3.23 show the distribution of CV in TP I and TP II of all electrically identified fibers (upper graph) and of the separate groups of fibers showing different types of ectopic activity - spontaneous activity (SA) and/or responses to mechanical and/or thermal stimuli applied to the nerve injury site (open parts in the upper graph and below graphs). Not surprising, these figures show that the CV distributions are similar for mechano-, cold, heat sensitivity and SA in view of combination of these properties discussed above (for review see chapter 3.1.2; Table 3.2, 3.3).

Figure 3.22 obviously indicates that in TP I most electrically identified A-fibers were silent and only few of them, mainly conducting in A δ -fibers range (2 - 16 m/s) developed ectopic activity. These A-fibers were either spontaneous active and/or mechanosensitive. At the same time period many electrically identified C-fibers exhibited different types of ectopic

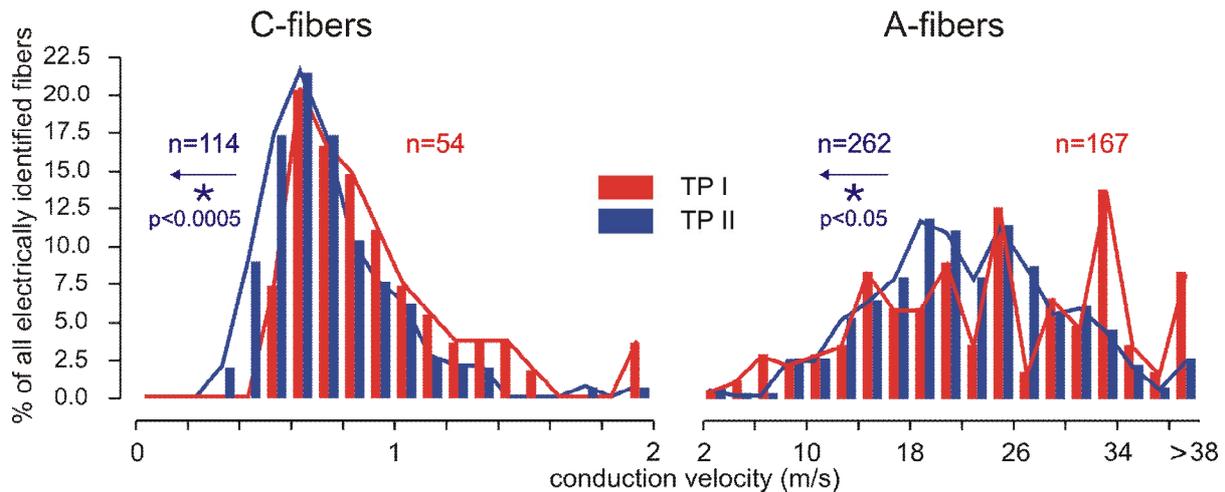


Fig. 3.21. Conduction velocities (CV) of the electrically identified A- and C-fibers recorded from sciatic nerve in the lateral gastrocnemius-soleus nerve in time period I (TP I) (4-13 hours after nerve crush; in red) and in time period II (TP II) (4-7 days after nerve crush; in blue). The CV of chronically lesioned (TP II) muscle afferent A- ($p<0.05$, Wilcoxon signed rank test) and C-fibers ($p<0.0005$, t-test) were significantly lower in comparison with the CV of acutely lesioned (TP I) ones.

activity, mainly mechano- and /or heat sensitivity. Cold sensitivity was almost absent in all acutely injured muscle fibers.

Figure 3.23 presents data from all experiments done on the chronically lesioned rats (TP II) recorded from sciatic nerve (40 rats, group A2 and B). In the group B rats (testing the responsiveness of lesioned muscle afferents to transient receptor potential [TRP] receptor agonists) we were recording only from fibers with ectopic activity, discarding those fibers which were silent. Thus the percentages of the fibers having either spontaneous activity and/or mechanosensitivity and/or thermosensitivity are higher in this figure in comparison to percentages in Table 3.1. Figure 3.23 demonstrates significantly increased proportions of ectopically active muscle injured A- and C-fibers in TP II and the development of novel cold and heat sensitivity in the myelinated fibers and novel cold sensitivity in the C-fibers.

b. Recording from dorsal root

Seventy-nine per cent of the A-fibers and 83% of the C-fibers with ectopic activity were recorded from filaments isolated from the sciatic nerve and the remaining fibers were isolated from the dorsal root L5 (see upper part of Tables 3.2 and 3.3, Fig. 3.24). In the measurements of the CV of the fibers which were isolated from the dorsal roots L4/L5 three to four groups of fibers were observed (Fig. 3.24) – (1) a group of slowly conducting C-fibers ($CV \leq 1.1$ m/s), (2) a group of fast conducting C-fibers together with a group of slowly

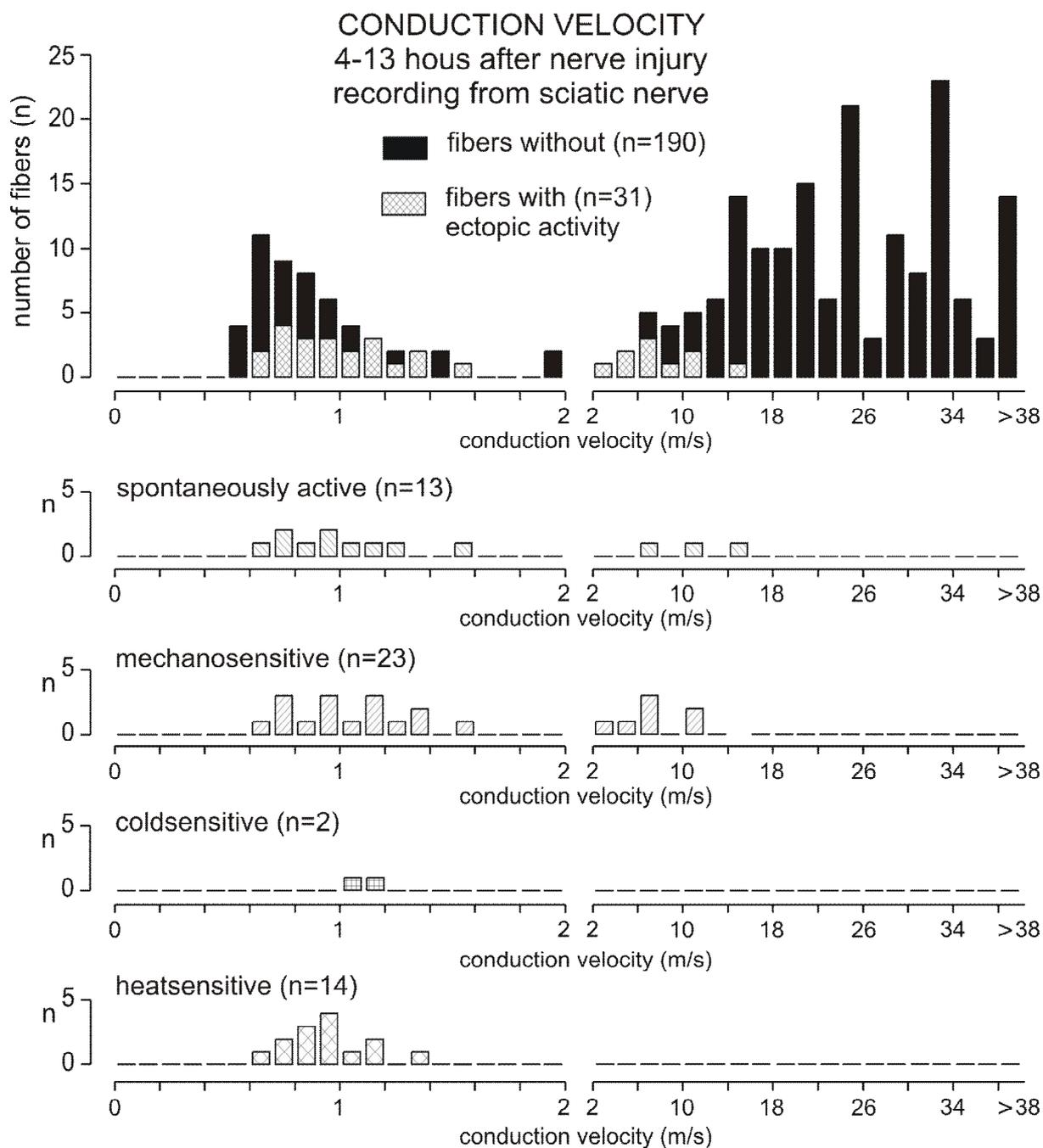


Fig. 3.22. Conduction velocity (CV) of electrically identified A- and C-fibers recorded from the sciatic nerve and projecting in the lateral gastrocnemius-soleus nerve in time period I (TP I) (4-13 hours after nerve crush). Upper graph is a summary of all electrically identified fibers. Open parts of the columns: Fibers showing spontaneous activity and/or responses to mechanical and/or thermal stimuli applied to the nerve injury site. Black parts of the columns: fibers without ectopic activity. Lower 4 graphs show distributions of the CV of the afferent fibers separated for the spontaneous activity, mechano-, cold or heat sensitivity.

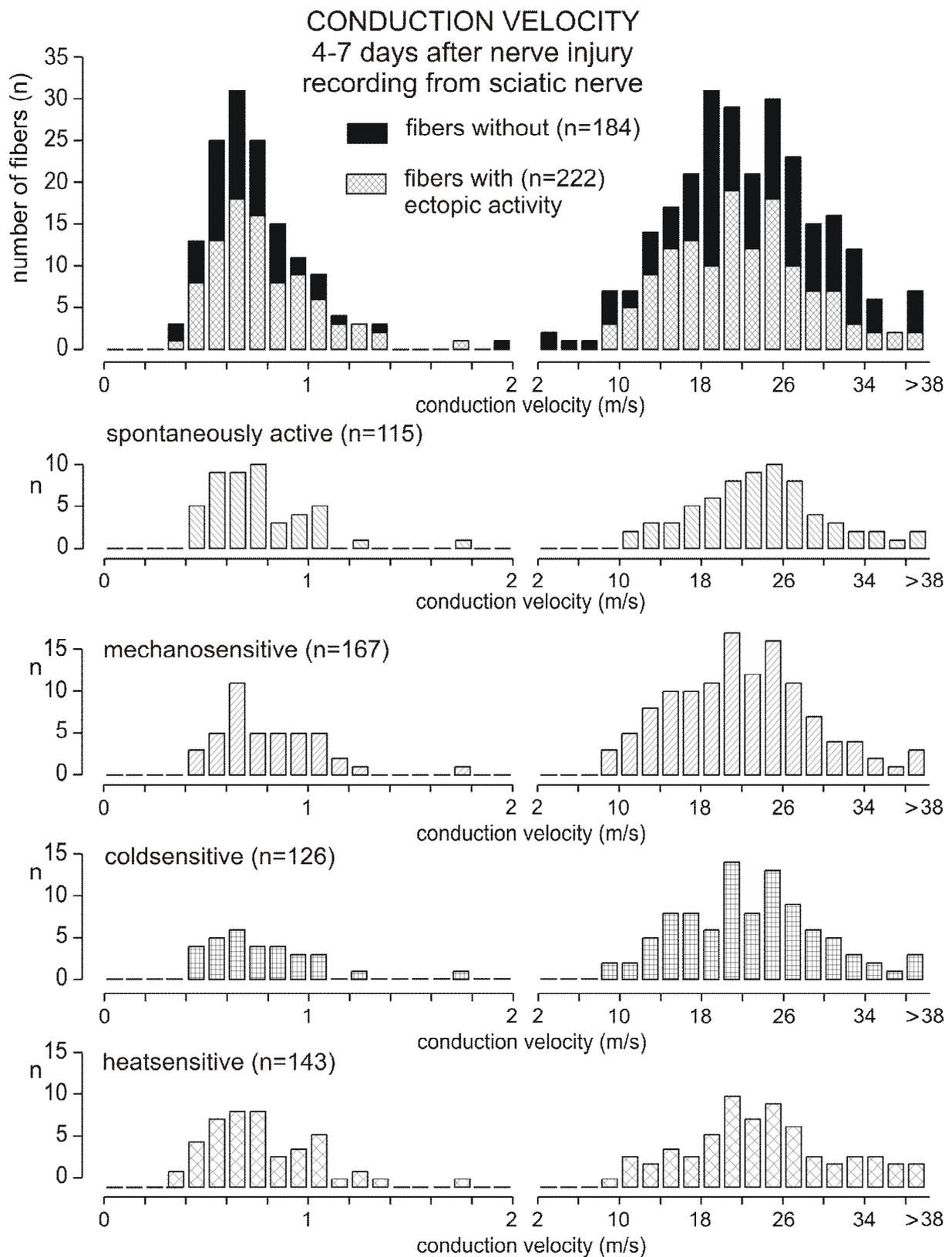


Fig. 3.23. Conduction velocity of electrically identified A- and C-fibers recorded from the sciatic nerve and projecting in the lateral gastrocnemius-soleus nerve in time period II (TP II) (4-7 days after nerve crush). Data from 40 experiments (group A2, and group B). For details see the legend of the figure 3.21.

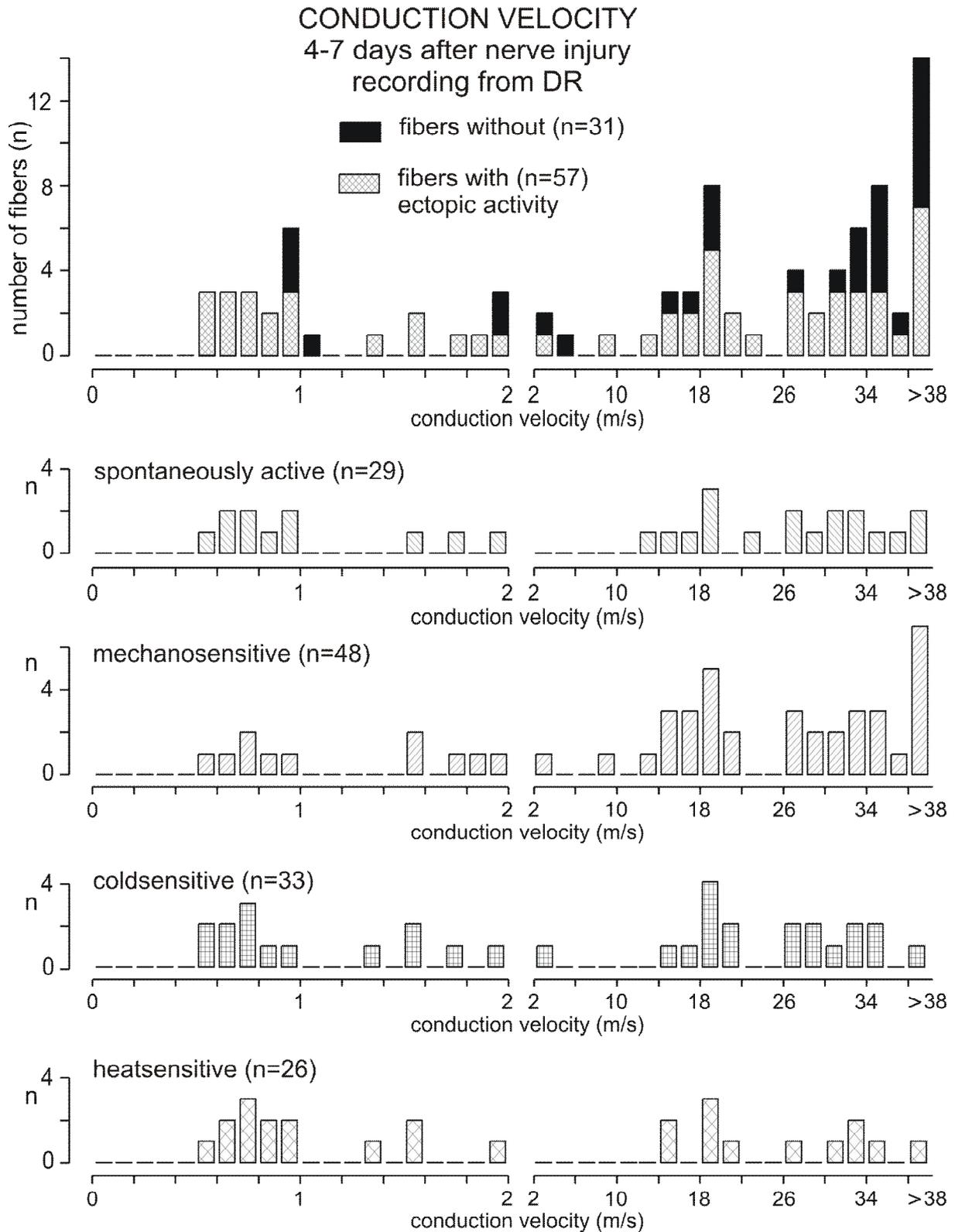


Fig. 3.24. Conduction velocity of electrically identified of A- and C-fibers projecting in the lateral gastrocnemius-soleus nerve and recorded from the dorsal root (DR) L4/L5 in time period II (TP II) (4-7 days after nerve crush). For details see the legend of the figure 3.21.

conducting A-fibers ($1.3 < CV < 6$ m/s) and (3-4) two groups of fast conducting A-fibers ($12 < CV < 24$ and $CV \geq 24$ m/s).

Thus in comparison to the measurements of the CVs done in the recordings from sciatic nerve fibers (where no division in sub-groups of the A-fibers could be seen [Figs. 3.21; 3.22, 3.23]), in the recordings from the dorsal roots L4/L5 the middle group of fast conducting C-fibers and slowly conducting A-fibers appeared. Mense and Meyer (1985) recorded from intact muscle afferents from dorsal roots in cats and compared the range of the CVs proximal and distal to the spinal ganglion. They showed that afferent unmyelinated fibers with low CVs could be found proximal to the spinal ganglion and myelinated fibers with middle CVs distal to the spinal ganglion. Therefore the fast conducting C- and slowly conducting A-fibers presumably must be myelinated distal to the spinal ganglion and unmyelinated proximal to the dorsal root ganglion. As in the dorsal root recordings the electric stimulation always occurred distal of the spinal ganglions, the CVs of the fast conducting and slowly conducting axons shifted to a middle CV range. The functional characteristics and the form of the action potential of the slowly conducting A-fibers did not differ from those of the fast conducting A-fibers. The functional characteristics and the form of the action potentials of the fast conducting C-fibers also did not differ from those of the slowly conducting C-fibers. Thus, taking in account the low numbers of the fibers of the middle range CV group, they were assigned either to the C- or to the A-fiber group. The division between A- and C-fibers was assumed to be 2 m/s like it was done in the recordings from the sciatic nerve fibers (Lawson and Waddell 1991, Lawson 2005).

3.1.5 Response patterns to physiological stimulation

The lower parts of Tables 3.2 and 3.3 show the discharge patterns in injured muscle afferent fibers in TP I and II. These patterns of activation are illustrated graphically in Fig. 3.25 for TP I and TP II and Fig. 3.26 for TP II separately. The following differences between TP I and TP II and between A- and C-fibers in TP II need to be emphasized:

a. Comparison of TP I and TP II:

- In the TP I most A-fibers (216/230) are silent, only few (6%) mainly A δ -fibers exhibit either spontaneous activity (5/230) and/or mechanosensitivity (9/230). A few A-fibers in TP I show a weak mechano- and/or thermosensitivity that did not reach the criteria.

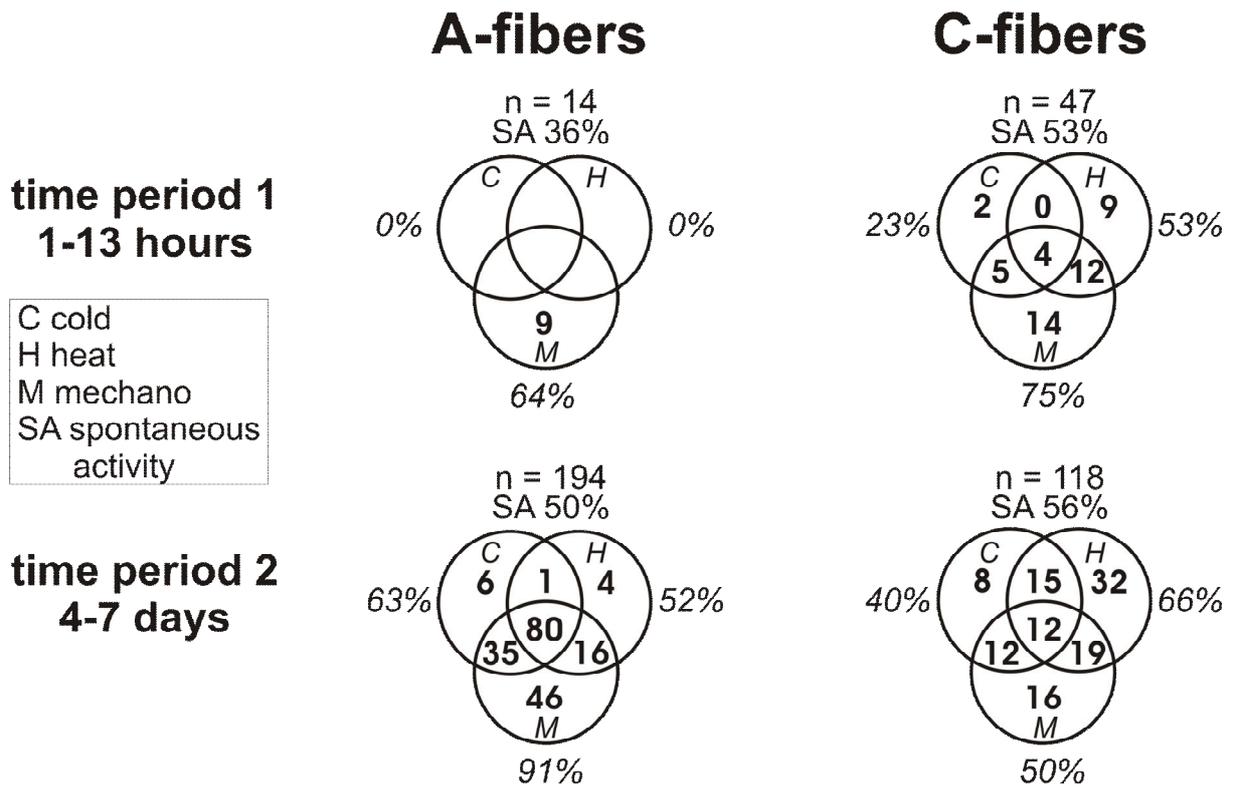


Fig. 3.25. Patterns of cold, heat and mechanosensitivity in A- and C-fibers projecting in the injured muscle nerve in TP I and TP II. The total numbers do not include afferents that have only ongoing activity (6 A-fibers and 4 C-fibers). The total numbers are therefore lower than in Tables 3.2 and 3.3. Percentages in italic show the proportions of cold-, heat- or mechanosensitive afferents.

- In TP I 41.6% (47/113) of the electrically identified C-fibers show ectopic activity. The largest groups are mechano- and/or heat-sensitive fibers.
- Cold sensitivity is almost absent in all acutely injured muscle fibers in TP I.
- In TP II 57% (194/339) of the electrically identified A-fibers and 64.1% (118/184) of the electrically identified C-fibers are ectopically active. Thus, the proportion of ectopically active afferent A- and C-fibers significantly increase in TP II compared to TP I.
- Practically all injured muscle afferents show ectopic activity in TP II, assuming that close to 50% of the myelinated and unmyelinated fibers in the gastrocnemius soleus nerve are somatomotor and postganglionic axons, respectively.
- In TP II many A-fibers develop novel cold and heat sensitivity. The percentages of the heat-sensitive and/or cold-sensitive C-fibers significantly increase compared to TP II.

b. Comparison of A-fibers and C-fibers in TP II:

- Cold sensitivity and mechanosensitivity have a significantly higher representation in A-fibers than in C-fibers ($p < 0.000$, χ^2 -test).
- Heat sensitivity has a significantly higher representation in C-fibers than in A-fibers ($p < 0.02$, χ^2 -test)
- Cold sensitivity or heat sensitivity is associated with mechanosensitivity in >90% of the cold- or heat-sensitive A-fibers, but in only 40 - 50% of the cold- or heat-sensitive C-fibers. This corresponds to the observation that 48% of the C-fibers are only thermo- (cold- and/or heat-) sensitive but not mechanosensitive compared to 5.9% of the A-fibers ($p < 0.000$, χ^2 -test).
- The patterns of discharge produced by mechanical or thermal stimuli are not significantly different in A-fibers conducting at <20 m/s (most of them probably being A δ - [or group III] afferents) and in A-fibers conducting at ≥ 20 m/s (all of them being A α/β - [or group II and I] afferents). However, the patterns of both are significantly different from those in C-fibers as shown for the total population of A-fibers in Fig. 3.26 and Table 3.9.

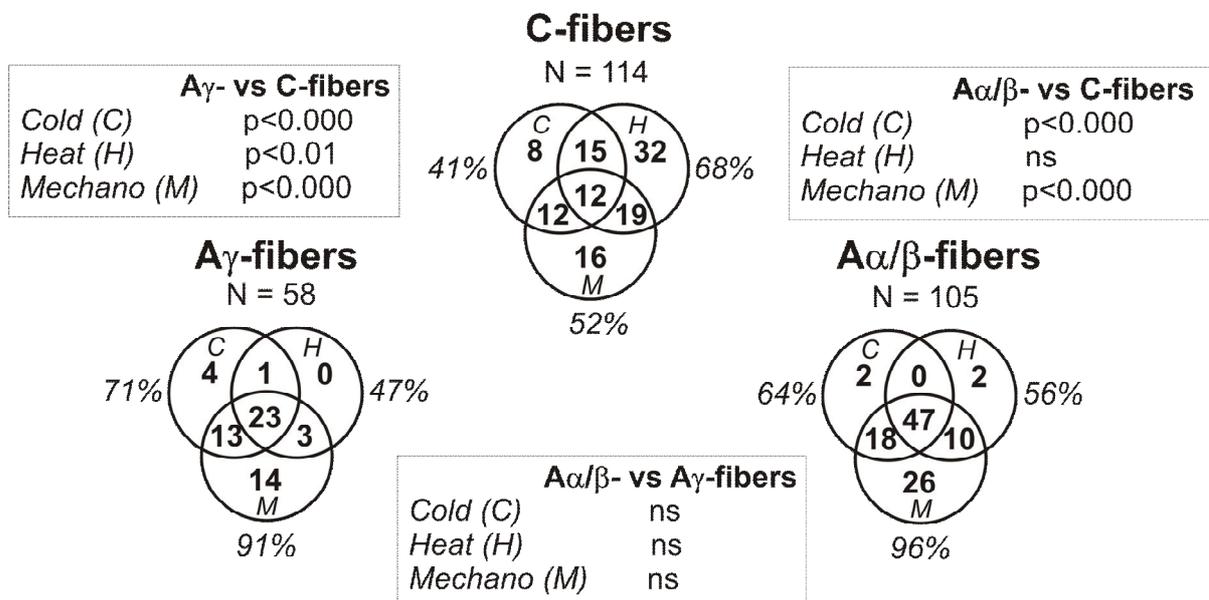


Fig. 3.26. Patterns of cold, heat and mechanosensitivity in A α/β , A δ - and C-fibers projecting in the injured muscle nerve in TP II. Percentages in italic show the proportions of cold-, heat- or mechanosensitive afferents. χ^2 -test for comparison of the frequencies of cold, heat or mechanosensitivity between A α/β -, A δ - and C-fibers.

	A δ -fibers (< 20m/s)	A β -fibers (\geq 20m/s)
	60	109
Ongoing activity (OA)	27 (45.0%)	54 (49.5%)
Mechanosensitivity	53 (88.3%)	101 (92.7%)
Cold sensitivity	40 (66.7%)	67 (61.5%)
Heat sensitivity	26 (43.3%)	59 (54.1%)

COMBINATIONS OF DISCHARGE CHARACTERISTICS

Mech only/OA	14/6 (23.3%)	26/6 (23.9%)
Cold only/OA	4/0 (6.7%)	2/1 (1.8%)
Heat only/OA	0 (0%)	2/0 (1.8%)
Mech&cold/OA	13/2 (21.7%)	18/2 (16.5%)
Mech&heat/OA	3/0 (5%)	10/4 (9.2%)
Cold&heat/OA	1/1 (1.7%)	0 (0%)
Mech,heat&cold/OA	23/16 (38.3%)	47/37 (43.1%)
OA only	2 (3.3%)	4 (3.7%)

Table 3.9. Individual discharge characteristics of A δ - and A β -fibers 4-7 days (time period II) after crush injury of the lateral gastrocnemius-soleus nerve.

3.2 Responses of the injured muscle afferents to the TRP channels agonists

In the previous chapter I described the responsiveness of myelinated and unmyelinated muscle afferents to mechanical and thermal stimuli applied to the injury site of the nerve 4-13 hours (TP I) and 4-7 days (TP II) after crush lesion of the *gastrocnemius-soleus* nerve in the rat. The overall result was that almost all injured muscle afferent A- and C-fibers develop spontaneous and/or evoked mechano-, cold and heat-sensitivity originating at the injury site (Kirillova et al 2011a). Not unexpectedly, most injured A-fibers and 50-80% of the injured C-fibers were mechanosensitive when compared to the response properties of intact muscle afferents (Mense 1993; Taguchi et al 2005). But the high frequency of cold and heat sensitivity of injured muscle afferents was remarkable. In comparison with intact muscle afferents the cold- and heat-sensitivity of A-fibers and cold-sensitivity of C-fibers are novel properties, which may contribute to the generation of neuropathic pain (Kirillova et al 2011a).

Which are the molecular transduction mechanisms underlying the thermosensitivity of injured (and intact) muscle afferents? This up to date has not been investigated. Neurophysiological studies have largely focussed on cutaneous nociception, although skin pain is clinically less relevant than muscle and visceral pain. Since many researchers focussed

in their studies on cutaneous pain, the knowledge about ectopic activity in cutaneous afferent fibers and the molecular transduction mechanisms underlying it are becoming more or less clear (Fig. 3.27, Belmonte and Viana 2008).

Heat sensitivity of muscle unmyelinated afferents may be dependent on the TRPV1 (transient receptor potential V1) channel (Caterina 2007). It has been reported that all small- to medium-sized rat dorsal root ganglion (DRG) neurons that respond to temperatures in the noxious range (average threshold 45.3°C) were also capsaicin-sensitive. There exists a second class of heat-sensitive neurons, large in size, with threshold of $\geq 51^{\circ}\text{C}$, which are insensitive to capsaicin, and presumably possess a heat-sensitive mechanism distinct from TRPV1 (Nagy and Rang 1999, Ringkamp et al 2001). Indeed, about 50% of the intact muscle C-fibers is capsaicin-sensitive (Hoheisel et al 2004). But it has to be shown whether injured heat-sensitive muscle A- and C-afferents can be excited and/or desensitized by capsaicin or similar agonists of the TRPV1 receptor.

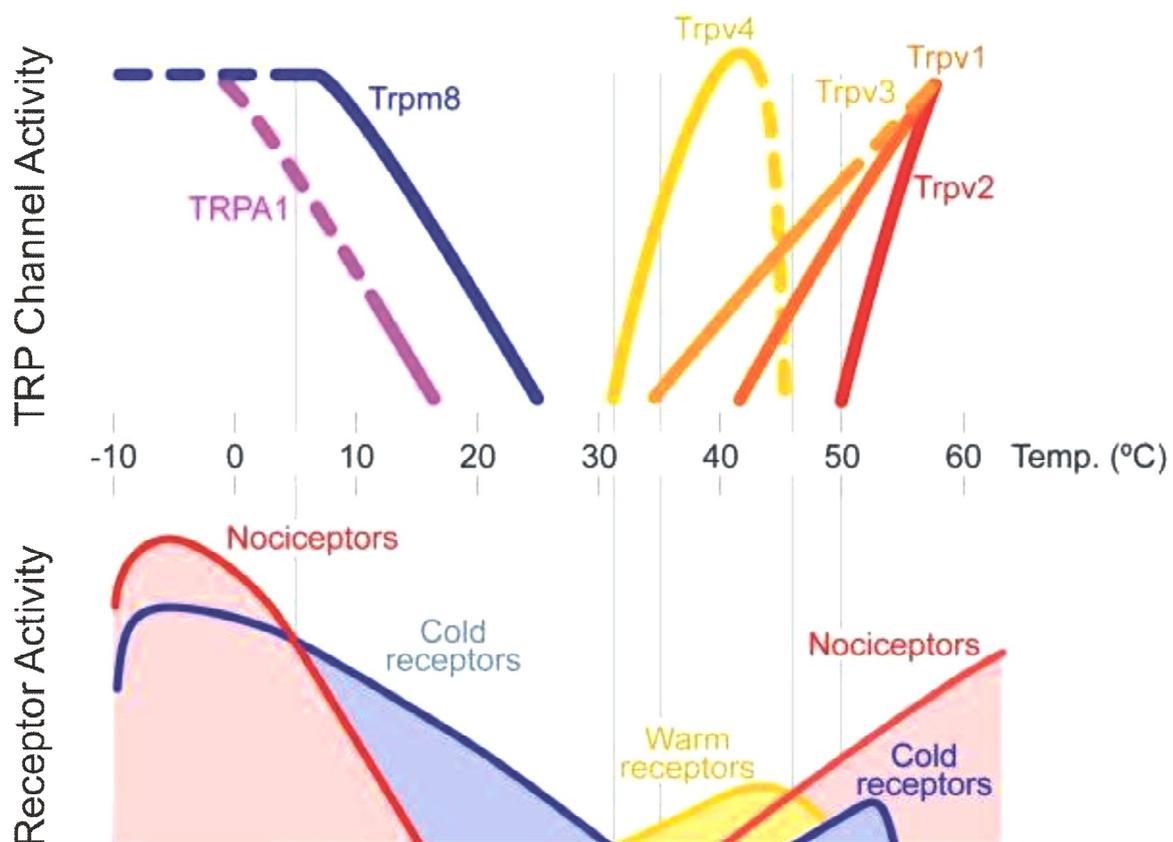


Fig. 3.27. Hypothetical correspondence between activation of TRP channels, body surface temperature and evoked sensations (adapted from Patapoutian et al 2003, Belmonte and Viana, 2008). Upper part: Schematic representation of the thermal activation profile of various TRP channels when expressed in recombinant systems. All of them have been located in sensory neurons and/or skin cells (e.g., keratinocytes). Lower part: Schematic representation of impulse activity in various cutaneous sensory receptors during application of temperature stimuli to their receptive fields.

TRPV1 is a good model of a heat-transduction molecule but it cannot be the only one since TRPV1 knock-down mice retain noxious heat responsiveness to a certain extent (Catarina et al 2000; Davis et al 2000). Other possible channels, which could be involved in heat sensitivity of muscle A and C-fibers are TRPV2, TRPV3, TRPV4 and TRPM3 (Belmonte and Viana 2008, Huang et al 2011, Park et al 2011, Peier et al 2002, Vriens et al 2011).

TRPV2 seems to be the best candidate to mediate heat-sensitivity of the myelinated fibers. TRPV2 is reported to be highly expressed in rat afferent cell bodies that are medium to large in diameter, many of which are nociceptors (Djouhri and Lawson 2004, Fang et al 2005) showing the capacity to develop heat-heat sensitization (Rau et al 2007, Park et al 2011). But it appears to be quite difficult to prove this hypothesis *in vivo* since a TRPV2-specific agonist has not been reported (Park et al 2011). TRPV3 and TRPV4 may make limited and strain-dependent contributions to innocuous warm perception or noxious heat sensation; however specific agonists for them do not exist (Huang et al 2011). TRPM3 is functionally expressed in a large subset of small-diameter sensory neurons in dorsal root ganglia implicating to be possibly involved in the detection of the noxious heat (Vriens et al 2011).

Cold-sensitivity of injured muscle afferents could be mediated by the menthol sensitive TRPM8 channel (Bandell et al 2004; Bautista et al 2007, Belmonte and Viana 2008, Belmonte et al 2009; Madrid et al 2006; McKemy et al 2002; Peier et al 2002) or by other channels, one being possibly the TRPA1 channel (Bandell et al 2004; Jordt et al 2004; Karashima et al 2009; Story et al 2003). But it will have to be shown whether or not cold-sensitive muscle A- and C-afferents can be excited by the agonist menthol, as it is the case in type I cold-sensitive cutaneous afferents (Teliban, 2012) and whether probably other channels (e.g., the TRPA1 channel) are involved in the novel cold sensitivity of injured muscle A- and C-afferents.

Studies in cell cultures show, that the sensing of innocuous cold and menthol by a functionally distinct population of small-diameter sensory neurons is correlated with the expression of TRPM8 (McKemy et al 2002; Peier et al 2002; Patapoutian et al, 2003; Thut et al, 2003). Activation of TRPA1 produces acute pain and neurogenic inflammation through peripheral release of neuropeptides (substance P and CGRP), purines, and other transmitters from activated nerve endings. This, in turn, produces robust hypersensitivity to thermal and mechanical stimuli. The expression of TRPA1 is referred to the sensitivity for mustard oil (MO) (Bandell et al 2004, Jordt et al 2004).

Thus, in the group B animals (25 rats) with injured muscle A- and C-fibers the following hypotheses were tested:

- whether the TRPV1 agonist capsaicin activates injured muscle heat-sensitive afferent nerve fibers but not cold- and/or mechanosensitive one's;
- whether the TRPM8 agonist menthol or the TRPA1 agonist mustard oil activates injured muscle cold-sensitive afferent nerve fibers but not heat- and/or mechanosensitive one's.

For this purpose the *gastrocnemius-soleus* nerve was injured 4-7 days before the neurophysiological experiments. The injured afferent C- and A-fibers were functionally characterized by their responses to the mechanical and thermal stimuli applied from 3mm proximal to 3mm distal to the injury site of the nerve. Then, these fibers were tested for their responses to capsaicin, menthol or mustard oil applied to their receptive fields in the nerve. After application of the TRP agonist the injured A-fibers and C-fibers were tested again for their responses to cold, heat and mechanical stimuli applied to the receptive fields in the nerve.

3.2.1 TRPV1 channel agonist capsaicin

44 functionally identified injured C-fibers and 39 functionally identified A-fibers were investigated in this study. Table 3.10 shows the individual functional properties of the injured muscle afferent nerve fibers tested for their responses to capsaicin.

C-fibers	Activation to CAP	No activation to CAP	Total
Heat-sensitive	31 (31)	3 (3)	34
Cold-sensitive only	1 [1]	5 [3]	6
Mechanosensitive only	2 [2]	2	4
Total	34 [3]	10 [3]	44

A-fibers	Activation to CAP	No activation to CAP	Total
Heat-sensitive with OA	10	12 (4)	22
Heat-sensitive without OA	0	13 (12)	13
Cold-sensitive only	0	1	1
Mechanosensitive only	0	3	3
Total	10	29	39

Table 3.10. Numbers of functional types of afferent neurons activated or not activated by capsaicin (CAP). Heat response of the fibers in round brackets was desensitized after application of capsaicin. Fibers in squared brackets were recruited to the heat response after application of capsaicin.

C-FIBERS:

Forty-four injured afferent C-fibers activated by physiological stimulation of the nerve were studied. Most heat-sensitive C-fibers were activated by capsaicin, applied to the receptive field in the nerve (31/34, 91%) (Table 3.10, Fig. 3.28, 3.29A, 3.30). Figure 3.29A shows the responses of a typical injured heat-sensitive C-fiber to capsaicin and to heat stimuli before and after application. The response to capsaicin consisted of a strong activation during application followed by a long-lasting irregular spontaneous activity. Three heat-sensitive C-fibers (3/34, 9%), which had high heat thresholds (48°C) were not excited by the highest dose of capsaicin (1mM), but desensitized for heat stimuli (Fig. 3.28).

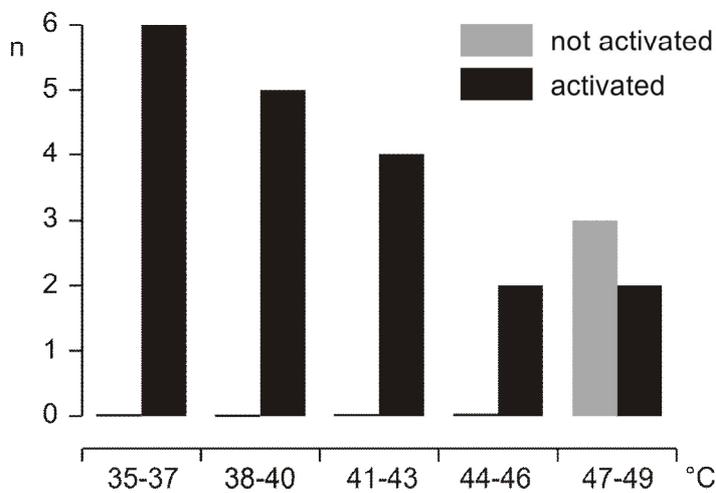


Fig. 3.28. Distribution of the thresholds of the heat-sensitive unmyelinated fibers, which were activated (black bars) or not activated (grey bars) during application of capsaicin to their receptive fields in the nerve ($n=22$). 3 fibers with high thresholds, which were not activated by capsaicin were desensitized for heat stimuli.

Most cold-sensitive and/or mechanosensitive but not heat-sensitive C-fibers were insensitive to capsaicin (7/10, Fig. 3.29B). Three C-fibers which were initially not heat-sensitive were weakly activated by capsaicin and exhibited heat sensitivity afterwards (3/10; Fig. 3.29C). Another three cold-sensitive neurons were not activated by capsaicin directly, but were excited by heat stimuli after application of the substance (Table 3.10). Thus in summary 6 initially heat-insensitive unmyelinated axons became heat-sensitive after the application of capsaicin (6/10). The magnitude of the cold and/or mechanical responses was not affected by capsaicin.

In general, the concentrations of capsaicin (50 μ M, 100 μ M and 1mM) were applied in ascending order until the acute reaction and/or desensitization of the fiber were observed. The activity of heat-sensitive C-fibers before, during and after application of different concentration of capsaicin and application of vehicle (Tyrode's solution) to the nerve are depicted in Figure 3.30. The spontaneous activity (SA) was measured 2 min before application of capsaicin as well as 4 min after the application (washing out using Tyrode's

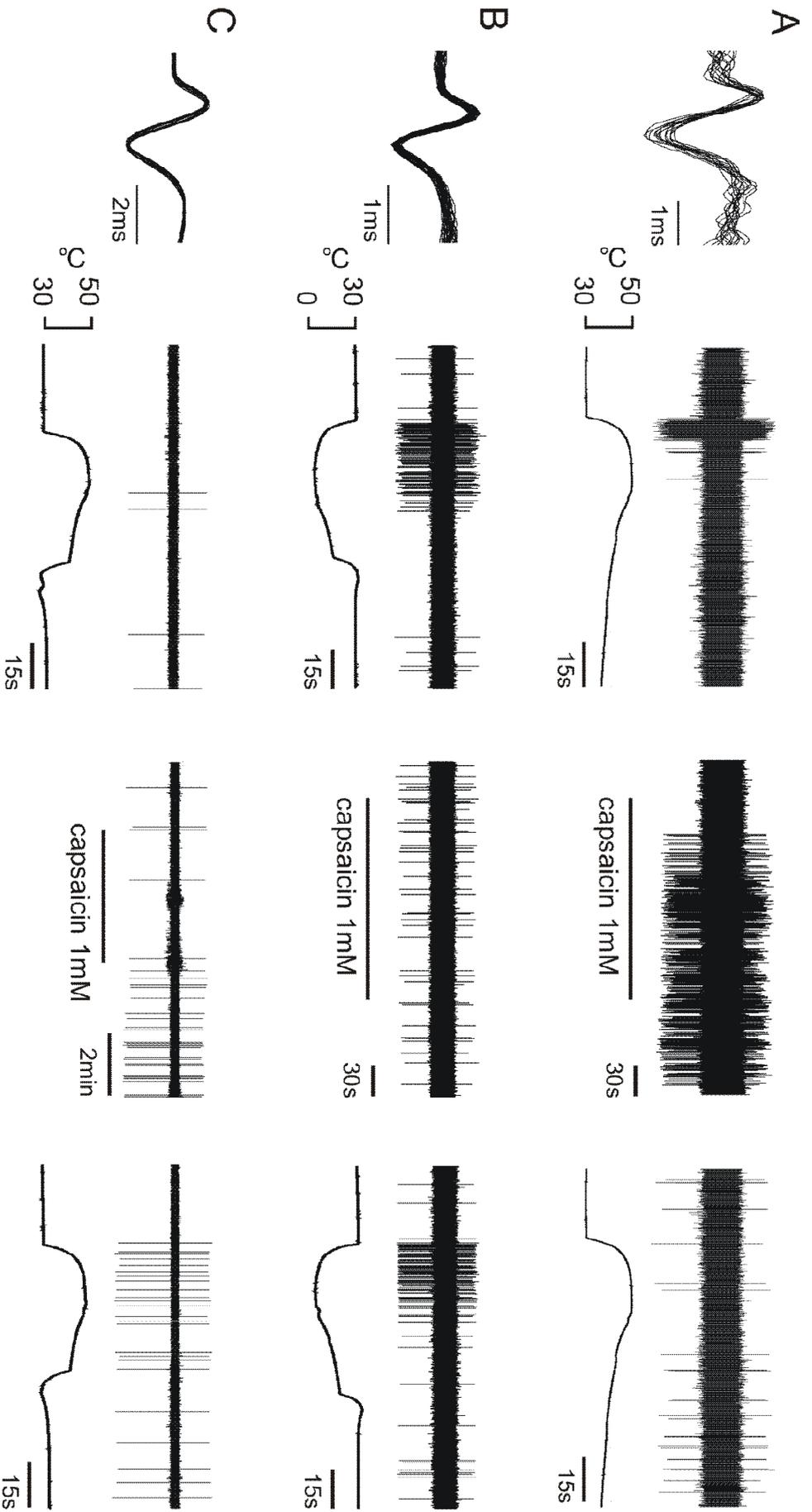


Fig. 3.29. Responses of the injured C-fibers to capsaicin (1mM) applied to the nerve injury site. A. A heat-sensitive C-fiber was activated by capsaicin, applied to the receptive field in the nerve, and lost its heat sensitivity after this application. Note that this fiber showed ongoing activity after capsaicin. B. A cold-sensitive but not heat-sensitive C-fiber was insensitive to capsaicin. Its cold response was not affected by capsaicin. C. A C-fiber which was initially not heat-sensitive was weakly activated by capsaicin and exhibited heat sensitivity afterwards. Cold (5°C) and heat stimuli (48°C) were applied to the injury site of the nerve. The temperature stimuli started from a preset temperature of about 30°C.

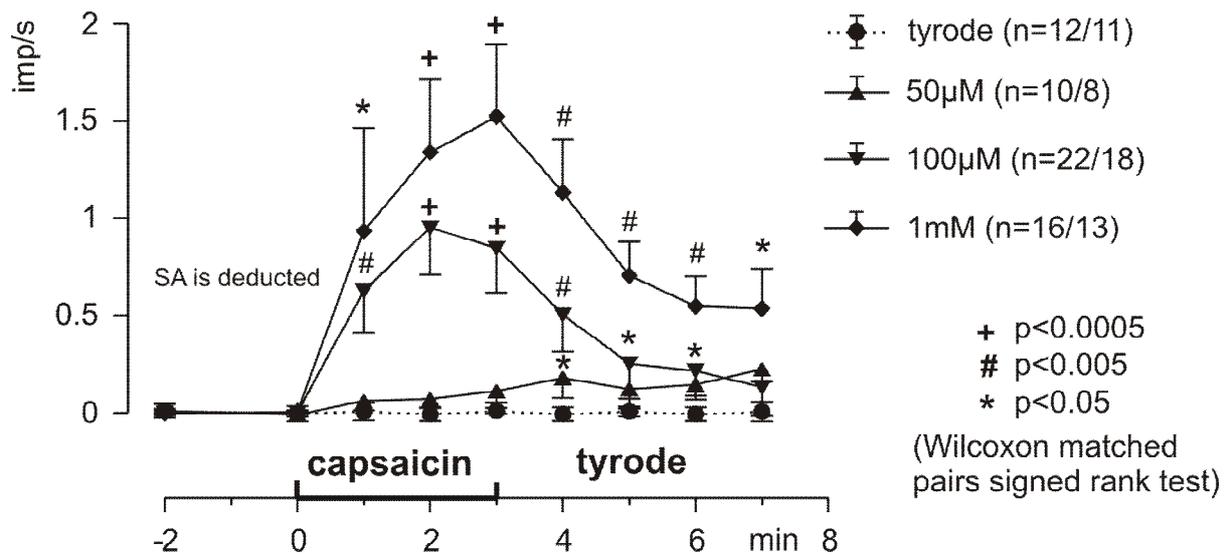


Fig. 3.30. Acute activation of heat-sensitive C-fibers during application of capsaicin to the nerve-injury site (Mean + SEM). The numbers of C-fibers with SA are given behind the slash. The rate of SA was subtracted from the response.

solution). The activity during application of capsaicin was recorded for 3 min. To summarize data obtained on spontaneously active and silent fibers, the mean value of SA before capsaicin application was deducted (Fig. 3.30).

The increase of activity during application of capsaicin was dose dependent and significantly higher when compared to the activity during application of vehicle starting in the 4th minute during application of 50µM capsaicin (*, $p < 0.05$) and 1st minute during application 100µM and 1mM (*, $p < 0.05$, #, $p < 0.005$, +, $p < 0.0005$, Wilcoxon matched pairs signed rank test). The activity after removal of capsaicin was still increased and significantly higher when compared to the activity after application of vehicle (* $p < 0.05$; # $p < 0.005$; Wilcoxon matched pairs signed rank test).

About 10 to 20 min after application of capsaicin, the injured heat-sensitive C-fibers were tested again for their responses to thermal and mechanical stimuli applied to the receptive fields in the nerve. The population responses of the injured heat-sensitive C-fibers to heat and cold stimuli applied to the nerve before (black circles) and after application of capsaicin (100µM black triangles; 1mM black squares) are shown in Figure 3.31. In order to observe the real change in the magnitude of the heat and cold response before and after application of the capsaicin the spontaneous activity was subtracted. The level of the spontaneous activity in the population of heat-sensitive fibers dose-dependently decreased after application of capsaicin (e.g., before 100µM capsaicin 0.59 ± 0.20 vs. after 100µM

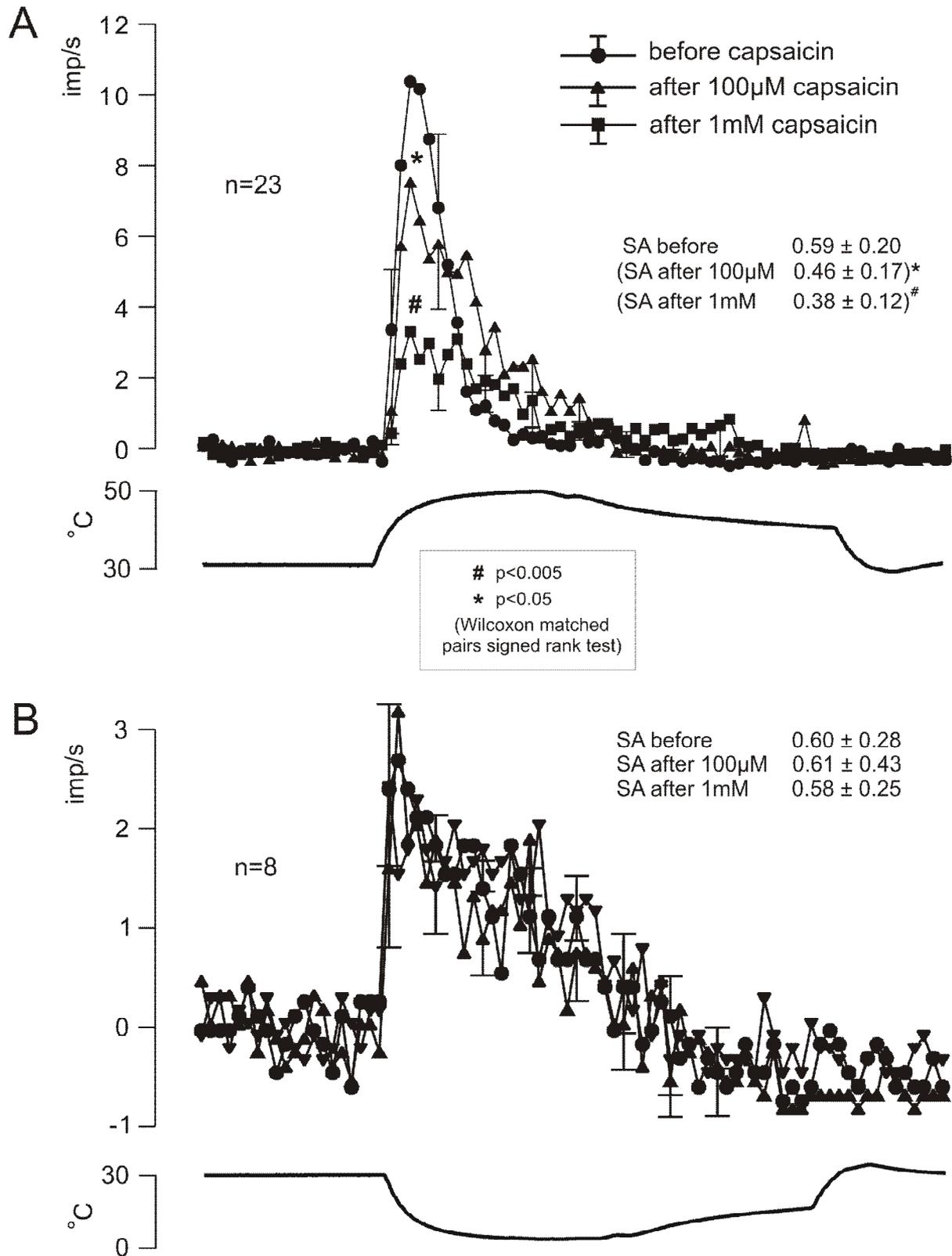


Fig. 3.31. Responses of the heat-sensitive C-fibers to heat stimuli (A, 48°C, n=23) and cold-sensitive C-fibers to cold stimuli (B, 5°C, n=8, 4/8 cold-heat-sensitive fibers) before (circles) and after application of capsaicin (100µM normal triangles; 1mM squares). The rate of SA was subtracted from the response. Inset: rates of SA before and after application of capsaicin (mean \pm SEM, imp/s). * $p < 0.05$, # $p < 0.005$ (Wilcoxon matched pairs signed rank test). The temperature stimuli started from a preset temperature of about 30°C. Temporal resolution 1s.

capsaicin 0.46 ± 0.17 (mean \pm S.E.M., imp/s), * $p < 0.05$; before 1mM capsaicin 0.59 ± 0.20 vs. after 1mM capsaicin 0.38 ± 0.12 (mean \pm S.E.M., imp/s), # $p < 0.005$; Wilcoxon matched pairs signed rank test), whereas the rate of spontaneous activity of the cold-sensitive fibers remained unchanged.

The heat response of all heat-sensitive C-fibers was desensitized after application of capsaicin (Table 3.10, Fig. 3.29A, 3.31A). Decrease of the response to heat stimuli was dose-dependent and significant when compared with the heat response before application of capsaicin (* $p < 0.05$; # $p < 0.005$; Wilcoxon matched pairs signed rank test). The activation to nerve cooling (Fig. 3.31B) or to mechanical stimulation (Fig. 3.32A; before capsaicin: mean 1.18 imp/s, median 1.05 imp/s vs. after 1mM capsaicin: mean 1.08 imp/s, median 0.9 imp/s, $p = 0.92$, Wilcoxon matched pairs signed rank test) were not changed by capsaicin.

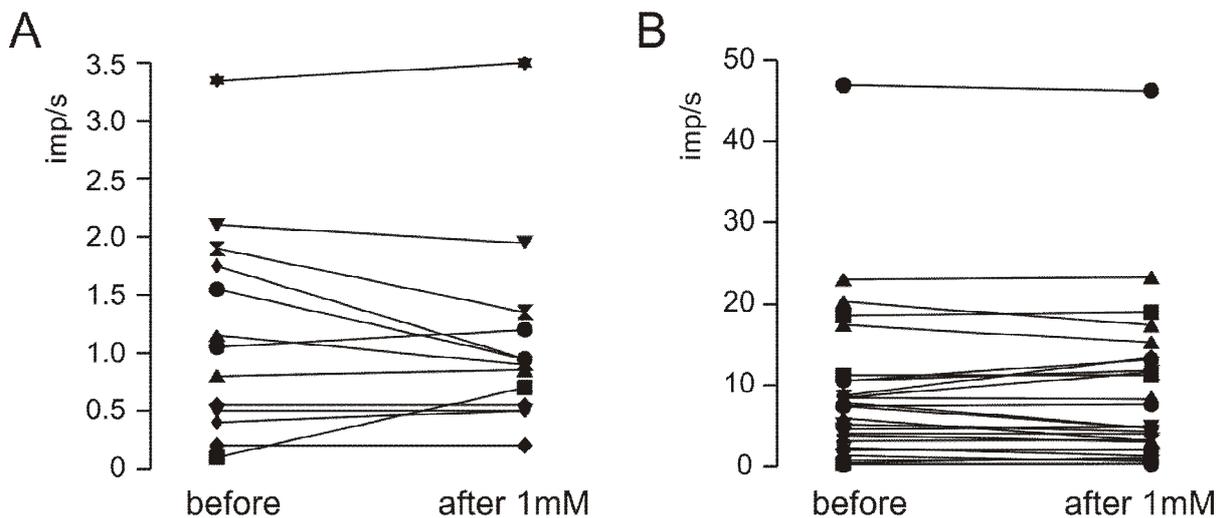


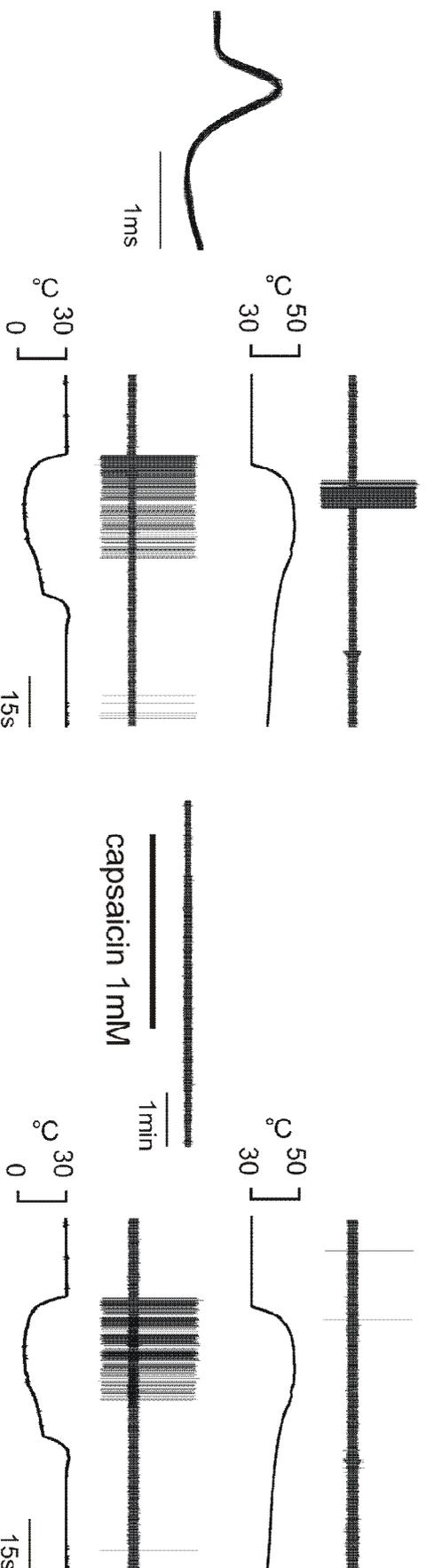
Fig. 3.32. Response magnitude to mechanical stimulation before and after application of capsaicin (1mM). Mechanical stimuli were applied by a fine blunt glass rod repetitively to the injury site of the nerve over 20 s at about 1 Hz. A. C-fibers (n=13). B. A-fibers (n=27). Ordinate scale is in imp/s during the stimulation period of 20s.

A-FIBERS:

Thirty-nine injured afferent A-fibers activated by physiological stimulation of the nerve injury site were studied. Note that injured muscle heat-sensitive A-fibers with spontaneous activity showed significantly stronger and longer responses and lower thresholds to heat stimulation than injured silent heat-sensitive A-fibers (for details see chapter 3.2.1c; Fig. 3.15). Similar observations were made when injured heat-sensitive A-fibers were tested for their activation during the application of the capsaicin on the nerve injury site.

A

A-fiber without SA: No activation and desensitization



B

A-fiber with SA: Activation and no desensitization

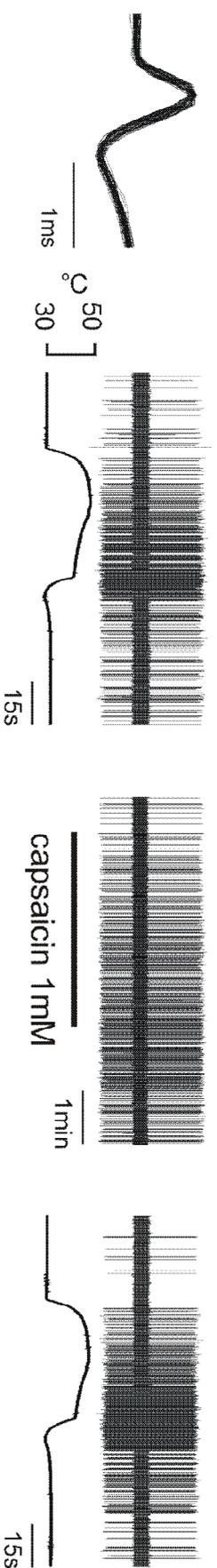


Fig. 3.33. Responses of A β -fibers before and after application of capsaicin (1 mM) to cold or heat stimulation. A. A cold- and heat-sensitive A-fiber without spontaneous activity (SA) showed no acute activation to capsaicin. After application of capsaicin the fiber could not any longer be activated by heat whereas the response to cold was unchanged. B. A heat-sensitive A-fiber with SA showing an activation to capsaicin and a weakly enhanced response to heat. Cold (5°C) or a heat stimulus (48°C) applied to the nerve injury site. The temperature stimuli started from a preset temperature of about 30°C.

Only spontaneously active heat-sensitive A-fibers were activated during local application of capsaicin (10/22; Fig. 3.33B, 3.34). Interestingly, these fibers did not show desensitization of their heat response after application of capsaicin (0/10), as present in heat-sensitive C-fibers. The remaining 12 spontaneously active heat-sensitive A-fibers were not activated by capsaicin, but 4 of them were desensitized for heat stimuli (4/12).

Heat-sensitive A-fibers without spontaneous activity (SA) were not activated by capsaicin (0/13) but showed dose-dependent desensitization after the application of capsaicin (12/13) (Table 3.10, Figs. 3.33A and 3.35A). All only cold-sensitive and/or mechanosensitive A-fibers were insensitive to capsaicin (4/4). The magnitude of the cold and/or mechanical response was not affected by capsaicin (Fig. 3.33A, 3.35B, 3.32B). A-fibers were not sensitized to heat stimuli by capsaicin as observed in some C-fibers (Fig. 3.29C).

The activity of 10 spontaneously active heat-sensitive A-fibers before, during and after application of two concentrations of capsaicin and application of vehicle (Tyrode's solution) to the nerve injury site is shown in Figure 3.34. The spontaneous activity was subtracted. The increase of the activity during application of capsaicin was dose dependent and significantly higher compared to the activity during application of vehicle starting in the 1st minute during application 100 μ M or 1mM capsaicin (*, $p < 0.05$; +, $p < 0.0005$, Wilcoxon matched pairs signed rank test).

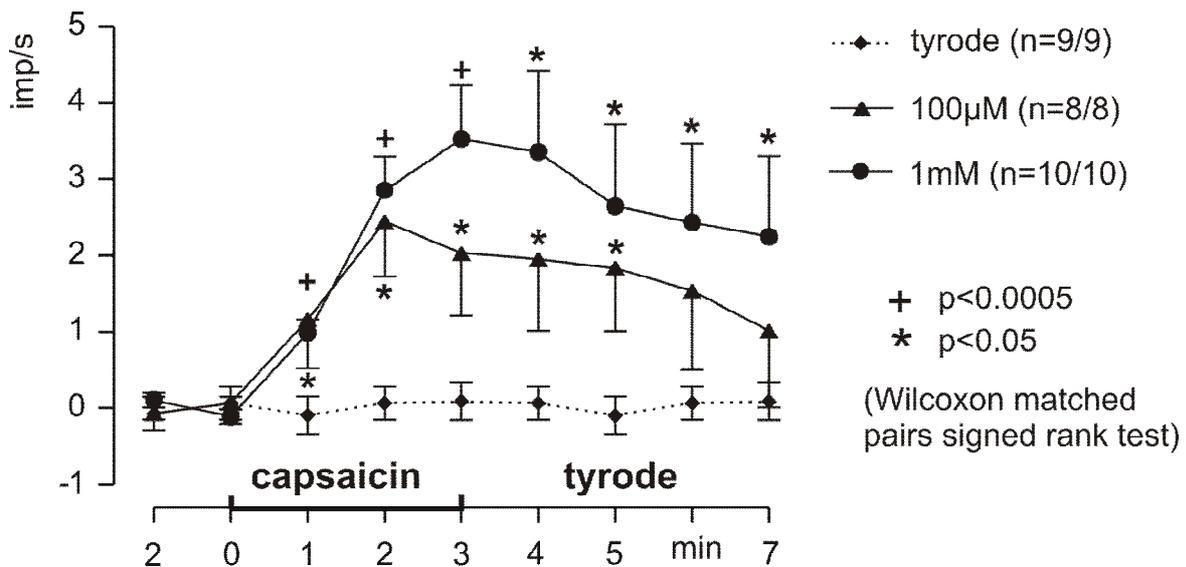


Fig. 3.34. Responses of heat-sensitive A-fibers (mean + SEM) to application of capsaicin to the nerve-injury site (100 μ M normal triangles, $n=8$; 1mM closed circles, $n=10$) or vehicle (Tyrode's solution diamonds, $n=9$). The numbers of A-fibers with spontaneous activity (SA) are given behind the slash. The rate of SA was subtracted from the response. * $p < 0.05$, + $p < 0.0005$ (Wilcoxon matched pairs signed rank test).

The activity after removal of capsaicin was still increased and significantly higher when compared to the activity after application of vehicle (* $p < 0.05$; Wilcoxon matched pairs signed rank test). 50 μ M of capsaicin had no effect on the activity of myelinated heat-sensitive A-fiber ($n=9$, data not shown).

10 to 30 minutes after application of capsaicin, the injured A-fibers were tested again for their responses to thermal or mechanical stimuli applied to the receptive fields in the nerve. The population responses of the injured heat-sensitive A-fibers (spontaneously active and silent fibers together) to heat or cold stimuli applied to the nerve before (black circles) and after application of capsaicin 100 μ M (black triangles) and 1mM (black squares) are shown in Figure 3.35. The spontaneous activity before thermal stimulation was subtracted. The rate of spontaneous activity in the population of injured myelinated fibers (most of them were polymodal mechano-cold-heat-sensitive A-fibers) dose-dependently decreased after application of capsaicin (e.g., before 100 μ M capsaicin 8.86 ± 1.68 [median 8.13] imp/s vs. after 100 μ M capsaicin 6.05 ± 1.86 [median 2.13], $p < 0.05$; before 100 μ M capsaicin 8.86 ± 1.68 [median 8.13] imp/s vs. after 1mM capsaicin 4.61 ± 1.83 [median 1.15] imp/s, $p < 0.005$; Wilcoxon matched pairs signed rank test).

Many heat-sensitive A-fibers (16/35) were desensitized after application of capsaicin for heat stimuli (Table 3.10, Figs. 3.33A, 3.35A,). Decrease of the response to heat stimuli was dose-dependent and significant at 1mM capsaicin when compared with the heat response before application of capsaicin (* $p < 0.05$; Wilcoxon matched pairs signed rank test). Neither the activation to cooling (Fig. 3.35B) nor to mechanical stimulation of the nerve were changed by capsaicin (Fig. 3.32B; before capsaicin: mean 8.93 imp/s, median 7.4 imp/s vs. after 1mM capsaicin: mean 8.78 imp/s, median 4.75 imp/s, $p > 0.5$, Wilcoxon matched pairs signed rank test).

In summary, most heat-sensitive C-fibers were dose-dependently activated and desensitized by capsaicin, but only about one third of the heat-sensitive A-fibers. These A-fibers were spontaneously active (SA) and had low heat thresholds. Heat-sensitive A-fibers without SA had high thresholds and were only dose-dependently desensitized by capsaicin. Cold- and mechano-sensitivity of C- and A-fibers were not affected by capsaicin. Figure 3.36 demonstrates a typical spontaneously active heat-sensitive C-fiber having an acute reaction to capsaicin and an A-fiber insensitive to capsaicin both recorded simultaneously in the same filament.

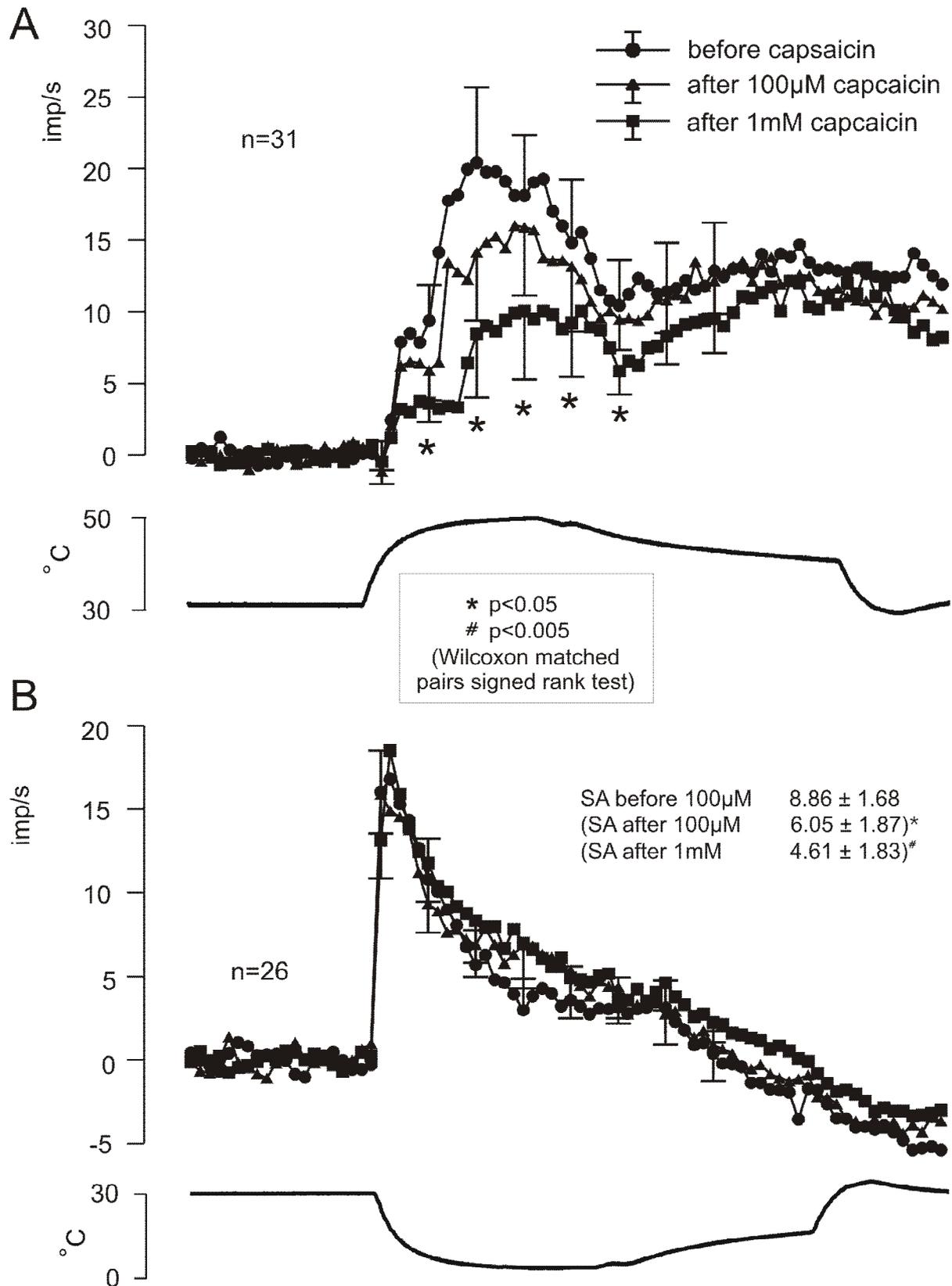


Fig. 3.35. Responses of injured thermosensitive A-fibers to heat stimuli (A, 48°C, n=31 fibers, 25/31 heat-cold-sensitive fibers, 6/31 heat-sensitive only) or cold stimuli (B, 5°C, n=26 fibers, 25/26 cold-heat-sensitive fibers, 1/26 cold-sensitive only) before (circles) and after application of capsaicin to the injury site (100µM normal triangles; 1mM squares). The rate of SA was subtracted from the response. Inset: SA before and after application capsaicin (n=14, mean ± SEM, imp/s). The temperature stimuli started from a preset temperature of about 30°C. Temporal resolution 1s.

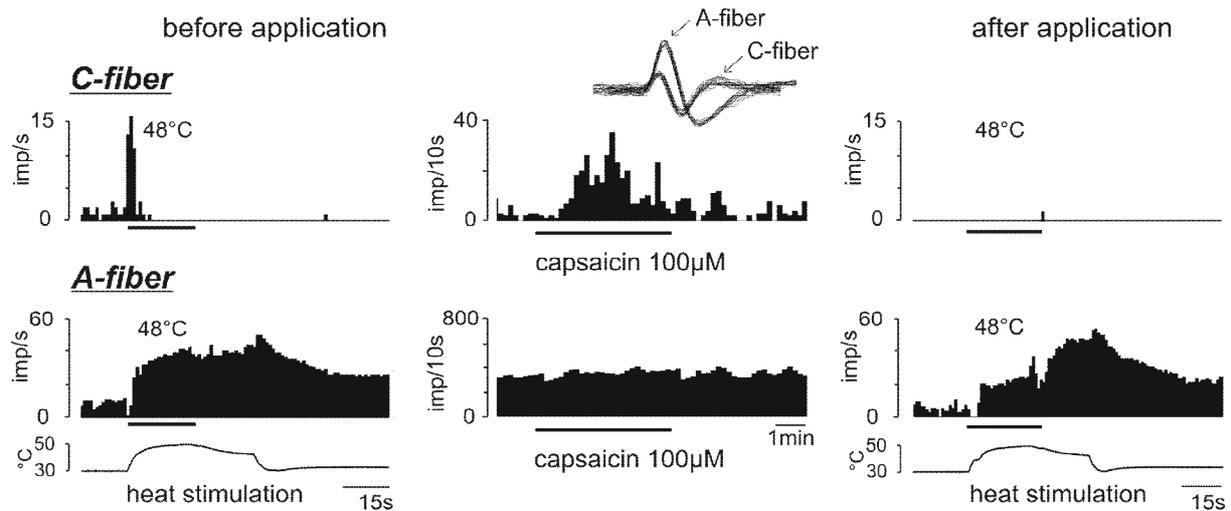


Fig. 3.36. Testing of an injured heat-sensitive C- and an injured A-fiber recorded simultaneously in the same filament by application of 100µM capsaicin to the injury site of the nerve. The unmyelinated fiber showed activation to capsaicin and desensitization of its heat response, while the activity of myelinated fiber was not changed during application of capsaicin and its heat response was decreased.

3.2.2 TRPM8 channel agonist menthol

36 functionally identified injured muscle C-fibers and 35 functionally identified A-fibers were investigated in this study. Table 3.9 shows the individual functional properties of the injured muscle afferent nerve fibers tested for their responses to menthol.

C-FIBERS:

All injured muscle cold-sensitive C-fibers tested were activated by menthol, applied to the lesion site in the nerve (15/15, 100%) (Table 3.11, Fig. 3.37A, 3.38). Figure 3.37A shows the responses of a typical cold-sensitive C-fiber to cold stimuli before and after application of menthol. The response to menthol consisted of a small immediate activation, probably due to vehicle (0.2% ethanol solution) application, and a large activation during application of menthol followed by a long-lasting enhanced spontaneous activity (SA).

Most heat-sensitive and/or mechanosensitive but not cold-sensitive C-fibers were insensitive to menthol (15/21, 71%; Fig. 3.37B). Six initially cold-insensitive C-fibers were activated by menthol (6/21, 29%) two of them showing cold-sensitivity afterwards (2/21, 10%).

C-fibers	Activation	No activation	Total
Cold-sensitive	15	0	15
Heat-sensitive	6 (2)*	13	19
Mechanosensitive only	0	2	2
Total	21	15	36

A-fibers	Activation (and depression)	No activation	Depression only	Total
Cold-sensitive without SA or with a low rate ≤ 5 imp/s of SA	12 (9)***	2**	2	16
Cold-sensitive with SA 5 imp/s < rate < 15 imp/s	-	-	4	4
Cold-sensitive with SA rate ≥ 15 imp/s	-	5	3	8
Heat-sensitive only		2		2
Mechanosensitive only	0	5	0	5
Total	12 (9)	14	9	35

Table 3.9. Numbers of functional types of afferent neurons activated or not activated by menthol.

* fibers in brackets were recruited to the cold response after application of menthol

** in both fibers the cold response was desensitized after application of menthol

*** fibers in brackets were significantly depressed after activation by menthol

The activity of cold-sensitive C-fibers before, during and after application different concentrations of menthol and application of vehicle (0.2% ethanol solution) to the nerve are depicted in Figure 3.38. The activity was measured 2 min before, 4 min during and 4 min after the application of menthol (washing out using Tyrode's solution). The increase of the activity during application of menthol was dose dependent and significantly higher when compared to the activity during application of vehicle starting in the 1st minute during application 100 μ M and 1mM (#, $p < 0.05$, +, $p < 0.005$, Wilcoxon matched pairs signed rank test). The activity after removal of menthol was still increased and significantly higher when compared to the activity after application of vehicle (#, $p < 0.05$; Wilcoxon matched pairs signed rank test).

After application of menthol, the injured muscle C-fibers were tested again for their responses to thermal and mechanical stimuli applied to the receptive fields in the nerve. The population responses of the injured cold- and/or heat-sensitive C-fibers to cold or heat stimuli applied to the nerve before (black circles) and after application of 4 mM menthol (black squares) are shown in Figure 3.39.

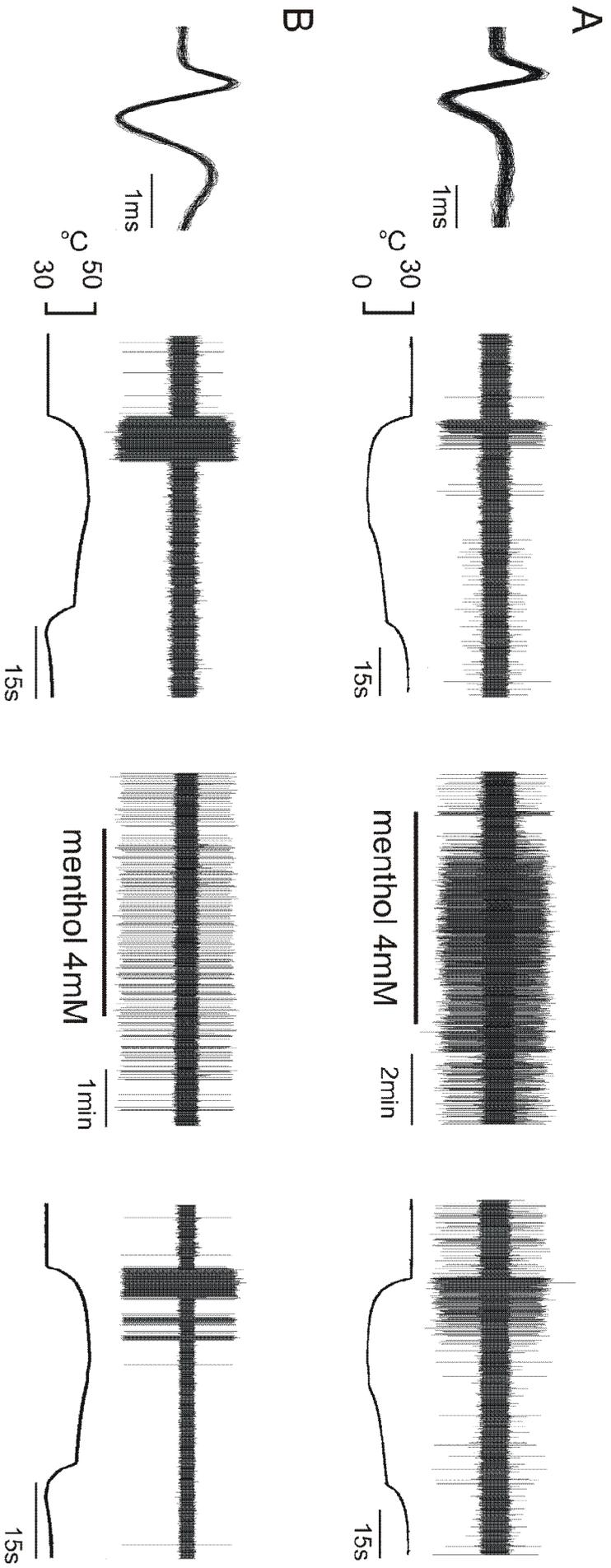


Fig. 3.37. Responses of two injured C-fibers to menthol (4 mM) applied to the injured nerve. A. Cold-sensitive C-fiber activated by menthol. The response to a cold stimulus (5°C) applied to the nerve was enhanced after menthol. B. Heat-sensitive C-fiber not activated by menthol. The response to a heat stimulus (48°C) applied to the nerve was unchanged. The temperature stimuli started from a preset temperature of about 30°C.

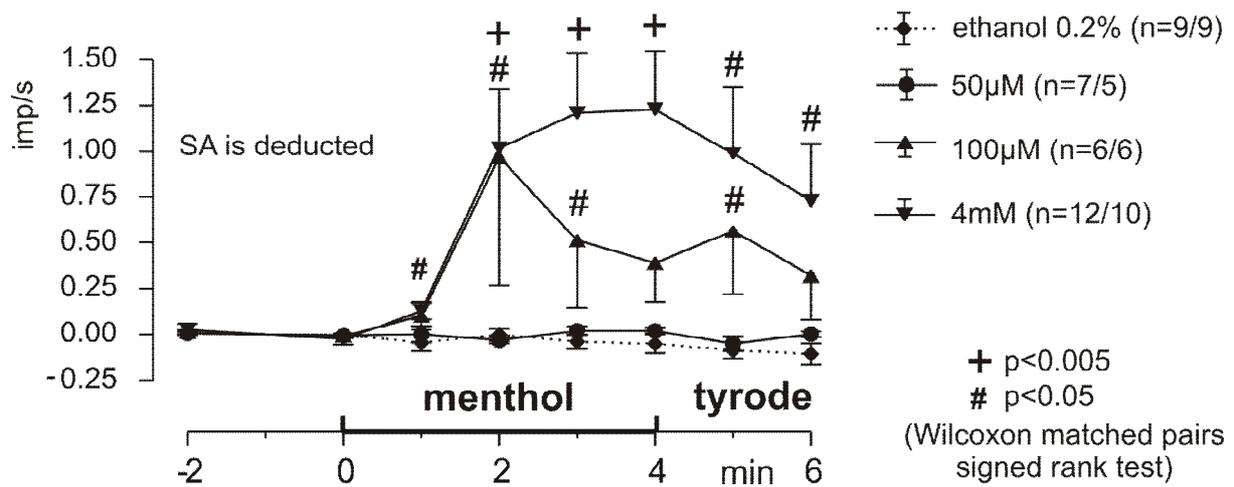


Fig. 3.38. Responses of injured cold-sensitive C-fibers to application of menthol to their receptive fields in the nerve. Mean + SEM of the activity of the cold-sensitive C-fibers before, during and after application of menthol 50µM (circles, n=7), 100µM (normal triangle, n=6) and 4mM (inverted triangle, n=12) to the nerve (fibers with spontaneous activity (SA) indicated by number behind the slash). The rate of SA was subtracted from the response. + p<0.05, # p<0.005 (Wilcoxon matched pairs signed rank test). Dotted line: activity in 9 cold-sensitive C-fibers before and during application of vehicle (ethanol 0.2%).

The cold response of the C-fibers was sensitized after application of menthol (Fig. 3.37A, 3.39A). The increase of the response to the cold stimuli was significant when compared to the cold response before application of menthol (#, p<0.05; +, p<0.005; Wilcoxon matched pairs signed rank test). The thresholds of the activation to the cold stimuli were significantly shifted to the low range after application of menthol (Fig. 3.40A). The heat response of the heat-sensitive fibers was significantly decreased after application of menthol (Fig. 3.39B). The activation thresholds to heat stimuli were unchanged (Fig. 3.40B). The rate of the spontaneous activity in the population of cold-sensitive or cold-heat-sensitive fibers dose-dependently increased after application of menthol (e.g., before application of menthol 0.48 ± 0.15 (mean \pm S.E.M., imp/s) vs. 30 minutes after 4mM menthol 0.91 ± 0.19 (mean \pm S.E.M., imp/s), p<0.005; Wilcoxon matched pairs signed rank test).

The activation to mechanical stimulation after application of menthol was not significantly different compared to the activation before (before menthol: mean 1.06 imp/s, median 0.6 imp/s vs. after 4mM menthol: mean 1.05 imp/s, median 0.85 imp/s, p=0.71, Wilcoxon matched pairs signed rank test) (Fig. 3.39C).

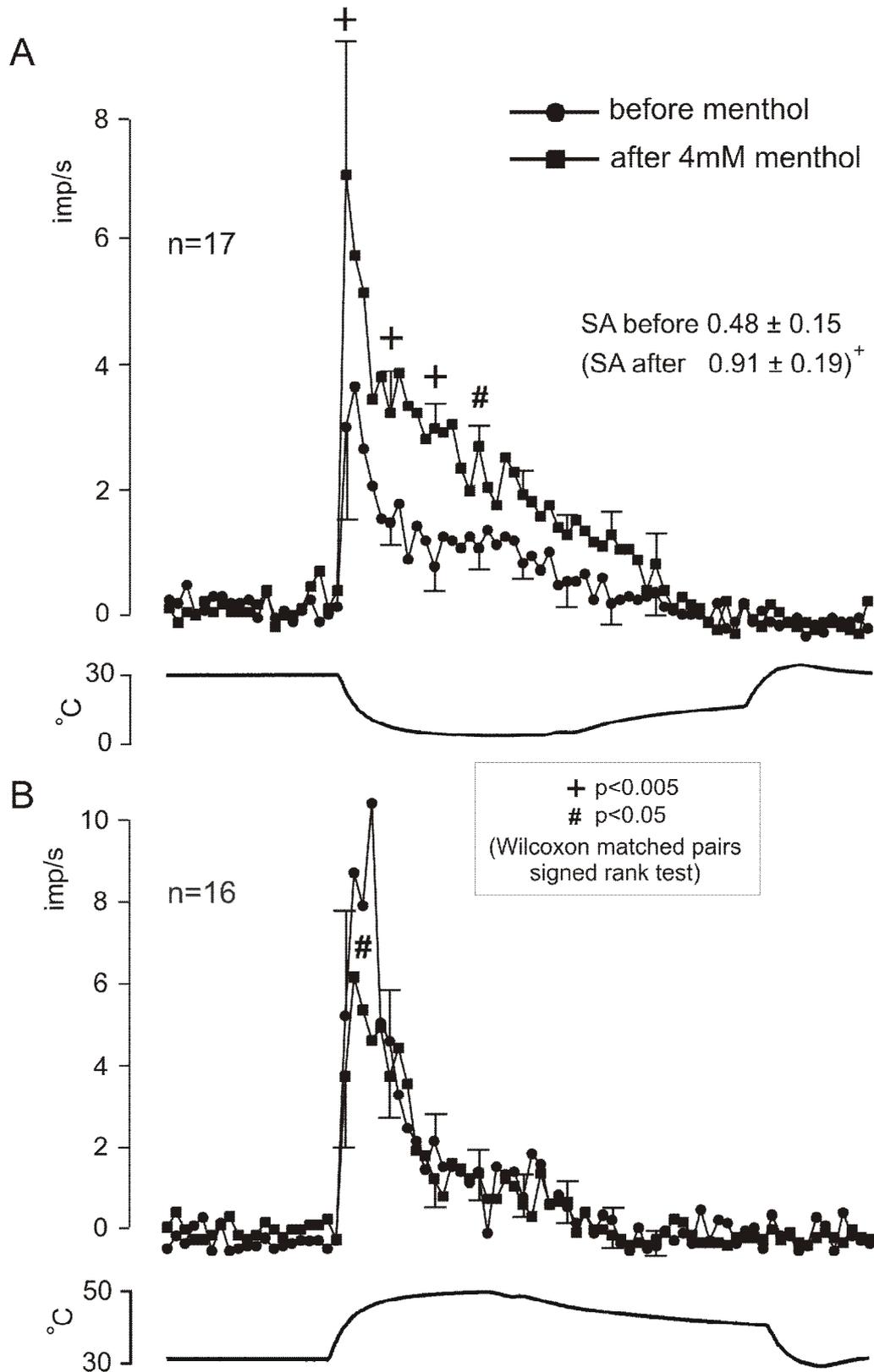


Fig. 3.39. Responses to cold stimuli of 5°C (17 fibers) and heat stimuli of 48°C (16 fibers, 8/16 heat-cold-sensitive fibers) before application of menthol (circles) and after application of menthol (squares). The rate of spontaneous activity (SA) was subtracted from the response. Inset: deduced rate of SA before application of the cold and heat stimulus (n=16, mean \pm SEM, imp/s). # p<0.05, + p<0.005 (Wilcoxon matched pairs signed rank test). The temperature stimuli started from a preset temperature of about 30°C. Temporal resolution 1s.

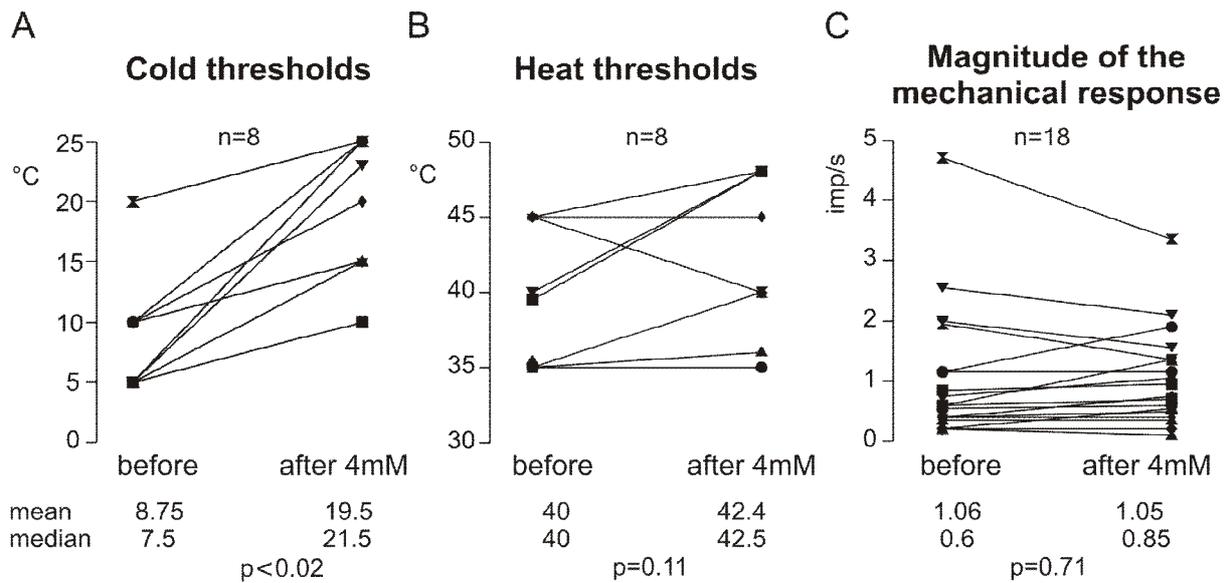


Fig. 3.40. Cold thresholds (A), heat thresholds (B) and magnitude of response to mechanical stimulation (C) of C-fibers before and after application of menthol (4 mM) to their receptive fields in the nerve. Mechanical stimuli applied by a fine blunt glass rod repetitively to the injury site of the nerve over 20 s at about 1 Hz. Ordinate scale is in imp/s during the stimulation period of 20 s. Statistics -Wilcoxon matched pairs signed rank test.

A-FIBERS:

In contrast to the menthol-sensitivity of injured muscle cold-sensitive C-fibers, most cold-sensitive injured muscle A-fibers seemed to be depressed by menthol. Depending on the rate of spontaneous activity and the reaction to application of menthol the population of cold-sensitive myelinated fibers was divided tentatively into (Table 3.11, Fig. 3.41):

- cold-sensitive fibers without spontaneous activity (SA) or with a low rate of SA (≤ 5 imp/s), 12 of which were activated by low concentrations of menthol (5 – 100 μ M) and/or were activated in the first minute of application of menthol 4 mM, followed by a dramatic depression of activity (12/16, Fig. 3.42); 2 fibers were not activated; in the remaining 2 afferents the SA was depressed. These fibers were desensitized for cold stimuli by menthol;
- cold-sensitive fibers with a middle rate of SA ($5 \text{ imp/s} < \text{rate} < 15 \text{ imp/s}$) were only depressed in their SA and were desensitized for cold stimuli after application of menthol (Fig. 3.43);
- cold-sensitive fibers with a high rate of SA ($\geq 15 \text{ imp/s}$) were not activated (5/8, Fig. 3.44) or were depressed and desensitized to cold stimuli (3/8).

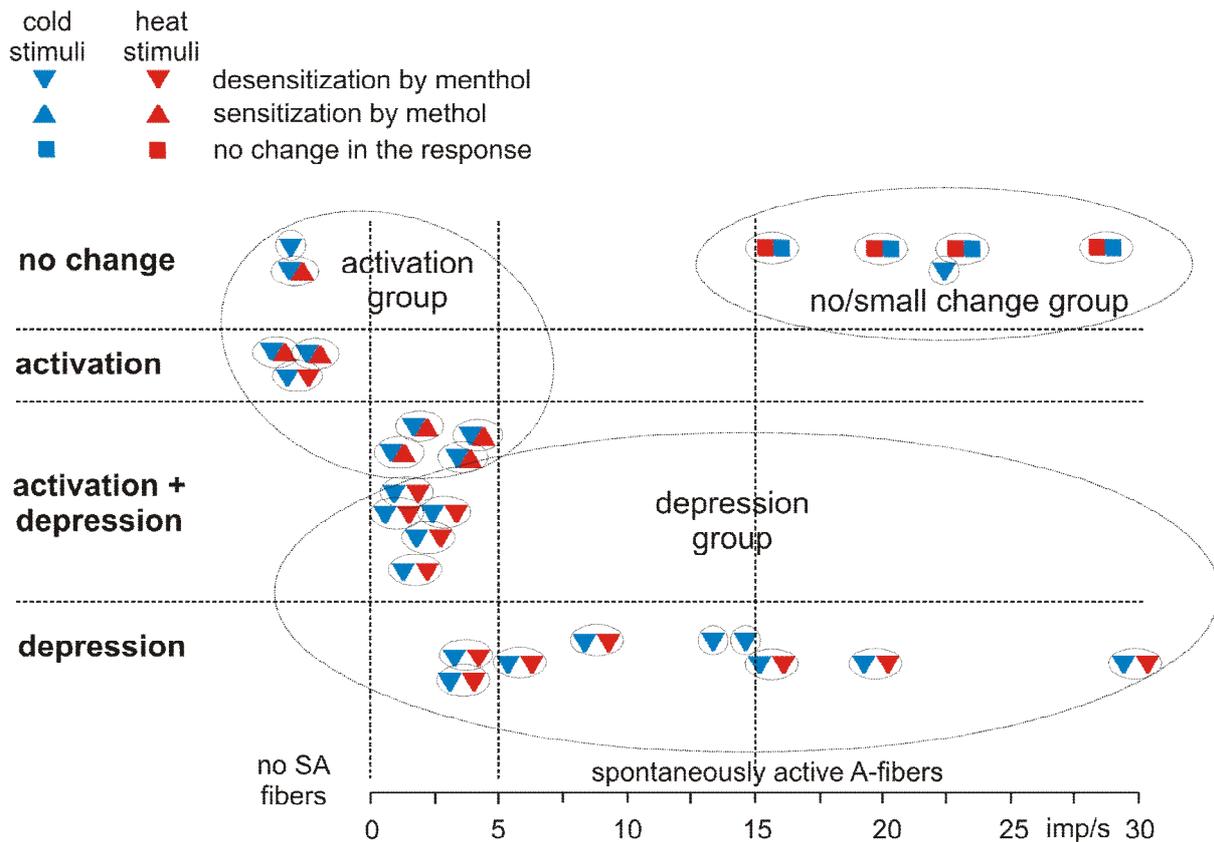


Fig. 3.41. Subgroups of cold-sensitive myelinated fibers ($n=28$ [small circles]; 24 fibers were cold-heat-sensitive, 4 fibers were cold-sensitive only) depending on the rate of spontaneous activity (SA) and the reaction to the application of menthol. Triangles in the circles show desensitization or sensitization of the fibers to cold and/or heat stimuli after application of menthol. Squares in the circles demonstrate no change in the response strength to thermal stimulation after application of menthol.

Neither heat- nor mechano-sensitive A-fibers which were cold-insensitive responded to menthol (7/7). Thus, most cold-sensitive A-fibers with SA were depressed in their activity by menthol (17/23). Activation was observed only in cold-sensitive A-fibers without or with a low rate of the SA (12/16) and this activation was followed by depression of SA. Cold-sensitive A-fibers with a high rate of SA were almost not affected by menthol (5/8).

According to the type of reaction to menthol I divided cold-sensitive A-fibers into three groups (Fig. 3.41, 3.45): activation group ($n=9$; Fig. 3.42), depression group ($n=14$; Fig. 3.43) and “no change” group ($n=5$; Fig. 3.44). This division collides with the above tentative division.

The activation group contained fibers without SA or with a low rate of SA, which showed acute activations during application of menthol, followed by a depression of the activity (particularly to high concentrations of menthol; the cold response decreased and the heat response increased after application menthol. Figure 3.42 gives an example of the

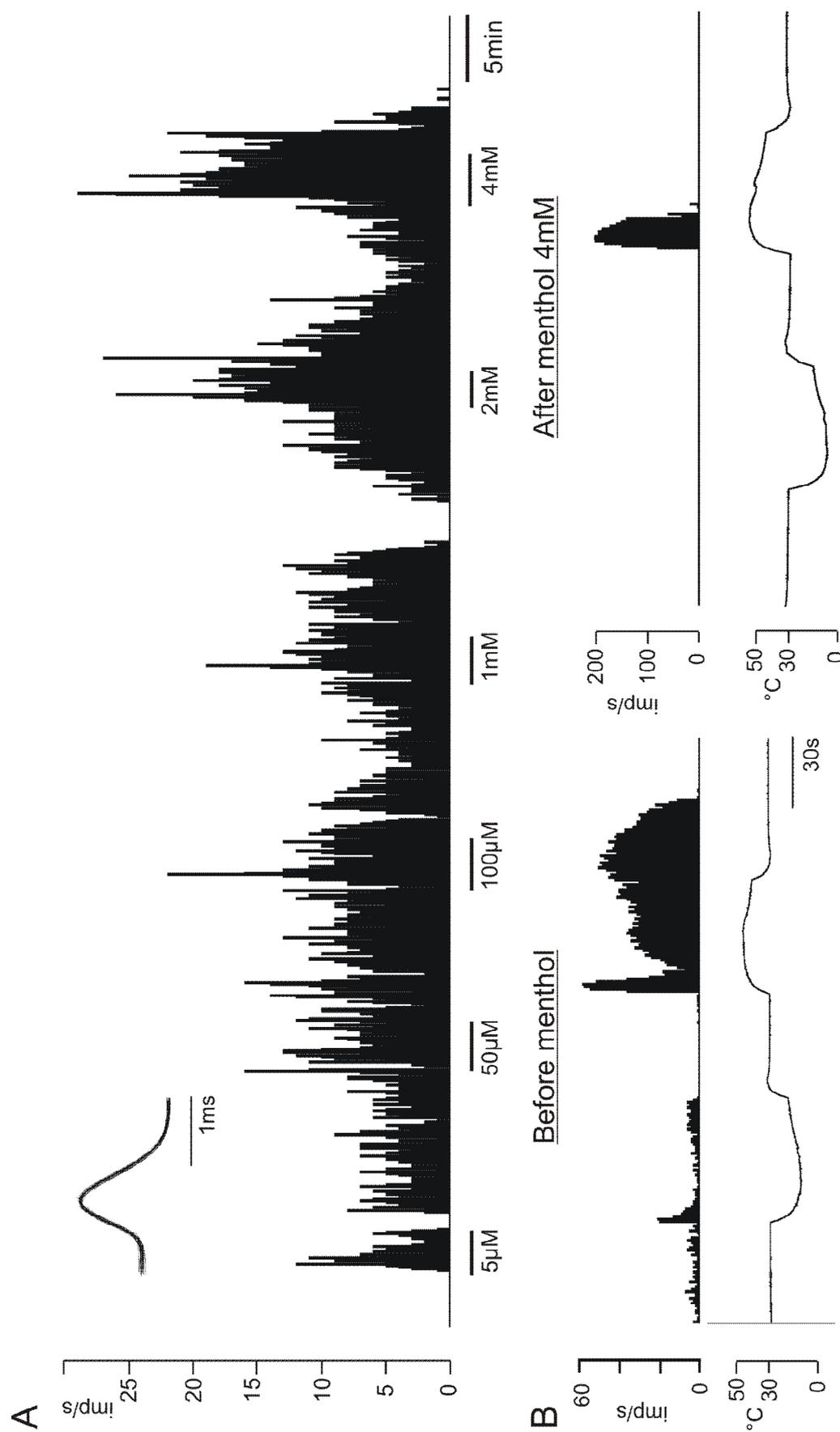


Fig. 3.42. Activation of an injured A β -fiber by menthol (5 μ M, 50 μ M, 100 μ M, 1mM, 2mM, 4mM) (A). The fiber had a low rate of the spontaneous activity, it was mechanosensitive, cold-sensitive and heat-sensitive. The response to mechanical stimulation was not depressed by menthol (not shown). The response to a cold stimulus (5°C) was depressed; the response to a heat stimulus (48°C) was enhanced (the initial part of the response) after application of menthol (B). The temperature stimuli started from a preset temperature of about 30°C. The fiber was not activated during application of vehicle (ethanol 0.2%, not shown) and was completely depressed during application of 4mM menthol (A).

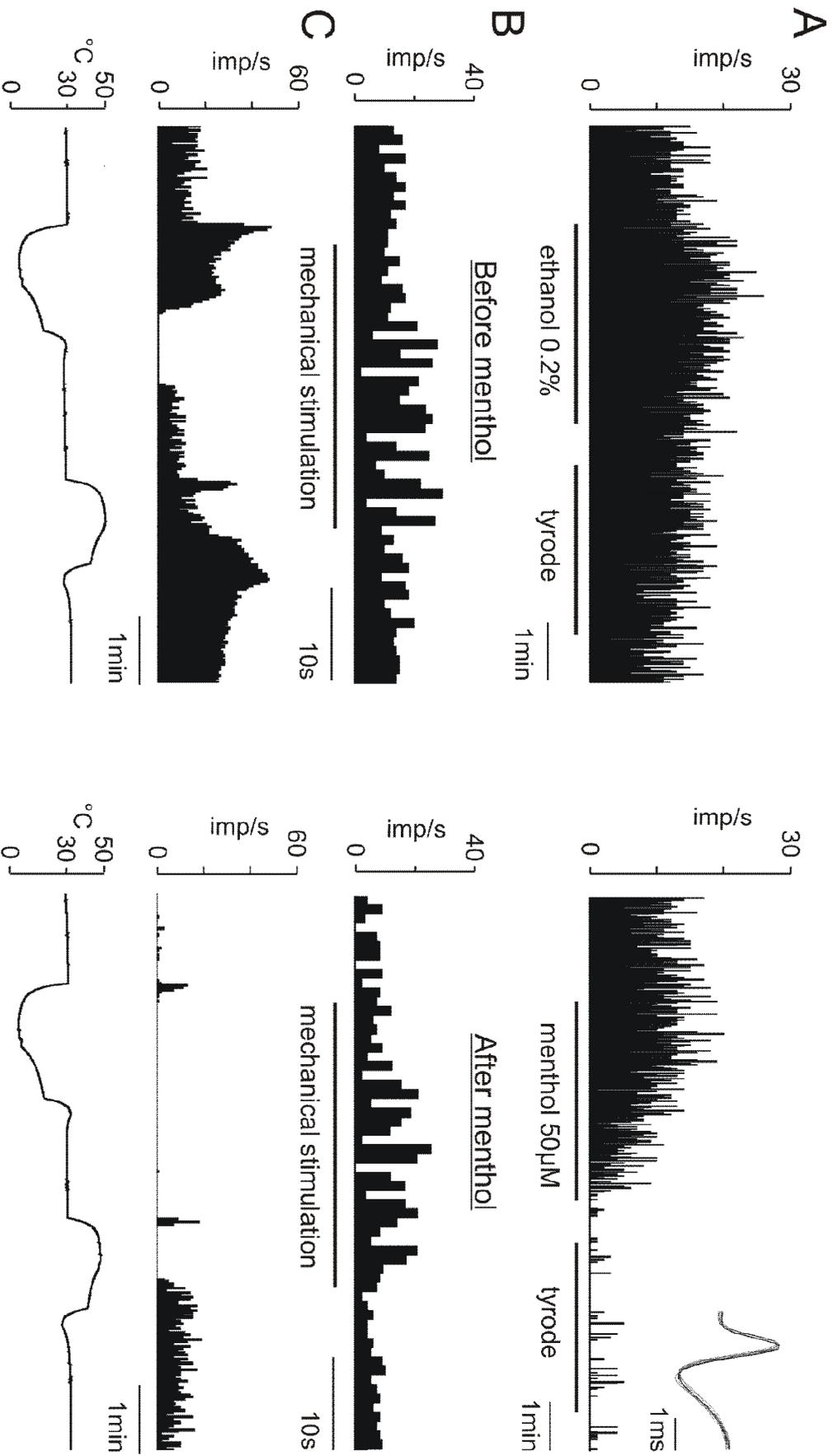


Fig. 3.43. Depression of spontaneous activity of an injured A β -fiber by menthol (50 μ M). The fiber was mechanosensitive, cold-sensitive and heat-sensitive. It showed a small activation during application of vehicle (ethanol 0.2%) and was almost completely depressed during application of menthol (A). The response to mechanical stimulation was not depressed by menthol (B). The responses to a cold (5°C) or a heat stimulus (48°C) were depressed after application of menthol (C). The temperature stimuli started from a preset temperature of about 30°C.

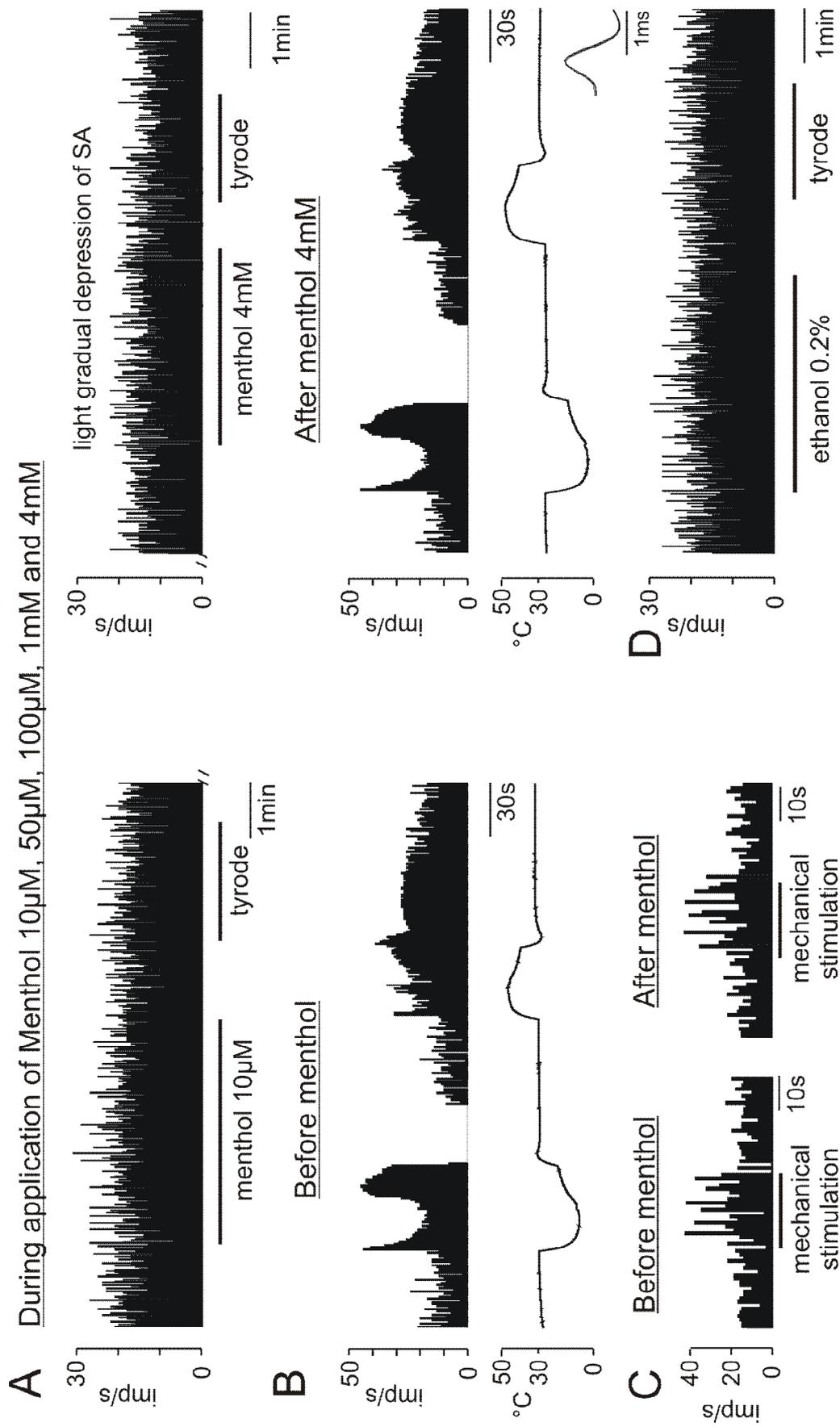


Fig. 3.44. Original record of an injured A β -fiber showing no response to menthol (no change group). The fiber was mechano-, cold- and heat-sensitive with a high rate of spontaneous activity and low thresholds to cold- and heat stimuli. It showed very small gradual depression of spontaneous activity during application of Menthol 10 μ M, 50 μ M, 100 μ M, 1mM and 4mM (A, application of 50 μ M, 100 μ M, 1mM not shown), no sign of sensitization or desensitization after the application of menthol (B, C) and no reaction on vehicle (ethanol 0.2%, D).

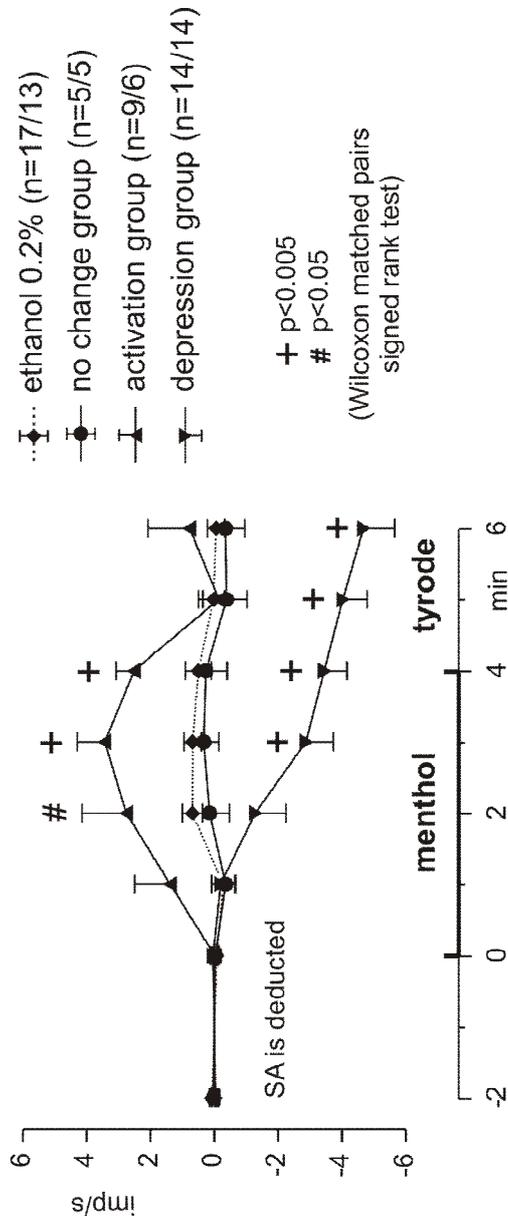
mechano-cold-heat-sensitive A-fiber with a low rate of SA (activation group). The fiber was activated during application of a very low dose of menthol (5 μ M) and showed graded response to higher doses of menthol (50 μ M, 100 μ M, 1mM, 2mM and 4mM). This evoked activity was completely suppressed by the highest menthol concentration applied (4mM). After application of menthol the cold response was decreased and the heat response was strongly increased, however the mechanosensitivity remained unchanged. We recorded 7 fibers of such type: 4 were spontaneously active, 3 silent (Fig 3.41).

The depression group contained spontaneously active cold-sensitive A-fibers, which after short activation during the first minute of menthol application showed a dramatic depression of their activity or were only depressed in their SA; these fibers were desensitized for cold stimuli by menthol. Figure 3.43 shows the responses of a typical mechano-cold-heat-sensitive A-fiber with a middle rate of spontaneously activity (SA) (depression group) to menthol and to mechanical, cold and heat stimulation before and after menthol application. The response to menthol was an almost complete depression of the SA of the fiber. After application of menthol the cold and heat responses were strongly decreased, however the mechanosensitivity remained unchanged. Note that vehicle (0.2% ethanol solution) caused a small activation of this injured myelinated fiber, but no depression. This ethanol-sensitivity of the injured muscle afferents is described in the next subchapter 3.2.3.

The “no change” group contained fibers with a high rate of SA, which were not affected by menthol. Figure 3.44 depicts a mechano-cold-heat-sensitive A-fiber with a high rate of SA. During application of different doses of menthol it showed a small gradual depression of SA. After application of menthol the fiber neither changed its threshold to the cold and heat stimuli, nor the magnitude of the response to the mechanical stimulation.

The activity of the cold-sensitive A-fibers, separated into the depression, activation and “no change” group, before, during and after application of menthol or application of vehicle (0.2% ethanol solution) to the nerve is shown in the figure 3.45A. The data is normalized with respect to menthol concentration. In the activation group the increase of the activity during application of menthol was significantly higher when compared to the activity during application of vehicle starting in the 2nd minute (#, $p < 0.05$; +, $p < 0.005$; Wilcoxon matched pairs signed rank test). The response was dose-dependent in the low range of the applied menthol concentrations (Fig 3.42). High concentrations of menthol (4mM) mostly depressed the evoked activity (Fig. 3.43). In the depression group the decrease of activity was dose dependent (Fig. 3.45B) and significant (Fig. 3.45A) starting in the 3^d minute during application of menthol. After removal of menthol the activity was still decreased (and in some

A Responses to menthol for the three groups of A-fibers



B Depression group over various doses

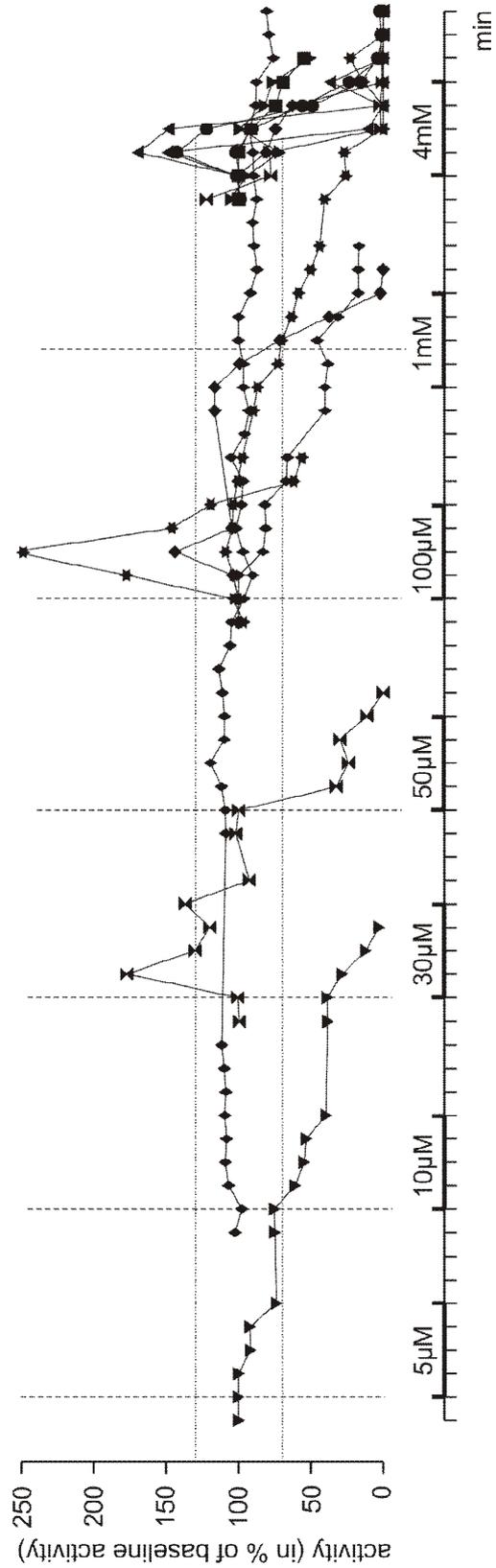


Fig. 3.45. Responses of injured cold-sensitive A-fibers to application of menthol to their injury site in the nerve. A. Mean + SEM of the activity of the whole population of the cold-sensitive A-fibers before, during and after application of menthol: activation group (normal triangles, n=9), no change group (circles, n=5) and depression group (inverted triangles, n=14). Numbers of fibers with SA are given after the slash. The rate of SA was subtracted from the response. Dotted line: activity in 17 cold-sensitive A-fibers before and during application of vehicle (0.2% ethanol). Temporal resolution is 1 min. B. Responses to the application of various doses of menthol to individual A-fibers of the depression group (n=14).

cases completely depressed) and significantly lower when compared to the activity after application of vehicle (+, $p < 0.005$; Wilcoxon matched pairs signed rank test). In the “no change” group the decrease of SA was not significantly different compared to the activity during application of vehicle ($p > 0.3$; Wilcoxon matched pairs signed rank test).

About 10 to 20 minutes after application of menthol, the injured muscle A-fibers were tested again for their responses to thermal and mechanical stimuli applied to the receptive fields in the nerve. The population responses separated for the three groups of A-fibers to cold and heat stimuli applied to the nerve before (black circles) and after application of menthol (black squares) are shown in figure 3.46. The spontaneous activity (SA) was not subtracted in order to show the differences among the groups.

- A-fibers from the activation group (no SA or low rate of the initial SA) showed desensitization of their cold responses and increase of their heat responses after application of the menthol. Only few fibers in this group had SA; therefore it was difficult to judge the changes of the rate of SA.
- A-fibers from the depression group (middle rate in the initial SA) significantly decreased the level of their SA after application of menthol ($p < 0.005$; Wilcoxon matched pairs signed rank test). Additionally their cold response was decreased.
- In the group of the A-fibers with high rates of SA there were not observed any significant shift in the level of the activity and responses to the cold or heat stimulus.

In general, cold response of the whole population of the cold-sensitive A-fibers was significantly decreased when compared with the cold response before application of menthol (#, $p < 0.05$, Wilcoxon matched pairs signed rank test) (Fig. 3.47). The thresholds of the activation to the cold and heat stimuli were significantly shifted to the higher range after application of menthol 4mM (Fig. 3.48A and B). Some fibers even completely lost their cold or heat sensitivity after menthol 4mM. The activation to mechanical stimulation was not significantly changed after application of menthol (Fig. 3.48C).

In summary, most injured cold-sensitive C-fibers are activated and sensitized for cold stimuli by menthol. Most injured cold-sensitive A-fibers are depressed in their activity by menthol and desensitized for cold stimuli.

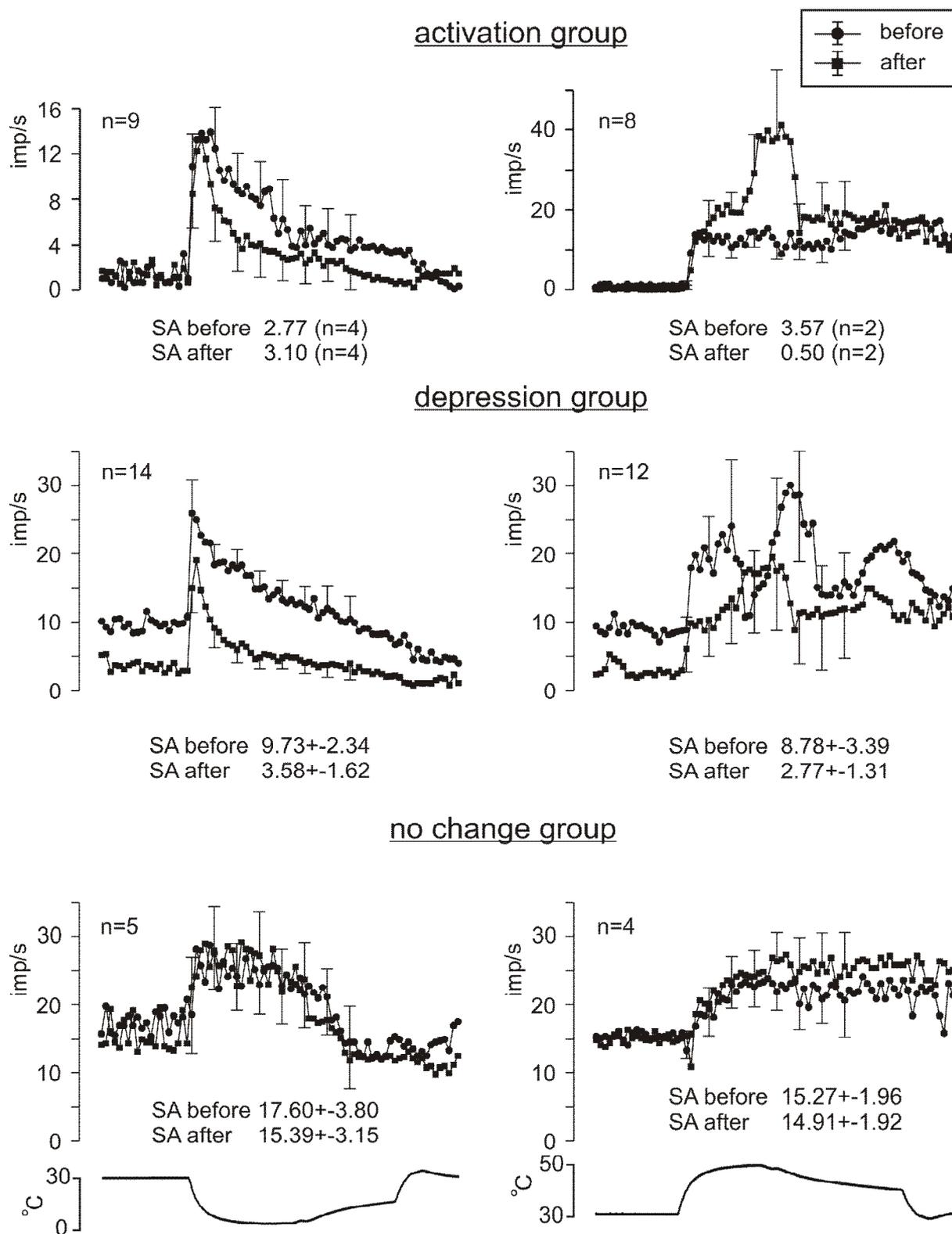


Fig. 3.46. Population responses to cold and heat separated for the three groups. Responses to cold stimuli of 5°C (left part) and to heat stimuli of 48°C (right part) before application of menthol (circles) and after application of menthol (squares). Spontaneous activity (SA) is not deducted in order to show its decrease after application of menthol. Inset: rates of spontaneous activity before and 30 min after application of menthol (mean ± SEM, imp/s). The temperature stimuli started from a preset temperature of about 30°C. Temporal resolution is 1s.

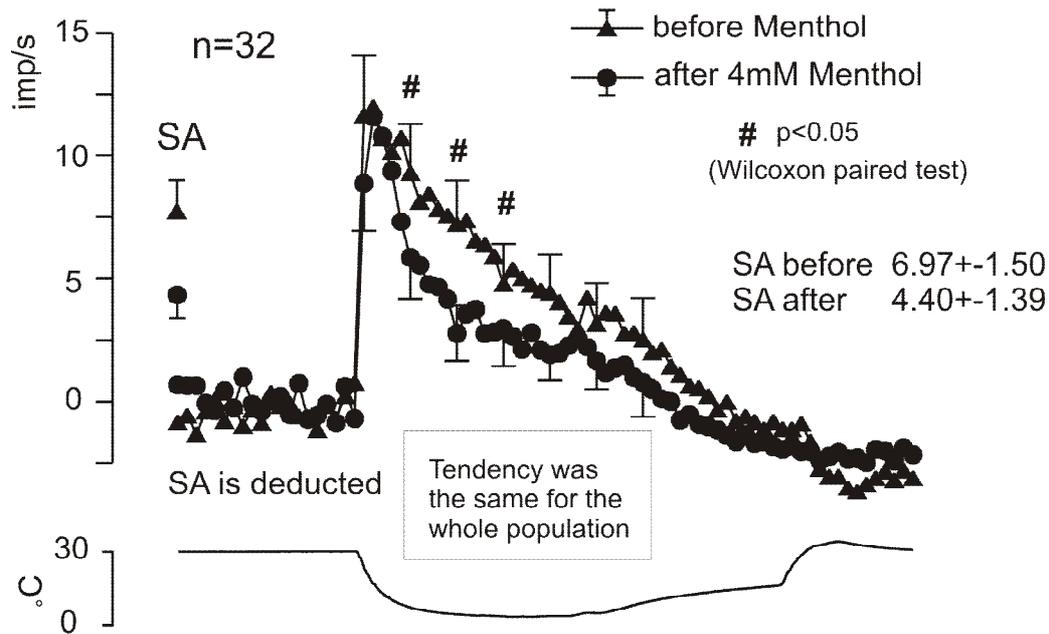


Fig. 3.47. Responses to cold stimuli of 5°C of the whole population cold-sensitive A-fibers (32 fibers) before application of menthol 4mM (triangles) and after application of menthol 4mM (circles). The rate of spontaneous activity (SA) was subtracted from the response. Inset: rates of SA before and after application of menthol (mean ± SEM, imp/s). # p<0.05 (Wilcoxon matched pairs signed rank test). The temperature stimuli started from a preset temperature of about 30°C. Temporal resolution is 1s.

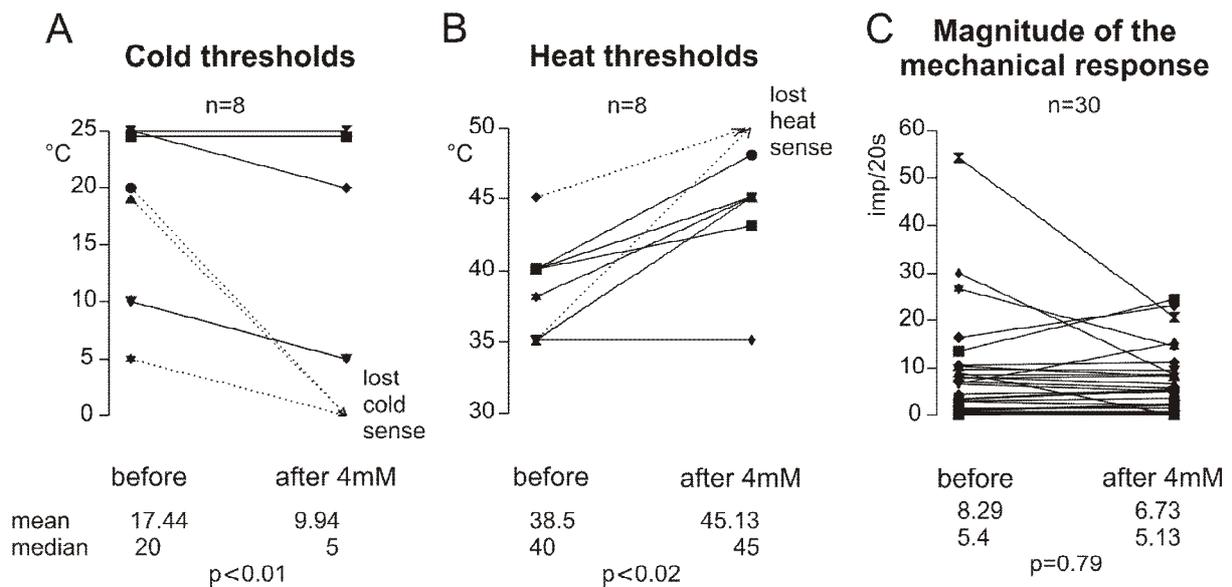


Fig. 3.48. Cold thresholds (A), heat thresholds (B) and magnitude of response to mechanical stimulation (C) of A-fibers before and after application of menthol (4 mM) to their receptive fields in the nerve. Mechanical stimuli were applied by a fine blunt glass rod repetitively to the injury site of the nerve over 20 s at about 1 Hz. Ordinate scale is in imp/s over the stimulation period of 20 s. Wilcoxon matched pairs signed rank test.

3.2.3 TRPA1 channel agonist mustard oil

The hypothesis of this study was to test whether the TRPA1 agonist mustard oil (MO) activates injured muscle cold-sensitive afferent nerve A- and/or C-fibers but not heat- and/or mechanosensitive one's. The work was complicated because of the high water insolubility of this substance. High concentrations of ethanol or dimethyl-sulfoxide (DMSO) are required to get MO into solution. Based on the personal communication with Prof. P. Reeh it was initially decided to use in the investigation 10mM, 1mM, 100 μ M MO solutions in 10%, 1%, 0.1% ethanol, respectively. However, most muscle injured fibers were activated during application of vehicle (Fig. 3.49, 3.50). Therefore I tried to solve 10mM, 1mM, 100 μ M MO in 1%, 0.1%, 0.01% ethanol respectively. Whether MO was solved remained unclear.

In summary 7 experiments were conducted in this investigation. In every experiment before or after most applications of MO we tested the effect of the corresponding solution of ethanol (0.1%, 0.2%, 0.5%, 1%, 2%, 10%) applied on the lesion site of the nerve fibers (Fig. 3.51). Injured muscle afferents were activated during application of ethanol at concentration of > 0.5%. The myelinated fibers appeared to be more sensitive to the application of ethanol (Fig. 3.49, 3.50) than the unmyelinated fibers. In the A-fibers the increase of the activity

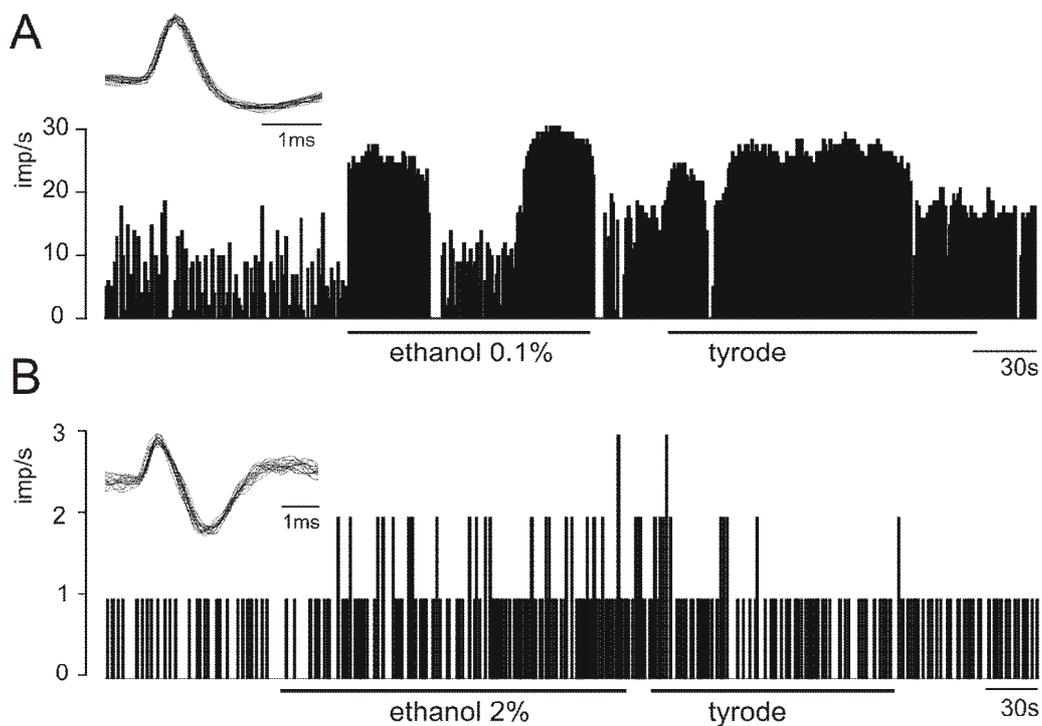


Fig. 3.49. Responses of an injured A- (A) and an injured C-fiber (B) to ethanol applied to the injury site. A. The myelinated fiber was already activated at a concentration of ethanol 0.1%. B. The unmyelinated fiber was activated by 2% ethanol.

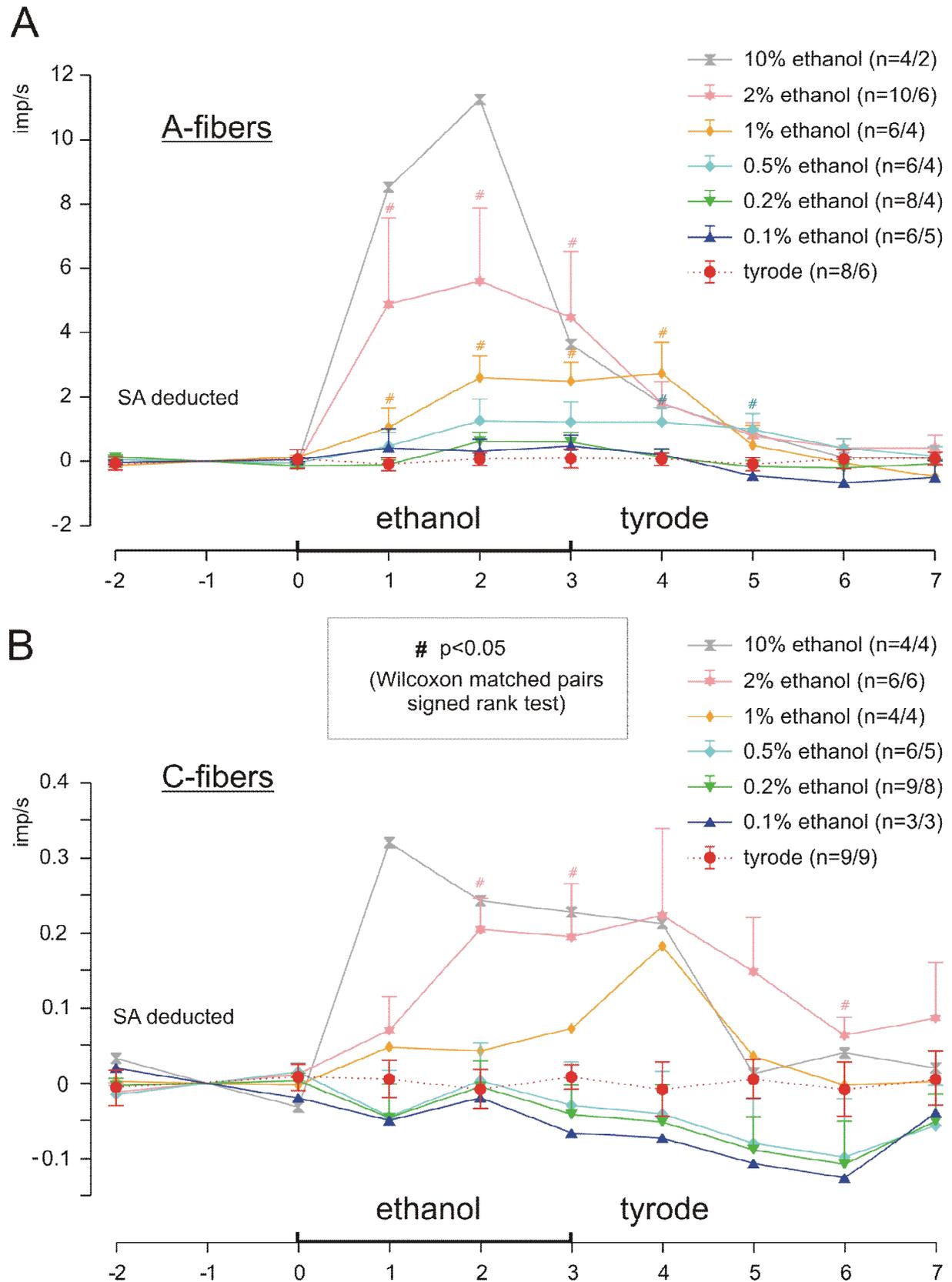


Fig. 3.49. Acute response of injured muscle A- (A) and C-fibers (B) during application of ethanol. Responses (mean + SEM) to application of ethanol to the nerve-injury site (0.1%, 0.2%, 0.5%, 1%, 2%, 10%) or vehicle (Tyrode's solution). The numbers of fibers with spontaneous activity (SA) are given behind the slash. The rate of SA was subtracted from the response. # p<0.05 (Wilcoxon matched pairs signed rank test).

during application of ethanol was dose-dependent and significant when compared to the activity during application of vehicle (Tyrode's solution) starting in the 4th minute during application of 0.5% ethanol and in the 1st minute during application of $\geq 1\%$ ethanol (Fig. 3.50A; #, $p < 0.05$, Wilcoxon matched pairs signed rank test). After removal of ethanol the activity recovered to baseline or slightly decreased. C-fibers were activated by ethanol starting at a concentration of 1% (Fig. 3.49B, 3.50B).

Moreover, it was noticed, that ethanol desensitized the afferent fibers for ethanol. Therefore, it was important to test fibers in the sequence "ethanol - MO". Finally MO strongly desensitized the afferent fibers for >100 minutes. Therefore it was not possible to study the dose-response functions of MO.

C-FIBERS:

19 C-fibers were tested to 100 μ M mustard oil (MO), solved in 0.1% or 0.01% ethanol. Most unmyelinated muscle fibers were insensitive to MO (17/19). Two cold-heat-sensitive neurons were activated by 100 μ M MO. Most neurons tested with MO were additionally examined for their reactions to the local application of menthol and/or capsaicin. Interestingly the same 2 cold-heat-sensitive neurons, which could be activated by MO, were excited by menthol as well as by capsaicin (Table 3.12).

C-fibers	N	Reaction to 100 μ M MO (0.1%* ethanol)	CAP-sensitivity	Menthol-sensitivity
Cold-heat-sensitive	6	2/6	4/4	5/5
Cold-sensitivite only	2	0/2	0/2	1/1
Heat-sensitive only	11	0/11	8/8	0/11
Total	19	2/19		

A-fibers	N	Reaction to 100 μ M MO (0.1%* ethanol)	CAP-insensitivity	Menthol-depression
Cold-heat-sensitive	15	7/15	3/3	6/9
Mechano-sensitivite only	4	0/4	n/t	0/4
Heat-sensitive only	3	0/3	n/t	n/t
Total	22	7/22		

Table 3.12. Numbers of functional types of afferent neurons activated or not activated by mustard oil (MO), capsaicin (CAP) or menthol. n/t, not tested. * In half of the cases 100 μ M MO was solved in 0.01% ethanol.

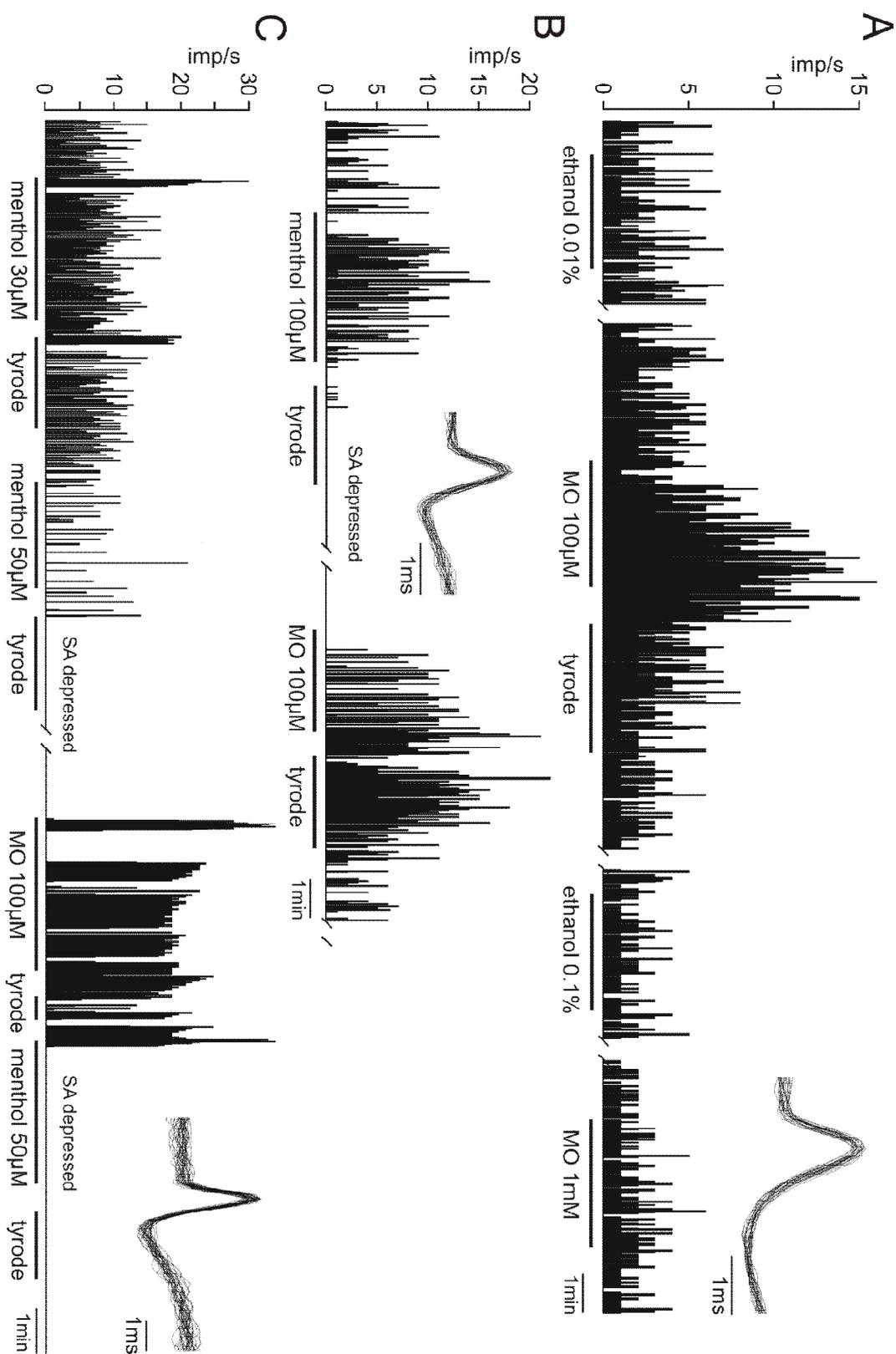


Fig. 3.51. Responses of three injured mechano-cold-heat-sensitive A-fibers to mustard oil (MO) applied to the injury site. A. Fiber activated by the application of 100 μ M MO. The response of the fiber to the second application of MO was much weaker. B and C. Two examples of typical A-fibers, in which the spontaneous activity (SA) was depressed by the application of menthol, but the same fibers were excited by the application of MO. Initial short activation in C is due to low threshold mechano-cold-sensitivity of the fiber.

A-FIBERS:

22 A-fibers, showing no response to application of vehicle (0.1% or 0.01% ethanol), were examined for their responses to local application of 100 μ M mustard oil (MO) to the lesion site. Mechano- and/or heat-sensitive myelinated fibers were not excited by the application of MO. About half of the cold-sensitive myelinated fibers (7/15) showed acute activation to 100 μ M MO (Table 3.12., Fig. 3.51). These acute reactions were large, but not long lasting. Due to desensitization the activation by MO could not be reproduced (Fig. 3.51A).

Four fibers which were activated by MO were tested for their reaction to menthol before application of MO. The spontaneous activity (SA) of these fibers was depressed by local application of menthol. MO reactivated these fibers after menthol (Fig. 3.51B and C). Comparison of the population responses of the injured cold-heat-sensitive A-fibers to heat and cold stimuli applied to the nerve before and after application of MO 100 μ M did not show any significant differences.

Thus, this study indicates that most unmyelinated injured muscle afferents are unresponsive to MO, whereas many myelinated fibers are activated and strongly desensitized by MO.

3.3. Responses of injured muscle afferents to electrical stimulation of the sympathetic supply (group C)

In 17 rats with chronic GS nerve lesion (4-7 days after nerve injury) the injured afferent muscle fibers were investigated for their responses to activation of postganglionic axons by electrical stimulation of the sympathetic chain (group C). The goal of this study was to test whether the spontaneous activity, mechano- and thermo-sensitivity of the injured muscle fibers can be changed by activation of the sympathetic innervation, thus to prove or disprove the presence of the sympathetic-afferent coupling 4-7 days after lesion of the muscle nerve.

Fine filaments were recorded from the dorsal root L5 or L4 (recording 1, Fig. 2.2). At the beginning of every experiment the strength of sympathetic chain stimulation (SCS) at 2-10 Hz to obtain a maximal decrease of relative blood flow through the plantar skin, using laser Doppler flowmetry, was determined. This strength was used throughout the rest of the experiments. The direct activation of vasoconstrictor neurons generated an increase of peripheral resistance and as a consequence – change in the arterial blood pressure (BP),

recorded in parallel: mostly increase of the BP (4 - 20%; Fig. 3.52A, 11 experiments) or decrease of BP (6 - 17%; Fig. 3.52B, 4 experiments).

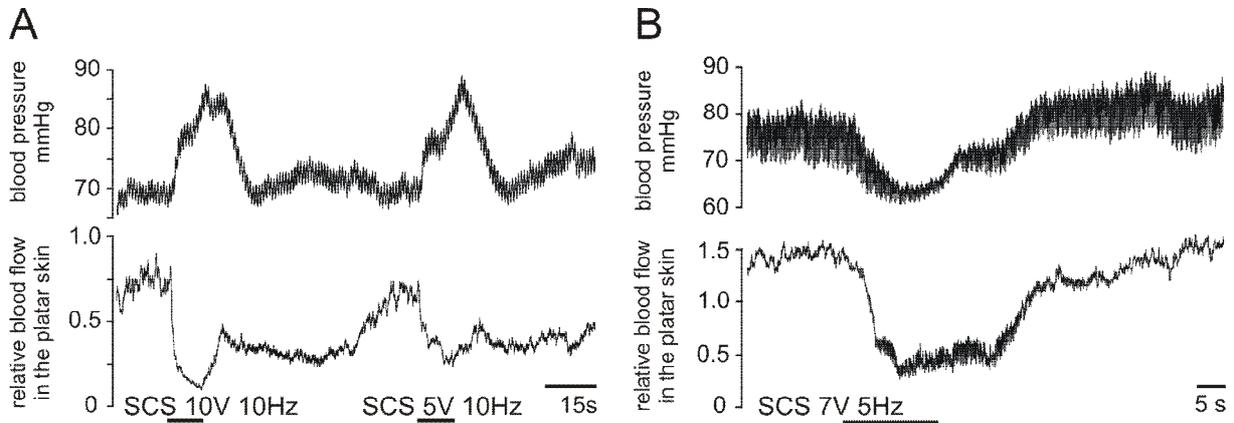


Fig. 3.52. Simultaneous measurement of the arterial blood pressure (mmHg) and relative blood flow within the L5 innervation territory on the plantar skin of the left hind paw before, during and after repetitive electrical stimulation of the sympathetic chain stimulation (SCS).

In total, 38 A-fibers and 25 injured muscle C-fibers were tested. Table 3.13 shows the numbers of injured A-fibers activated or depressed by SCS. Activity of 11 out of 38 tested A-fibers could be modified by SCS (29%). Most A-fiber neurons with spontaneous activity (SA) and very few A-fibers without SA increased their activity (Fig. 3.53A1 and A2) or enhanced their response to the mechanical (Fig. 3.53B) or thermal stimuli (Fig. 3.53C) during STS. Activation of the injured A-fibers required high-frequency stimulation of the sympathetic neurons at 5 to 10Hz.

Numbers of tested A-fibers	Numbers of fibers changed by SCS	Spontaneous activity change	Mechano-sensitivity change	Thermo-sensitivity change
38	11	7↑	3↑	1↑

Table 3.13. Numbers of injured A-fibers activated by sympathetic chain stimulation (SCS). The most common reaction on SCS was an increase of the rate of the spontaneous activity and a lowering of mechanical and thermal threshold.

Injured unmyelinated muscle nerve fibers were not activated by SCS in this study (0/25). One C-fiber was activated by SCS. However, this fiber did not project in the injured muscle nerve and could not be identified by physiological (mechanical or thermal) stimuli (Fig. 3.54).

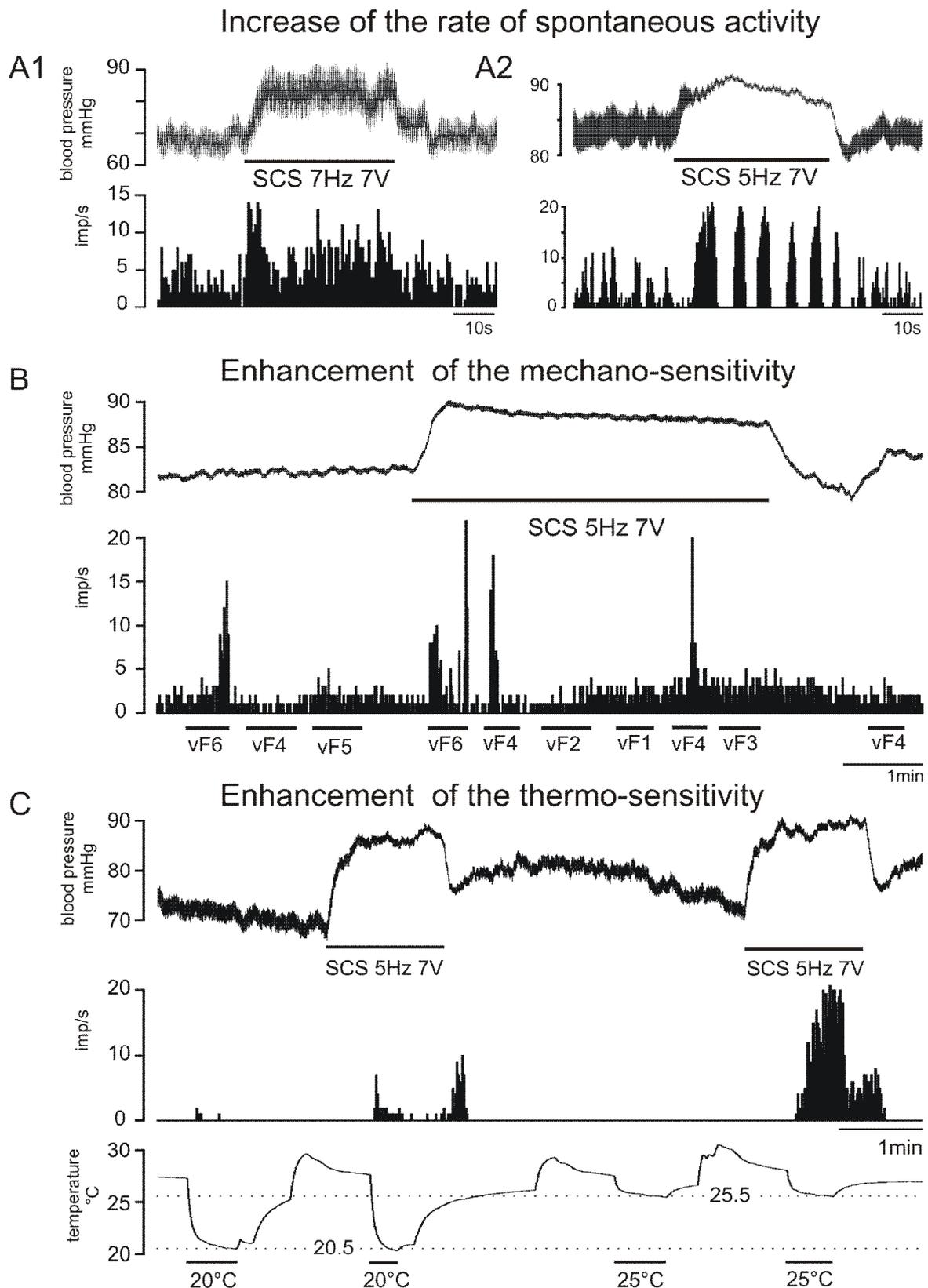


Fig. 3.53. Characterization of the injured myelinated fibers by their ectopic excitability and the activation thresholds in relation to the sympathetic chain stimulation (SCS). A1 and A2. Activation by SCS. B. Change of the activation threshold to mechanical stimuli. Before SCS the mechanical threshold was von Frey filament (vF) №6 (7.5 mN); during SCS the threshold was vF №4 (1mN). C. Decrease of the activation threshold to the cold stimulation by SCS.

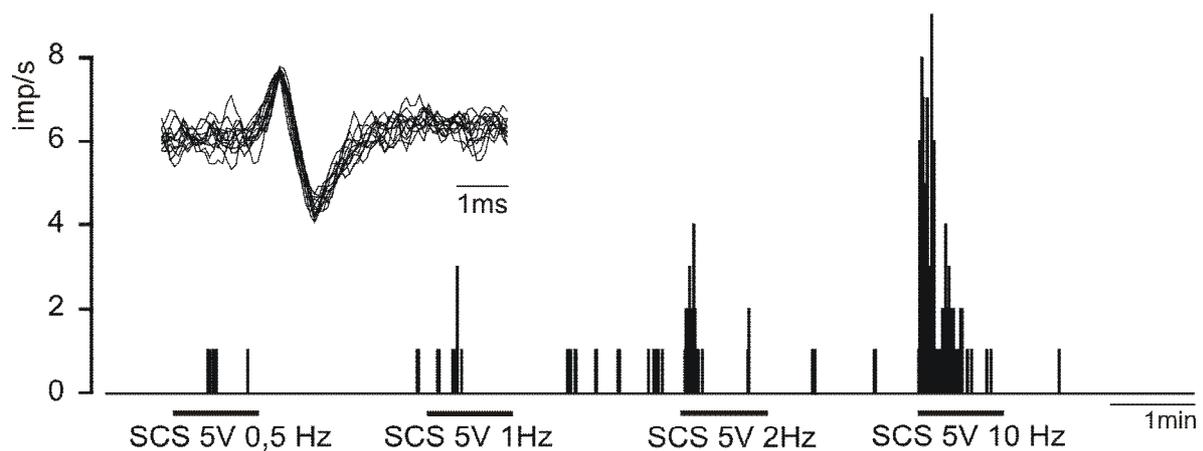


Fig. 3.54. Excitation of an unmyelinated afferent fiber by electrical stimulation of the sympathetic chain (SCS). The multiunit filament contained 2 unmyelinated fibers. One C-fiber was spontaneously active and mechano-heat-sensitive, could be identified by electrical stimulation of the lesioned gastrocnemius-soleus nerve, but was not activated by SCS (not shown). The second C-fiber (shown here) was irregularly spontaneously active and activated by SCS at 0.5-10 Hz. The fiber was not activated by electrical or physiological stimulation of the lesioned gastrocnemius-soleus nerve.

3.4. Afterdischarge to electrical stimulation of the muscle nerve (groups D1 and D2)

During identification in the unmyelinated fibers range to electrical stimulation of the gastrocnemius soleus nerve (1-40V, 0.3-1Hz, 0.5ms) some 20% myelinated muscle afferent fibers (for which this strength of the stimulation was 10 – 100 times suprathreshold) exhibited afterdischarges (AD) at a very high rate. In this section the characteristics of this afterdischarge and its selective blockade by lidocaine are described.

3.4.1. Proportions of fibers with afterdischarge before and after nerve injury

In total, from 72 electrically identified intact muscle myelinated afferents 27 could be activated by light stretching the gastrocnemius muscle (Table 3.14). 17 of them exhibited AD on suprathreshold stimulation of the muscle nerve (STS). Most fibers were activated by STS, three fibers showed depression of the activity during STS (Fig. 3.55A1 and A2). With one exception (weak activation during STS; Fig. 3.55B) all unmyelinated fibers tested (0/58 intact C-fibers [no lesion]; 0/103 injured C-fibers [0-12 hours after nerve injury]; 1/184 injured C-fibers [4-7 days after nerve injury]) did not exhibit AD.

	Number of electr. identified fibers	Number of physiol. activated fibers	Number of fibers with AD	Peak discharge rate, imp/s	Range of the duration of the discharge, s	Mean rate of the whole discharge, imp/s
no lesion	72	27/14 (37.5%)	17/8 ¹ (23.6%)	76.7 ± 17.5 (17.6 – 132.9)	39.4 ± 30.5 (3.9 – 222.4)	33.9 ± 8.5 (5.5 – 68.0)
0 - 4 hours after crush	60	2/1 (3.3%)	4/1 (6.7%)	40.8 ± 5.3 (34.0 – 51.3)	5.9 ± 0.6 (4.9 – 7.0)	22.4 ± 2.9 (16.5 – 25.6)
4 - 12 hours after crush	278	14/6 (5.0%)	40/3 (14.4%)	42.7 ± 6.5 (2.4 – 144.9)*	18.5 ± 5.7 (2.4 – 163.7)	18.5 ± 4.3 (0.2 – 125.3)
4 - 7 days after crush	95	51/23 (53.7%)	18/9 (18.9%)	34.9 ± 8.8 (4.5 – 111.4)*	21.1 ± 8.2 (2.0 – 90.1)	22.6 ± 6.4 (1.6 - 76)

¹ Three additional fibers were depressed in their activity probably because of contraction of extrafusal musculature

Table 3.14. Numbers of intact and injured (crush lesion of the gastrocnemius soleus [GS] nerve) muscle A-fibers tested for afterdischarge (AD) generated by suprathreshold electrical stimulation (STS) of the GS nerve. First column: total numbers of electrically identified fibers in every tested time period. Second column shows numbers and percentages of fibers with spontaneous activity and/or activity evoked by mechanical or thermal stimuli activity. Third column: numbers and percentages of fibers showing afterdischarge to STS. The numbers behind the slash indicate the numbers of fibers with spontaneous activity (SA) (included in the number before the slash). The percentages are expressed in respect to the total number of electrically identified fibers. Last three columns: qualitative description of the discharge peak rate, range of duration and mean rate of the whole discharge (mean ± S.E.M., range). * $p < 0.05$; Wilcoxon matched pairs signed rank test.

In most myelinated fibers the AD started with the first stimulus of the train (5 stimuli 30V 1Hz 0.5ms), increased from the first to the fifth stimulus and lasted 4 - 222.4 seconds after the stimulus train (Table 3.14). The peak of activation usually came at the end of the stimulus train; lasted about 3 seconds (2.6 ± 0.8 s, not in the table) and reached up to 130 Hz (17.6 – 132.9 Hz). The thresholds of activation ranged from 4 to 14 Volts. The response was strength-dependent (Fig. 3.56).

After nerve injury the ability of A-fibers to exhibit long-lasting high-rate afterdischarges in response to STS is quantitatively and qualitatively smaller than before nerve injury. Particularly in the first 4 hours after crush injury of the nerve only 7% of all tested A-fibers showed AD. Figure 3.57 shows a long lasting testing of a mechanosensitive A-fiber for AD before and after crush lesion of the nerve. The AD of the fiber had an activation threshold of 8 Volts. Crush lesion activated the fiber. Immediate application of STS after nerve injury elicited only weak AD. Two hours after crush injury the fiber could again be excited by STS, but much weaker in comparison to the initial AD.

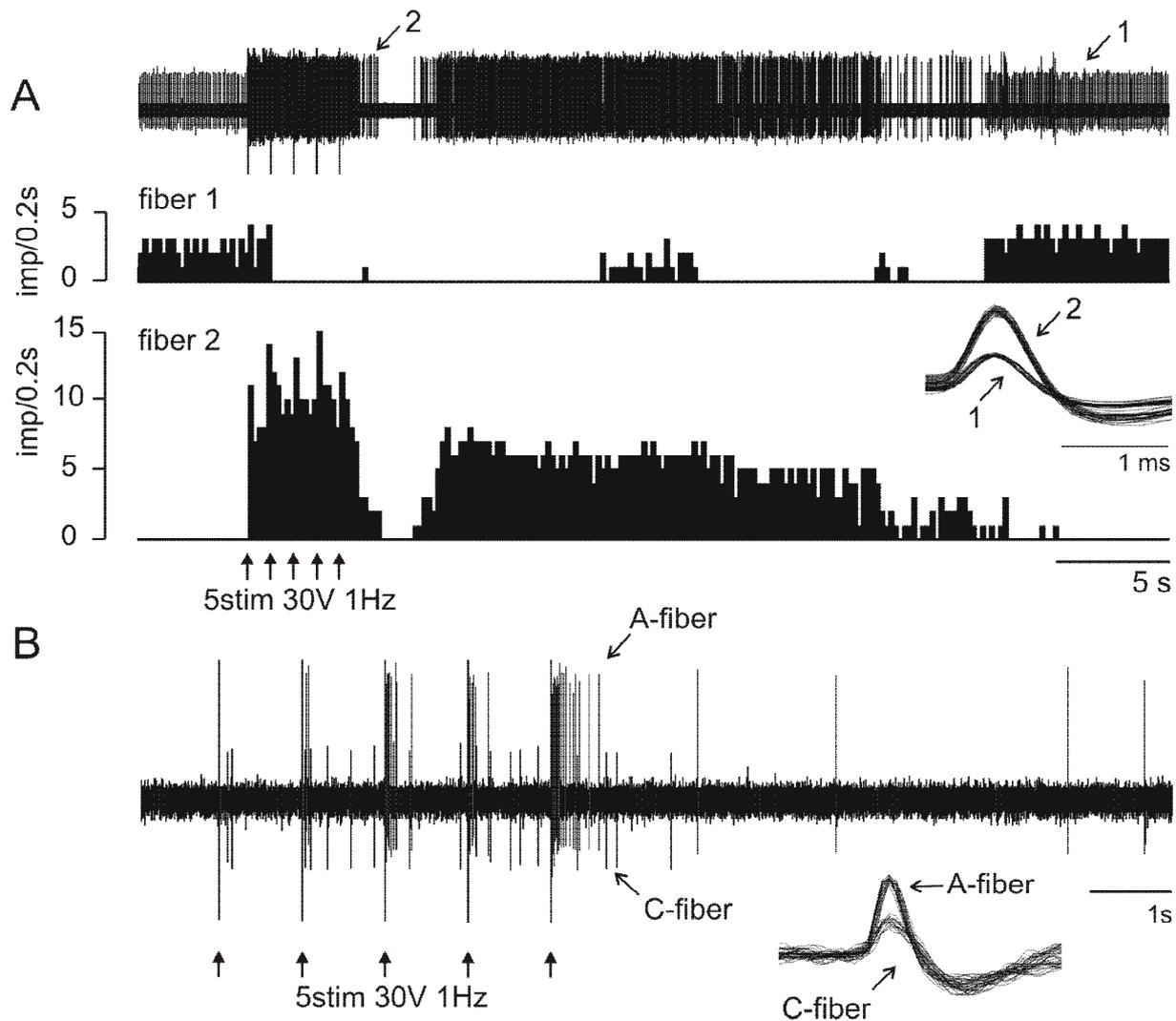


Fig. 3.55. Original records of intact (A) and injured (B) muscle afferent fibers before, during and after suprathreshold stimulation (STS) of the muscle nerve. A. Simultaneous recording of two intact myelinated fibers in one filament. Fiber 1 was spontaneously active and was depressed by STS. Fiber 2 was not spontaneously active and exhibited afterdischarge (AD) in response to STS. B. Simultaneous recording of an A-fiber and a C-fiber in one filament 6 days after nerve injury. Both had weak AD in response to STS.

4-12 hours after injury of the muscle nerve 14% of the A-fibers exhibited AD. The incidence of AD was three times more frequent than the incidence of ectopic activity. 4 to 7 days after nerve injury 19% of the A-fiber had AD. However the peak rate of AD (* $p < 0.05$; Wilcoxon matched pairs signed rank test), the duration and mean rate of AD were smaller after injury in comparison to the responses of intact muscle A-fibers (Table 3.14).

Most chronically injured muscle A-fibers which exhibited AD had unstable irregular spontaneous activity showing spontaneous bursts with intraburst frequencies of up to 150 Hz (Fig. 3.20, B I and II). The responses of these A-fibers to thermal stimuli were not reproducible and were followed by irregular bursts (Fig. 3.20, B II).

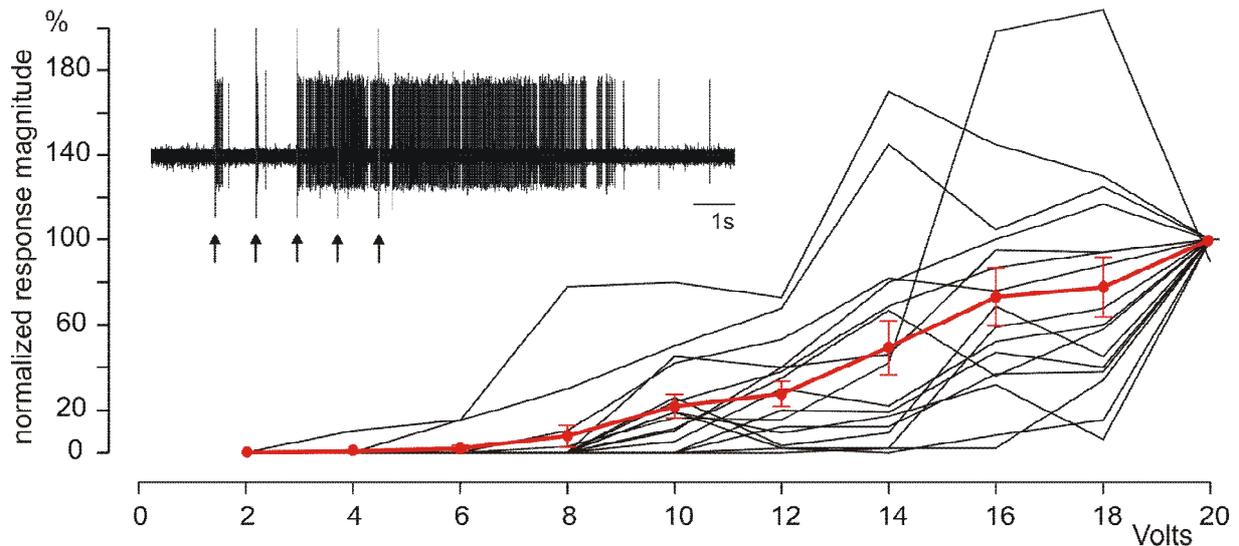


Fig. 3.56. Stimulus-response-functions of intact muscle A-fibers to suprathreshold stimulation (STS; 5 stimuli, 1Hz, 0,5ms, 2-20Volts) of the muscle GS nerve ($n=16$). The population response (mean \pm SEM) is shown in red and individual responses of single fibers in black thin lines. The magnitude of the response was measured during the first 10s after the first stimulus and then normalized in relation to the magnitude of the response during maximal stimuli of 20 Volt. The inset shows a typical response of an intact A-fiber to STS of the muscle nerve (5 stimuli, 30V, 1Hz).

3.4.2. Blocking of the afterdischarge by local application of lidocaine

In 10 experiments the hypothesis was tested whether the AD in muscle afferent fibers generated by electrical stimulation of the muscle nerve is dependent on the presence of sodium channels in the nerve fibers. To answer this question lidocaine (a sodium channel blocker) in concentrations of 0.001, 0.01, 0.1 and 1mg/mL (corresponding to solutions of approximately 0.004, 0.04, 0.4 and 4mM/L) was applied locally to the stimulation electrode site of the GS nerve for 4 min in the same way as described for the application of the TRP agonists (see 2.5.2). After the application lidocaine was washed out with Tyrode's solution and the response of the afferent fiber to STS of the nerve (5 stimuli, 30V, 0,5ms, 1Hz) was tested repeatedly every 30 min.

In total, 10 intact myelinated muscle fibers with AD were tested. In 3 fibers three concentrations of lidocaine were tested starting with the lowest dose of 4×10^{-7} M (Fig. 3.58). Lidocaine applied in this way neither caused a conduction block of the fibers, nor affected the rate of spontaneous activity of the fibers, but dose-dependently decreased the strength of the AD in the fibers to STS. After application of 4×10^{-4} M lidocaine the ability of fibers to exhibit AD was completely blocked, moreover STS now caused depression of the spontaneous activity of the fibers (Fig. 3.58, 3.59). With time the depression of spontaneous activity

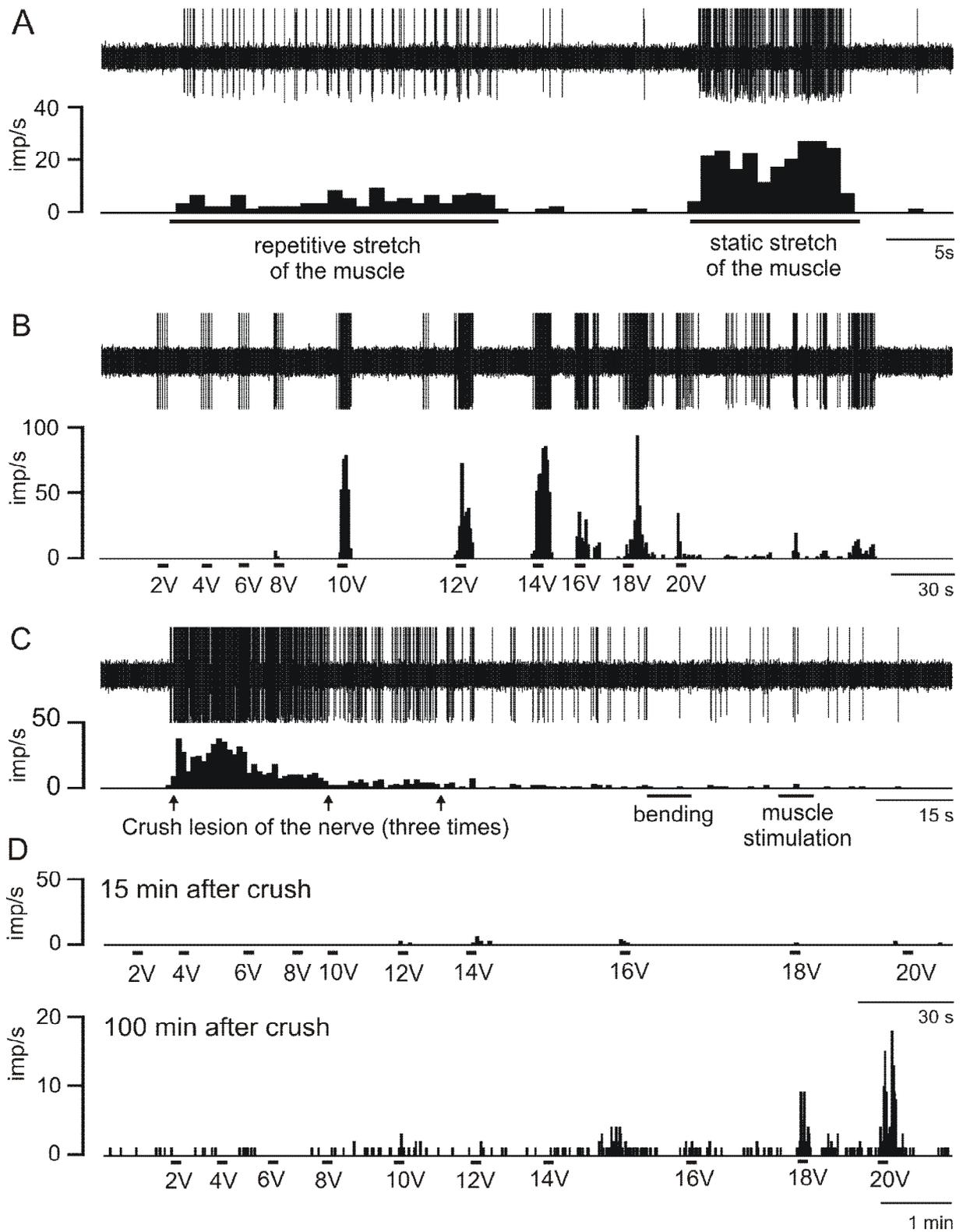


Fig. 3.57. Afterdischarge (AD) of a myelinated muscle afferent fiber before and after crush lesion of the gastrocnemius-soleus (GS) nerve. A. The A-fiber was activated by light stretching of the muscle. B. AD in response to suprathereshold stimulation (STS) of the muscle GS nerve. C. Acute firing in response to crush lesion of the GS nerve. D. STS of the muscle nerve 15 and 100 minutes after crush lesion.

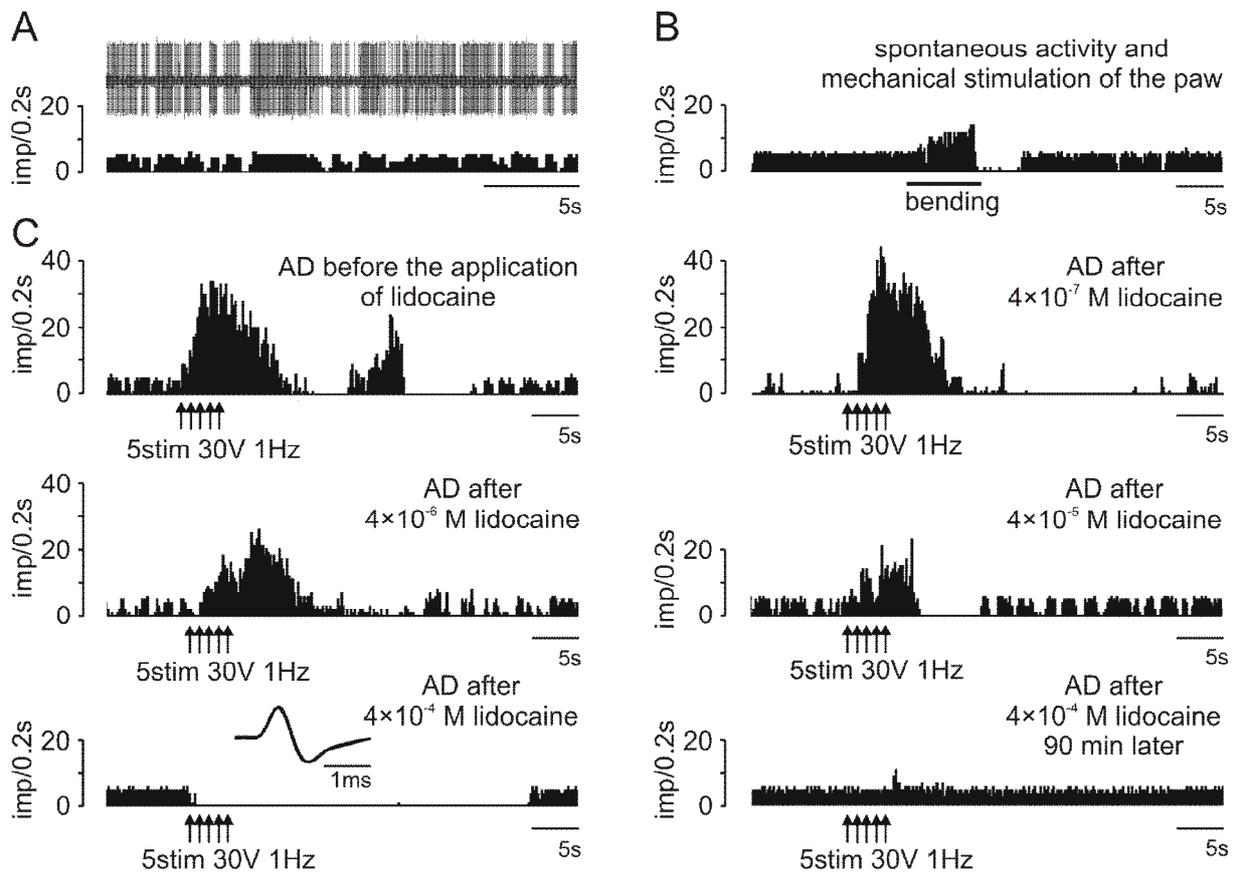


Fig. 3.58. Block of the afterdischarge (AD) of an intact muscle afferent A-fiber by local application of lidocaine, applied to the stimulation electrode site. A. Spontaneous activity. B. Response to bending of the paw. C. AD in response to the suprathreshold stimulation (STS; 5 stimuli 30V 1Hz 0.5ms) of the muscle GS nerve (C, first graph). Local application of lidocaine in ascending order (4×10^{-7} , 4×10^{-6} , 4×10^{-5} , 4×10^{-4} M) to the stimulation electrode site dose-dependently blocked the AD. After the application of 1mM lidocaine STS caused depression of the spontaneous activity of the fiber. This depression disappeared yet the AD could not be elicited ≥ 90 min after application of 4×10^{-4} M lidocaine.

became shorter. Up to 10 hours after the first local application of lidocaine to the stimulation electrode site myelinated fibers isolated from the GS nerve did not exhibit AD to STS. In one fiber 2×10^{-3} M lidocaine was tested (Fig. 3.60). At this high concentration of lidocaine the conduction of the fiber was blocked, but recovered 10 minutes later. The block of the afterdischarge to STS lasted throughout the experiment (≥ 120 min).

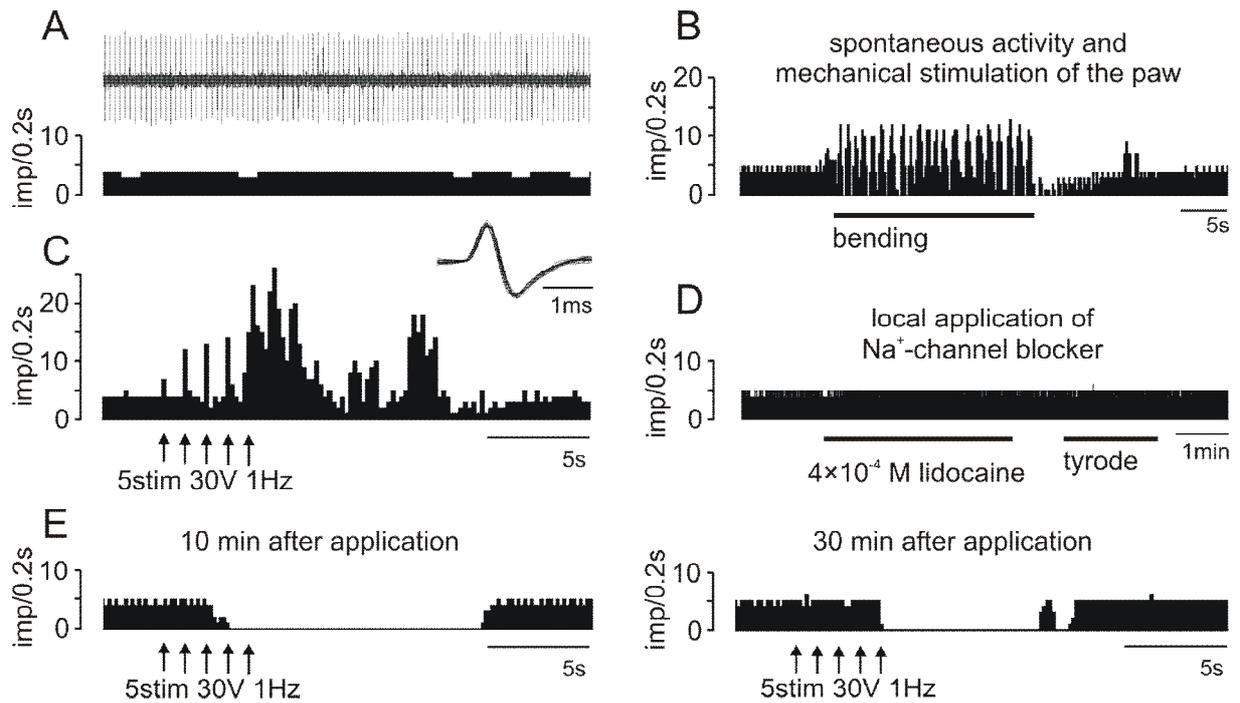


Fig. 3.59. Block of the afterdischarge (AD) of an intact muscle afferent A-fiber by local application of 4×10^{-4} M lidocaine to the stimulation electrode site. The fiber was spontaneously active (A) and activated on bending of the paw (B). This fiber exhibited AD in response to suprathereshold stimulation (STS; 5 stimuli 30V 1Hz 0.5ms) of the muscle GS nerve (C). D. Local application of 4×10^{-4} M lidocaine to the stimulation electrode site blocked the AD without affecting the spontaneous activity of the fiber (i.e. the conduction). E. Testing of the fiber for the AD 10 and 30 minutes after application of lidocaine. After application of lidocaine STS depressed the spontaneous activity of the fiber. This effect decreased with time.

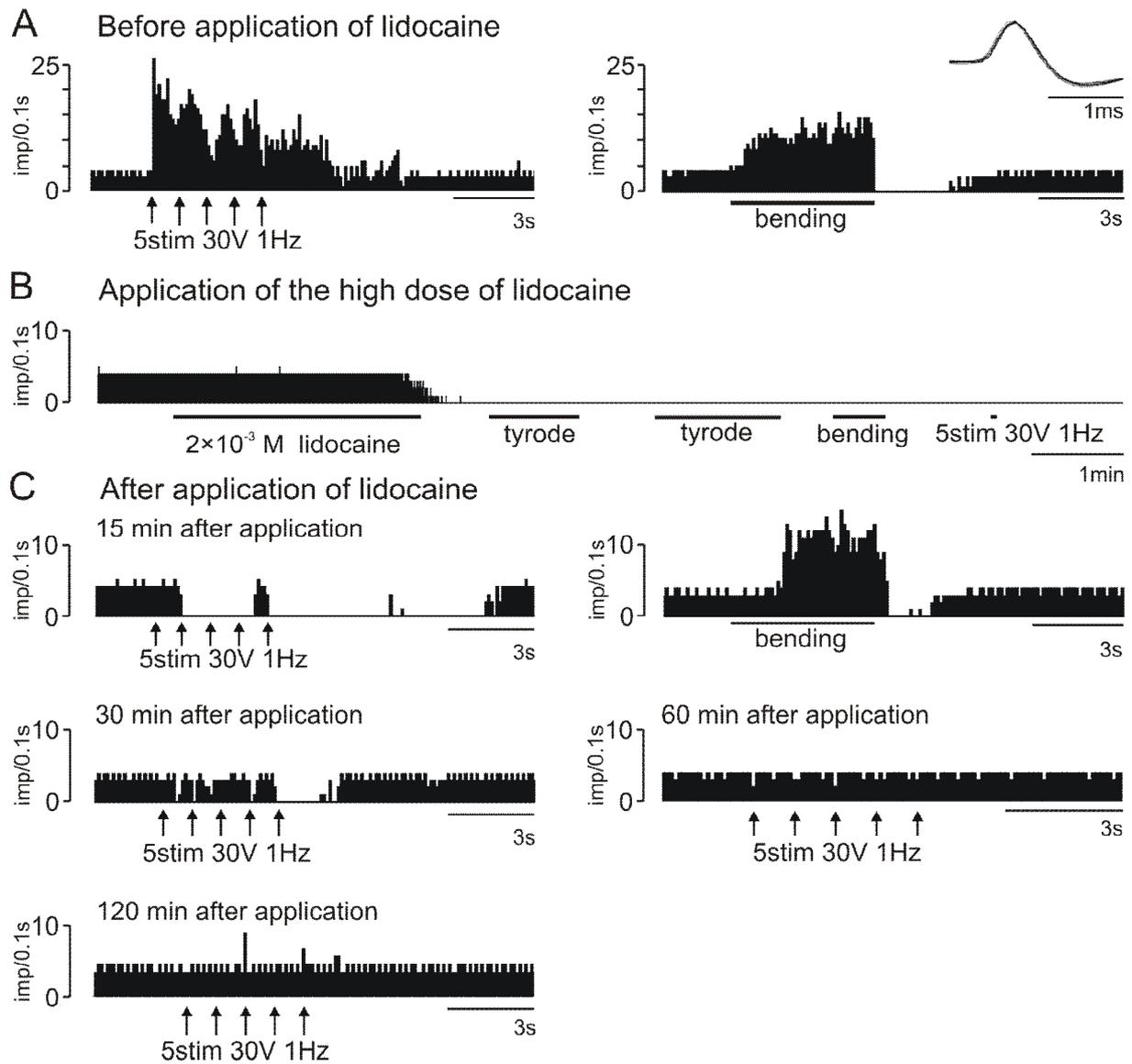


Fig. 3.60. Block of the afterdischarge (AD) of an intact muscle afferent A-fiber with spontaneous activity by local application of a high dose of lidocaine (0.5%, 2×10^{-3} M) to the stimulation electrode site. A. AD to electrical stimulation of the lateral GS nerve by 5 stimuli of 30 V at 1 Hz. Activation by muscle stretch generated by bending of the paw. B. Local application of 2×10^{-3} M lidocaine to the stimulation electrode site caused a conduction block, which recovered in 10 minutes. C. Testing of the fiber for the AD 15, 30, 60, 120 minutes after application of lidocaine. The AD did not recover for ≥ 120 min after application of lidocaine although the conduction fully recovered (see response to muscle stretch). The depression of the spontaneous activity elicited by STS lasted for about 40 min.

Chapter 4

Discussion

4.1. Ectopic sensitivity of the injured muscle afferents (group A1 and A2)

Here I have described for the first time the responsiveness of myelinated and unmyelinated muscle afferents to mechanical and thermal stimuli applied to the injury site of the nerve 4-13 hours (TP I) and 4-7 days (TP II) after crush lesion of the muscle nerve. The overall results are:

- Almost all afferent A- and C-fibers develop ongoing activity and/or mechano- and/or thermosensitivity.
- A high proportion of C-fibers but very few A-fibers show mechanosensitivity and/or thermosensitivity already hours after nerve injury.
- Mechanosensitivity is particularly prominent in A-fibers.
- A high proportion of both A- and C-fibers exhibits cold and/or heat sensitivity which is mostly combined with mechanosensitivity.
- Afferent A-fibers being thermo-sensitive only are rare, but afferent C-fibers being thermosensitive only are common.
- Ongoing activity is present in close to 50% of the A-fibers (in TP II) and in >50% of the C-fibers.
- The functional characteristics of A-fibers conducting at <20 m/s are significantly different from those of C-fibers.

4.1.1. Proportions of injured fibers with ectopic activity

About 50% of the myelinated fibers in the gastrocnemius-soleus nerve are somatomotor fibers (Mitchell and Schmidt 1983; Peyronnard et al 1986; Sittiracha and McLachlan 1986) and 45% of the unmyelinated fibers are postganglionic (Baron et al 1988). These motor fibers do not develop ectopic ongoing activity or responsiveness to physiological stimuli when injured (Schmelz et al 1998; Schmidt et al 1995, Struck 2012). Therefore, practically all injured muscle afferent A- and C-fibers show at least one type of activity elicited by mechanical or thermal stimulation or generated spontaneously (Table 4.1).

4.1.2. Functional response characteristics of injured and intact muscle afferents

In TPI most A-fibers are silent (Fig. 4.1B, blue columns). This phenomenon must be connected with the demyelination associated with shifts in the normal distribution of ion-selective channels. The main reason is that the inward Na^+ current provided by nearby nodes is not sufficient to overcome the capacitative and resistive load created by the now naked intermodal membrane (Devor 1984). Within a few days of experimental demyelination, old and newly synthesized functional Na^+ -channels redistribute and insert in the intermodal membrane and the fibers begin to conduct.

a. Mechanosensitivity:

Not unexpectedly, most injured A-fibers and 50-80% of the injured C-fibers were mechanosensitive when compared to the response properties of intact muscle afferents (Fig. 4.1, 4.2, blue columns; Mense 1993; Taguchi et al 2005). The high degree of mechanosensitivity of injured myelinated muscle afferents as reflected by their low threshold to mechanical stimulation with von Frey filaments and in their high stretch sensitivity (Fig. 3.4) has already been demonstrated by Johnson and Munson (1992) and Proske et al (1995). The high mechanosensitivity of afferent C-fibers in an injured muscle nerve is described here for the first time. The thresholds clearly show a bimodal distribution both in A- and C-fibers with modal values at 0.3 to 0.45 mN and 7.5 mN (Fig. 3.3C). Whether this is related to low threshold (non-nociceptive) and high threshold (nociceptive) mechanosensitive intact muscle afferents we do not know and needs to be studied.

Table 4.1

Comparison of injured muscle afferents (4-7 days after nerve crush) and injured cutaneous afferents (4-14 days after nerve crush)

	A-fibers			C-fibers		
	Muscle N. gastrocnem.-soleus lat. N=195	Skin N. suralis N=43	U-test or χ^2 -test	Muscle N. gastrocnem.-soleus lat. N=116	Skin N. suralis N=135	U-test or χ^2 -test
% efferent axons	50% somatomotor	a few somatomotor	##	49% postganglionic	17% postganglionic	##
% electr. activated fibers with ectop. act.	46%	42%	##	56%	49%	##
<i>Spontaneous activity</i> rate	0.01-38.8 i/s (9.5 i/s), N=96	0.4, 2.8, 6.4 i/s		0.02-7.0 i/s (0.32 i/s), N=65	<i>C1</i> : 0.12-10 i/s (2.2 i/s), N=28 <i>C2</i> : 0.1-1.9 i/s (0.3 i/s), N=50	ns
frequency	48.7%	7%	p<0.001	56%	57.7%	ns
<i>Mechanosensitivity</i> threshold frequency	0.3->10mN (1mN), N=76 90.8%	0.7-70mN (7.5mN), N=38 98%	p<0.001 ns	0.3->10mN (0.7mN), N=43 49.1%	0.45->70mN (10 mN), N=73 54%	p<0.001 ns
<i>Cold sensitivity</i> threshold frequency	<i>SA</i> : 10-27°C (22.5°C), N=16 <i>silent</i> : 5-26°C (12°C), N=36 63.1%	3-14°C (7°C), N=5 11.6%	p<0.001	5-20°C (15°C), N=36 38.8%	<i>C1</i> : 17-22°C (18.5°C), N=29 <i>C2</i> : 3-21°C (5°C), N=29 42.8%	ns
<i>Heat sensitivity</i> threshold frequency	<i>SA</i> : 35-50°C (40°C), N=14 <i>silent</i> : 35-50°C (45°C), N=12 51.8%	36-48°C (40°C), N=7 16.3%	p<0.001	35-50°C (41°C), N=35 67.2%	35-49°C (41.5°C), N=74 62.2%	ns ns

ns, not significant; range (median); SA, afferents with spontaneous activity; silent: silent afferents; i/s impulses/second;

type C1/C2: non-noxious/noxious cold sensitivity (see Grossman et al 2009, Jänig et al 2009). Data on cutaneous afferents from Jänig et al (2009). All percentages (frequencies of afferent fibers with a particular functional property) are expressed with respect to the total number of fibers.

The number of afferent fibers with ectopic activity is significantly higher in the injured muscle nerve than in the injured sural nerve since 50% and 49% of the axons in the muscle nerve are somatomotor or postganglionic, respectively, yet very few and 17% in the sural nerve.

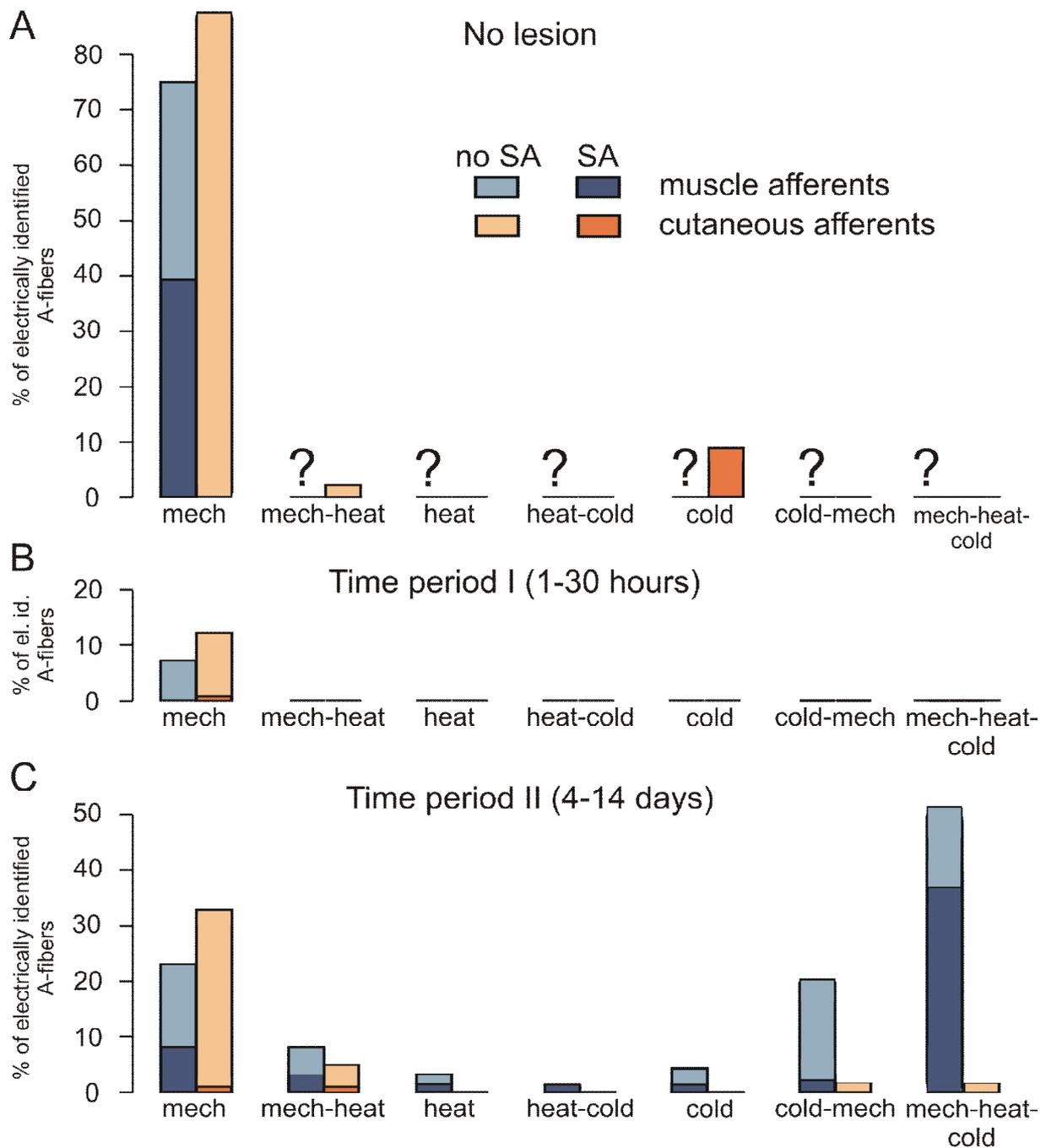


Fig. 4.1. Proportions of intact and lesioned myelinated muscle (blue columns) and cutaneous afferents (orange columns) with mechanosensitivity (mech), heat or cold-sensitivity or combinations of these sensitivities. Dark part of the column represents spontaneously active (SA) fibers, light part – fibers without SA. Numbers of somatomotor axons are deducted (muscle nerve, 50%). A. Intact fibers. Numbers of cutaneous fibers are taken from Jänig et al (2009). Numbers of mechanosensitive muscle A-fibers are taken from the study of group D1 and D2. The thermo-sensitivity of the intact muscle afferents is unknown. B. Time period I (1-13 hours after crush lesion [muscle nerve] and 1-30 hours [skin nerve]). Data on cutaneous afferents are taken from Michaelis et al (1995). C. Time period II (4-7 days after crush lesion [muscle nerve] and 4-14 days [skin nerve]). Data on cutaneous afferents are taken from Jänig et al (2009).

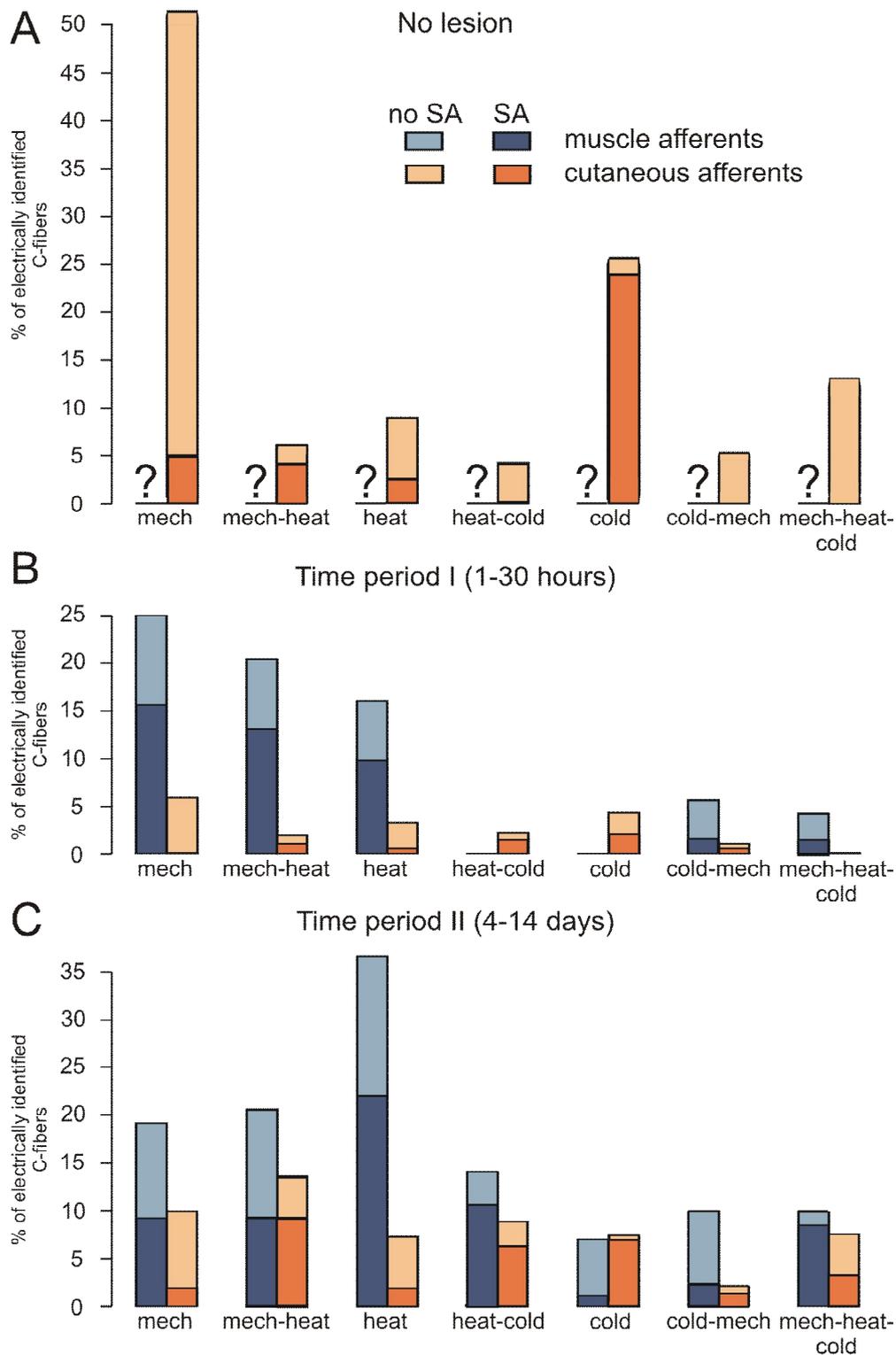


Fig. 4.2. Proportions of intact and lesioned unmyelinated muscle (blue columns) and cutaneous (orange columns) afferents with mechanosensitivity (mech), heat or cold-sensitivity or combinations of these sensitivities. Dark part of the column represents spontaneously active (SA) fibers, light part – fibers without SA. Numbers of postganglionic (muscle nerve 49%, skin nerve 17%, Baron et al 1988) are deducted. A. Intact fibers. Numbers of cutaneous fibers taken from Jänig et al (2009). Intact muscle C-fibers were not systematically investigated for their thermosensitivity and spontaneous activity (Table 1.1; for review see Hoheisel et al 2005, Mense 1993, Taguchi et al 2005). B. Time period I (1-13 hours after crush lesion [muscle nerve] and 1-30 hours [skin nerve]). Data on cutaneous afferents from Michaelis et al (1999). C. Time period II (4-7 days after crush lesion [muscle nerve] and 4-14 days [skin nerve]). Data on cutaneous afferents are taken from Jänig et al (2009).

b. Thermosensitivity:

The high frequency of cold and heat sensitivity of injured muscle afferents is remarkable (Fig. 4.1, 4.2, blue columns). These thermal sensitivities are mostly combined with mechanosensitivity although some 50% of the C-fibers are only thermo-sensitive. Heat sensitivity is already present in TP I (i.e. hours after nerve crush) in about 50% of the C-fibers with ectopic activity and this frequency remains high in TP II, the thresholds ranging from 35 to 50°C (Fig. 3.16). Heat sensitivity is absent in A-fibers in TP I but expressed in some 50% of these afferent fibers in TP II. Heat sensitivity has been reported in about 40 to 60% of intact muscle mechanosensitive C-fibers, but unfortunately was not investigated systematically (Fig. 4.2.A, query; Kumazawa and Mizumura 1977; Taguchi et al 2005). Furthermore, about 50% of the intact muscle mechanosensitive C-fibers are capsaicin-sensitive (Hoheisel et al 2004). Activation of intact muscle A-fibers by heating the muscle has not been reported in the literature (Fig. 4.1.A, query). Thus, heat sensitivity must be a novel functional characteristic of injured muscle A-fibers but not of injured muscle C-fibers. Whether heat sensitivity of injured muscle afferents contributes to neuropathic pain awaits further studies.

In TP I cold sensitivity is absent in A-fibers and present in some 20% of C-fibers with ectopic activity. Its frequency increases to some 40 - 60% in both groups of fibers in TP II. Cold sensitivity has not been reported in intact muscle A-fibers and only in a few intact muscle C-fibers (Fig. 4.1.A; 4.2.A; Kumazawa and Mizumura 1977; Mense 1986, 1993; Taguchi et al 2005). Thus, cold sensitivity must be a novel functional property of injured muscle afferents. The cold thresholds to excite the A- or C-fibers are largely in the noxious range, although some cold-sensitive A-fibers with ongoing activity can be excited by stimuli of 25 - 30°C. Whether the cold sensitivity of injured muscle afferents contributes to neuropathic pain or deep somatic dys- and paresthesias needs to be studied.

c. Spontaneous activity:

About 50% of the injured muscle A- and C-afferents show spontaneous activity. Whether non-injured muscle afferents have ongoing activity is very much debated. Some studies reported no ongoing activity in their recorded intact muscle afferents in humans (Marchettini et al 1996, Simone et al 1994). However, it has been shown by some groups in dogs and cats that intact muscle A δ -afferents (Kumazawa and Mizumura 1977) and up to 90% of the intact muscle C-fibers exhibit a low rate of spontaneous activity of ≤ 0.02 impulses per second (Berberich et al 1988; Diehl et al 1993, Kumazawa and Mizumura 1977; Taguchi

et al 2005). Thus, the rates of the spontaneous activity of injured muscle afferents are significantly higher than in intact ones.

The rates and frequencies of spontaneous activity are the same in the populations of injured muscle A- and C-fibers isolated from the sciatic nerve and in the populations of injured A- and C-fibers isolated from the dorsal root L4 or L5. This supports the notion that spontaneous activity in injured muscle afferent neurons may primarily originate in the periphery at the injury site and not in the dorsal root ganglion (DRG) (Devor 2006; Wall and Devor 1983). This agrees with the finding that peripheral nerve injury occurring remotely from the DRG is followed by spontaneous activity originating in the DRG in some 20% of muscle afferent neurons which have small diameter myelinated axons but not in the DRG cells of muscle afferent neurons with unmyelinated axons (and not in the DRG cells of cutaneous afferent neurons). The rate of spontaneous activity originating in the DRG in muscle afferent neurons is 0.1 – 15 imp/s (mean about 2 imp/s) in neurons with irregular discharges and 2.2 – 7.1 imp/s (mean 5.4 imp/s) in neurons with bursting discharges (Michaelis et al 2000). These rates of spontaneous activity originating in the DRG in injured muscle A-fiber neurons are lower than those originating in peripheral injured A-fibers as reported here.

4.1.3. Comparison of injured muscle A- and C-fibers

Muscle afferents consist functionally of three large groups: large-diameter myelinated ($A\alpha/\beta$ -) fibers, small diameter myelinated ($A\delta$ -) fibers and C-fibers. The muscle $A\delta$ - and C-fibers are involved in functions related to activation of the cardiovascular and respiratory system during exercise (“ergoreceptor” function), deep pressure sensation, muscle fatigue, and nociception and pain (Graven-Nielsen et al 2004; Rivers and Head 1908; Kaufman and Forster 1996; Mense 1993; Mitchell et al 1983; Simone et al 1994; Torebjörk et al 1984). Thus, these small-diameter muscle afferents have common functions which are different from those of the large-diameter muscle afferents. Therefore we expected that slowly conducting injured myelinated afferents exhibit discharge patterns similar to injured C-fibers. To our surprise injured myelinated afferents conducting at <20 m/s and those conducting at ≥ 20 m/s were functionally practical identical. But both populations of myelinated fibers were significantly different in their frequency of cold, heat and mechanosensitivity as well as rate of spontaneous activity from the population of injured C-fibers (Tables 3.2, 3.3, 3.7, Fig.

3.25). The same obvious similarity in sensory receptive properties of A δ and A β -fibers was observed in the cutaneous nerves (Djoughri and Lawson 2004).

We have divided the nerve fibers according to Waddell & Lawson (1991) and Waddell et al (1989) into A-fibers (conduction velocity >2 m/s) and C-fibers (conduction velocity ≤ 2 m/s). Our distribution of conduction velocity is very similar to that shown by Waddell et al (1989) for afferent fibers that have their cell bodies in the dorsal root ganglia L4 or L5 which also contain the cell bodies of the afferent neurons projecting in the lateral gastrocnemius-soleus nerve (Swett et al 1991). Waddell et al (1989) and Waddell & Lawson (1991) describe that A δ -fibers conduct at 2-12 m/s and A α/β -fibers at >12 m/s, however in rats with a weight ranging from 120 to 190 gram. The weight of our rats was more than double as high. The division between A δ -fibers and A α/β -fibers may therefore be closer to 20 m/s in our group of rats. For this reason we have compared the functional properties of A-fibers conducting at <20 m/s with those of C-fibers.

4.1.4. Importance of A-fibers in the nociception

The results of our work give strong support for the important role of the myelinated fibers in hyperalgesia and allodynia associated with muscle nerve injury. In spite of the strong evidence for the existence of myelinated nociceptive afferents fibers with conduction velocities in the A β range in different species in the skin nerve (Djoughri and Lawson 2004), A β -fiber nociceptive neurons are often ignored in the literature, and textbooks often promote the view that nociceptors conduct only in the C- and A δ -fiber range. This misleading view is perhaps due to:

- the fact that the conduction velocities of A-fiber nociceptors tend to peak in the A δ -range, albeit at the upper end of the range,
- the inappropriate use of A δ -fiber upper conduction velocities limits that are too high; for instance an upper limit for A δ -fibers of 30 m/s is sometimes somewhat arbitrarily, and incorrectly, applied to rat studies (e.g., Simone and Kajander 1997),
- the proportion of fast A α/β -fibers that are nociceptive is fairly small (about 20% in rat) (Djoughri and Lawson 2004), and
- conduction velocities measured towards the periphery conduct more slowly than the same fibers measured more centrally, and thus the A δ /A β conduction velocity border

is accordingly lower, a factor that is not often taken into account (Mense and Meyer 1985, Djouhri and Lawson 2004).

Thus, the possibility that A β -fibers might contribute to abnormal pain has not generally been considered. For instance, after the nerve injury a slight decrease in their mechanical thresholds, especially of moderate pressure receptors, might render them responsive to innocuous stimuli. Furthermore, greater firing in moderate pressure receptors once threshold is reached could contribute to allodynia. The same could apply to the abnormal responses of the injured muscle myelinated fibers to moderate heat or cold stimuli, which possibly contributes to heat hyperalgesia and cold allodynia.

4.1.5. Comparison of functional characteristics of injured muscle afferents and injured cutaneous afferents

Transection and ligating or crush injury of peripheral nerves triggers a complex regenerative process that concerns the dorsal root ganglion neuron as well as its central and peripheral axon. In most cases the peripheral axon starts to sprout in order to regain access to the target tissue. If regeneration is successful, the afferent neurons seem to obtain their normal physiological properties. Part of the sprouts is trapped and contributes to neuroma formation (mostly unmyelinated fibers) and the other part (mainly myelinated fibers) regenerates with a speed around 1mm/day. The permanent separation of the afferent axons from their target tissue generates permanent changes in the affected neurons. Cell bodies and axons of many surviving afferent neurons shrink; additionally there is a loss of sensory ganglion cells that probably affects mainly afferent neurons with unmyelinated axons. According to the investigation of Gorodetskaya et al (2009), and based on the investigation of Hu and McLachlan (2003) after transection and suturing of the sural nerve about 38% of C fibers regenerate to their target tissue, about 32% die and about 20% or so do not regenerate.

The neuropeptide content changes in the cell bodies and consequently also in central as well as peripheral endings of the afferent neurons. As a result, peripheral sprouts may become an ectopic source of the neuronal afferent activity – spontaneous activity, mechano-sensitivity and thermo-sensitivity. The generation of spontaneous ectopic impulses is not a direct consequence of the transection of the axons but appears hours and possibly days after the nerve lesion and shows differences between various nerve types, e.g. between a skin nerve and a muscle nerve.

a. Time period I:

Figures 4.1B and 4.2B compares the ectopic activities (spontaneous activity [SA], mechanosensitivity, thermosensitivity) developed by myelinated and unmyelinated muscle afferents and cutaneous afferents in the first hours following nerve crush (see Blenk et al 1996, Michaelis et al 1995 and 1999 for data obtained on injured cutaneous afferents). The percentages express the proportion of fibers with at least a single ectopic property with respect to all electrically identified fibers. The numbers of somatomotor axons (muscle nerve, 50%, Mitchell and Schmidt 1983, Peyronnard et al 1986, Sittiracha and McLachlan 1986) and of postganglionic axons (muscle nerve 49%, skin nerve 17%, Baron et al 1988) are deducted. Injured efferent (postganglionic and somatomotor) axons cannot be activated by physiological (mechanical or thermal) stimuli (Struck 2012). Mechano- and thermosensitivity were tested by physiological stimuli applied to the nerve at or distal to the injury site. For comparison only the numbers of injured muscle fibers recorded from the sciatic nerve are taken:

- Both muscle and skin afferent nerve fibers start to generate ectopic impulse activity already within the first hours after lesion of the nerve.
- In the first 13 hours after nerve injury the frequency of muscle C-fibers with ectopic activity (ongoing activity, mechano- and thermosensitivity) is significantly higher in a muscle nerve (74%) than in a skin nerve (18.5%) whereas the frequency of A-fibers with mechanical ectopic activity is equal in an injured skin nerve (13%) and in an injured muscle nerve (11%) (Blenk et al 1996; Michaelis et al 1995, 1999).
- Most injured myelinated fibers are silent in both nerves in TP I. 4 - 10% of the fibers are mechanosensitive; SA is almost absent (0.7% skin nerve, 0.2% muscle nerve); thermosensitivity is absent.
- In the both skin and muscle nerves the percentage of myelinated fibers excited by mechanical stimulation of the lesion point of the nerve increased with time after axotomy.
- Mechanical excitability at the site of the nerve lesion is a novel property of the axotomized fibers (Michaelis et al 1995, Teliban et al 2011). The development of mechanical excitability in axotomized A-fibers presumably depends on cellular components conveyed via the fast axonal transport (Koschorke et al 1994).
- The incidence and the rates of spontaneous activity are significantly higher in muscle C-fibers than in cutaneous C-fibers (muscle nerve: 42% of C-fibers, 0.13-7.0 imp/s, median 0.5 imp/s, [n=25] vs. skin nerve: 6% of C-fibers, 0.05-3.4 imp/s, median 0.3 imp/s, [n=52]).

- In both, injured cutaneous and muscle C-fibers, the rate of spontaneous discharge significantly decreased with time after axotomy in TP I (e.g.: within first 9h the mean discharge rate in cutaneous C-fibers was 0.81 imp/s (n=18), 0.4 imp/s (n=20) in 9-18 h after axotomy, after 22h – 0.18 imp/s (n=7) (Michaelis et al 1999).
- The numbers of the muscle cold-sensitive and heat-sensitive C-fibers in TP I are significantly higher in comparison with the acutely lesioned thermosensitive cutaneous nerve C-fibers (muscle nerve: 10% of C-fibers were cold-sensitive and 42% - heat-sensitive; skin nerve: 5.6% of C-fibers were cold-sensitive and 6.1% - heat-sensitive; Blenk et al 1996*).
- Acutely injured unmyelinated fibers like intact ones are predominantly mechano- and/or heat-sensitive (Kumazawa and Mizumura 1977; Taguchi et al 2005).

b. Time period II:

Table 4.1, figures 4.1C and 4.2C, 4.3 compare the ectopic activities (spontaneous activity [SA], mechanosensitivity, thermosensitivity) developed by myelinated and unmyelinated muscle afferents and cutaneous afferents 4-14 days after nerve crush (see Grossmann et al 2009a, Jänig et al 2009 for data obtained on injured cutaneous afferents). In the time intervals after nerve crush injury chosen the nerve fibers did not regenerate to their target tissues assuming maximal regeneration rates of 1-4 mm/day (Sunderland 1978, Sunderland 1991). Mechano- and thermosensitivity were tested by physiological stimuli applied to the nerve at or distal to the injury site. For comparison only the numbers of injured muscle fibers recorded from sciatic nerve are used:

- In the first two weeks after a nerve injury almost all muscle A-afferents and C-afferents exhibit ectopic activity but only about 40-60% of the cutaneous afferents.
- Qualitatively there is no difference in discharge pattern of C-fibers between skeletal muscle and skin.

* It is quite difficult to compare the results of our study on a muscle nerve and the results of Blenk et al (1996) on a skin nerve in the TP I, since different temperature were used to test the termsensitivity of the injured nerves. In our investigation we used 5°-25°C and 35°-50°C. In the work of Blenk et al (1996) the temperatures applied were in the range of 8-15°C and 35-50°C. Temperature stimuli of 8-15°C could be not enough to excite high threshold (type 2) cold-sensitive unmyelinated fibers (Grossman et al 2009, Jänig et al 2009). Taking this in account, Blenk et al (1996) possibly missed high threshold (type 2) cold-sensitive unmyelinated fibers. The percentage of cold-sensitive C-fibers was therefore lower, than our percentage, but the percentage of heat-sensitive C-fibers.

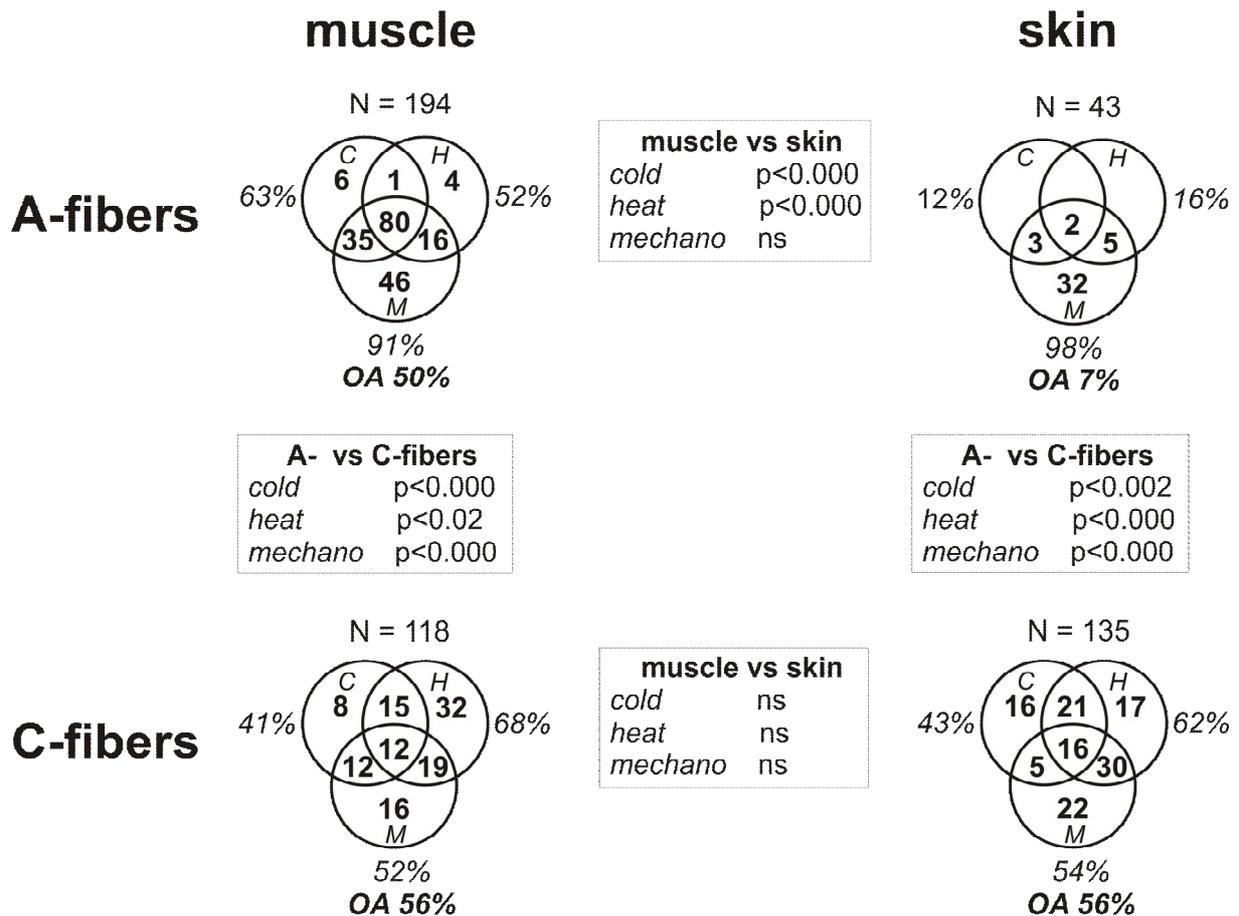


Fig. 4.3. Pattern of cold (C), heat (H), and mechanosensitivity (M) of A- and C-fibers projecting in an injured muscle nerve (gastrocnemius-soleus nerve) or in an injured skin nerve (sural nerve) 4-7 days (muscle) or 4-14 days (skin) after crush injury. The numbers in the Venn diagrams do not include afferents that have only spontaneous activity (SA) (muscle nerve: 6 A-fibers, 4 C-fibers; skin nerve: 1 A-fiber, 8 C-fibers). Numbers in italic show the frequencies of cold-, heat-, or mechanosensitive afferents. χ^2 -test was used for comparison of the frequencies of cold, heat, or mechanosensitivity between A- and C-fibers and between muscle and skin afferents. Data on cutaneous fibers is taken from Jänig et al (2009).

- More than 90% of the A-fibers are mechanosensitive in both the muscle and the skin nerve. This frequency is significantly higher than the frequency of mechanosensitive C-fibers in both.
- The mechanical thresholds are significantly lower in injured muscle A- and C- afferents than in injured cutaneous A- and C- afferents, respectively (A-fibers: 0.3->10mN [median 1 mN] vs. 0.7—70mN [median 7.5 mN] p<0.001; C-fibers: 0.3->10mN [median 0.7 mN] vs. 0.45->70Mn [median 10mN] p<0.001) (Gorodetskaya et al 2003, Jänig et al 2009).
- Cold and heat sensitivity have a significantly higher representation in injured muscle A-fibers than in an injured cutaneous A-fibers.

- Heat-sensitive injured muscle A- and C-fibers and cutaneous C-fibers have a significantly higher incidence of OA than heat-insensitive injured afferents.
- The cold sensitivity of cutaneous C-fibers was divided into type 1 cold sensitivity and type 2 cold sensitivity (see Grossmann et al 2009a, b; Jänig et al 2009, Teliban 2012). Type 1 cold-sensitive C-fibers are menthol-sensitive and behave like (non-nociceptive) cool receptors. Most of them have a high rate of ongoing activity at temperatures of 30°C on the surface of the nerve, are activated by small cooling steps and inhibited by heating stimuli. Some are additionally activated by strong heat stimuli. They are with a few exceptions mechanoinsensitive (Grossmann et al 2009a). Type 2 cold-sensitive C-fibers are menthol-insensitive and behave like nociceptive cold receptors. This division into non-nociceptive and nociceptive cold-sensitive afferent neurons does not seem to exist in skeletal muscle.
- The frequency of A- and C-fibers with ongoing activity is higher in an injured muscle nerve (55.4% of A-fibers, 62.5% of C-fibers) than in an injured skin nerve (2% of A-fibers, 31% of C-fibers). The rates of ongoing activity are not significantly different between injured muscle and cutaneous C-fibers.
- Injured cutaneous afferents show transduction characteristics for physiological stimuli that are qualitatively and quantitatively similar to those of intact cutaneous afferents. Thus, the frequencies of the functionally different populations of cutaneous afferent fibers are the same for intact and injured afferent neurons (Gorodetskaya et al 2009; Jänig et al 2009). Whether this rule applies to the muscle afferents as well has to be examined. According to the existing literature (for review see [Kumazawa and Mizumura 1977, Mense 1993, Taguchi et al 2005] activation of intact muscle A-fibers by heating or cooling the muscle has not been reported. 50-60% of the intact C-fibers are heat-sensitive and/or very few are cold-sensitive. Thus, cold sensitivity is probably a novel functional property of injured muscle C-fibers, while heat and cold sensitivity are probably a novel functional characteristic of injured muscle A-fibers. Whether the developed thermosensitivity of injured muscle afferents contributes to neuropathic pain or deep somatic dys- and paresthesias needs to be studied.
- Cutaneous afferent neurons with C-fibers shrink and may die after axotomy; injured cutaneous afferent A-neurons only shrink (Degn et al 1999; Hu and McLachlan 2003; Jänig and McLachlan 1984; Lekan et al 1997; Tandrup et al 2000). These cellular changes do not occur in axotomized primary afferent neurons innervating skeletal muscle (Hu and McLachlan 2003). The differential pathobiological changes in

cutaneous and muscle afferent neurons following axotomy may imply that activity in injured muscle afferent neurons is becoming more important in the generation of pain, dysesthesias and paresthesias than activity in injured cutaneous afferent neurons in chronic conditions.

These differences and similarities between injured muscle and cutaneous afferent fibers are remarkable. They indicate that neuropathic pain, paresthesias and dysesthesias in patients with nerve injury may also be related to the activity in injured muscle afferents and possibly other injured deep somatic afferents.

c. Long-term changes:

In the collaborative experimental work with Jan Tode and Vanessa Rausch we investigated long-term changes of the injured muscle afferents (Tode 2012). Since the gastrocnemius soleus (GS) nerve was too short (around 10mm) to investigate long term regeneration processes after nerve crush, we made a cross-union of the central stump of the lateral GS nerve with the distal stump of the sural nerve. 20 and 80 days later we tested the ectopic discharge characteristics of the muscle afferents in the cross-unioned nerve. Figure 4.4 summarizes the functional regeneration of injured cutaneous and muscle afferent A- and C-fibers to the target tissues 4-14 days after nerve injury (TP II), when the time interval for regeneration to the target tissue was too short, and months after nerve injury when regeneration to the target tissues was possible. The criterion for regeneration was the responsiveness of the afferent fibers to physiological (mechanical or thermal) stimulation of the target tissues, the nerve injury site or the nerve distal to the injury site. The nerve injury was either a crush lesion or a cross-union of the proximal stump of the lateral gastrocnemius-soleus nerve to the distal stump of the sural nerve. The last column in figure 4.4 shows the percentages of A- and C-fibers, in the skin or muscle nerve, which are efferent (sympathetic postganglionic for C-fibers, somatomotor for A-fibers). These percentages were subtracted in the calculation of the percentages of afferent fibers activated by the physiological stimuli applied to the nerve or the target tissues.

- Afferent cutaneous and muscle A-fibers exhibit powerful regeneration after injury whereas the regeneration of injured afferent C-fibers is overall poor, even after crush lesion which leaves the laminal tubes along which nerve fibers sprout intact.

time after crush lesion (CL) or cross-union (CU)	1 number of fibers	2 afferents activated from nerve ¹	3 afferents activated from target ¹	4 efferent ² fibers
skin 7-14 d C-neurons CL A-neurons	135/258 (52%) 43/98 (44%)	63% 44%	0% ³ 0%	17% postgangl no somatomotor
muscle 4 - 7 d C-neurons CL A-neurons	52/86 (60%) 79/155 (51%)	100% 100%	0% ³ 0%	49% postgangl 50% somatomotor
skin 10-15 m C-neurons CL A-neurons	107/257 (42%) 82/158 (52%)	32% ⁴ 5%	38% ⁴ 95%	17% postgangl no somatomotor
muscle ~ 80 d CU A-neurons	40/87 (46%) 52/135 (39%)	92% 30%	8% 70%	49% postgangl 50% somatomotor

skin: sural nerve; muscle: lat. gastroc. nerve: 4-7 d after CL; 80 d after CU to the sural nerve; d, days; m, months
¹ activated by physiological stimuli; ² Baron et al (1988), Mitchell & Schmidt (1983); ³ nerve fibers have not regenerated to target tissue; ⁴ >30% of injured cut. C-fibers die (Hu & McLachlan 2003)

Fig. 4.4. Activation of injured myelinated (A) and unmyelinated (C) nerve fibers by physiological stimuli applied to the nerve injury site and the (reinnervated) target (taken from Jänig and Kirillova 2012). The afferent nerve fibers were tested for their responses to physiological (mechanical or thermal) stimuli and for their ongoing activity 4-14 days after crush lesion (CL) of the sural nerve, 10-15 months after crush of the sural nerve, 4-7 days after crush of the lateral gastrocnemius-soleus nerve or 80 days after cross-union (CU) of the proximal stump of the lateral gastrocnemius-soleus nerve to the distal stump of the sural nerve. Column 1: Proportion of electrically identified nerve fibers which exhibited ectopic ongoing and/or evoked activity. Column 2: Percentage of afferent fibers activated by physiological stimulation of the injured nerve or having ongoing activity only. Column 3: Percentages of afferent fibers activated from the (reinnervated) target (including those which could additionally be activated from the nerve). The numbers of efferent fibers activated from the were taken into account. Column 4: Percentages of axons which are postganglionic (Baron et al 1988) or somatomotor axons (see column 4) and Schmidt 1983, Peyronnard et al 1986, Sittiracha and McLachlan 1986). Note: 4-7 and 4-14 days after crush lesion nerve fibers do not regenerate to their targets. 10-15 months after crush lesion or 80 days after cross-union afferent fibers can regenerate to the peripheral target tissue assuming a regeneration rate of 1-4 mm/day (Sunderland 1978, 1991); the frequency of regeneration of C-fibers is poor compared to the frequency of regeneration of A-fibers. Data from Gorodetskaya et al 2009, Jänig et al 2009, Kirillova et al 2011 and Tode 2012.

- Almost all injured cutaneous (95%) and many muscle A-fibers (70%) which can be activated by physiological stimulation reinnervated the skin (or a few deep tissues of the paw) 10 to 15 months after crush lesion of the skin nerve and 80 days after cross-union of the GS nerve and sural nerve.
- Only about a third of the injured cutaneous C-fibers, which can be activated by physiological stimuli, reinnervate the target tissues (skin and a few deep tissues). One third of the cutaneous C-fibers can only be activated from the injured nerve (i.e. have stopped sprouting) and about one third of the cutaneous C-fibers die long-term after nerve injury (Hu and McLachlan 2003).
- Practically all injured muscle afferent C-fibers and half of the cutaneous ones can be activated by physiological stimuli long-term after nerve injury. However, more than 90% of them are activated from the nerve injury site or rarely from the nerve distal to the injury site. Less than 10% can be activated from the skin.
- Almost all ectopically active injured muscle and skin A-fibers are mechanosensitive. Only a small proportion of these fibers are additionally thermosensitive.
- In TP II and long-term after nerve injury the thresholds of the mechanosensitive injured muscle A-fibers were significantly lower than thresholds of the cutaneous ones (muscle nerve A-fibers: activated from nerve – 0.7mN, activated from skin - 0.85mN, skin nerve A-fibers: activated from the nerve – 4.8mN, activated from the skin – 10-20mN [medians]).
- The frequency of thermosensitive A-fibers significantly decreased with time after nerve injury (muscle nerve: TP II – 84% of A-fibers, 80 days – 23%; skin nerve: TP II – 10% of A-fibers, 10-15 months – 5%).
- Thermosensitivity of chronically injured C-fibers in both muscle and skin nerves remains on the same high level days and months after nerve injury (70-80% of all ectopically active fibers).

4.1.6. Axonal mechano- and thermosensitivity

Cold and heat sensitivity of cutaneous afferent neurons with C-fibers may extend over the axon membrane in the nerve but not mechanosensitivity (Hoffmann et al 2008; Teliban et al 2011). Almost all non-nociceptive cold-sensitive cutaneous C-fibers (type 1 cold-sensitive

C-fibers) exhibit axonal cold sensitivity. About 30 to 40% of the nociceptive cold- or heat-sensitive C-fibers show axonal cold or heat sensitivity (Teliban et al 2011). Whether this axonal thermosensitivity is also present in muscle afferent neurons with C-fibers or A-fibers we do not know. In TP I (4-13 hours after nerve crush) heat and cold sensitivity of muscle A-fibers was absent and only very few A-fibers were mechanosensitive (Table 3.2). This shows indirectly that axonal mechano- and thermosensitivity are probably absent in myelinated muscle afferents since we tested the axons up to about 3 mm rostral to the injury site. However, axonal mechano- and thermosensitivity of muscle A- and C-afferents need to be investigated systematically.

Recently it has been shown that perineural inflammation generated by complete Freund's adjuvant applied to the sciatic nerve of rats is followed by axonal mechanosensitivity of C- and A δ -afferents innervating deep somatic tissues but not of those innervating skin (Bove et al 2003; Dilley and Bove 2008; Dilley et al 2005). This ectopic axonal mechanosensitivity during neuritis may be related to the high mechanosensitivity of injured muscle afferents as shown by us.

4.1.7. Mechanisms underlying ectopic spontaneous activity and mechanosensitivity

The mechanism of abnormal electrogenesis must lie in the change of the selective permeability of the axon membrane to various ions (Na⁺, K⁺, Cl⁻ and Ca²⁺). It is possible that Na⁺-channels originally destined for the distal axon become incorporated into the membrane proximally, altering its electrogenic properties. It is known, that regenerating nerves acquire many more Na⁺ channels than could possibly be supplied by rearranging those already present in the parent axon (Ritchie 1982). The number of channels incorporated in relation to the other axon parameters, and their kinetic properties, play an important role in determining the degree of excitability of neuroma sprouts.

Spontaneous activity of primary afferent neurons is related to several components, an important one being the voltage-sensitive sodium channels (Amir et al 2006, Cummins et al 2007, Gold and Caterina 2009, Rogers et al 2006, Wood 2009, Kirillova et al 2011b). This idea is supported by our study in which we showed that ectopic spontaneous activity in injured afferent cutaneous and muscle A- and C-fibers is inhibited dose-dependently by local or i.v. application of lidocaine (Na⁺ channel blocker) at doses that do not affect conduction and responses to physiological stimulation of the afferent fibers (Kirillova et al 2011b). Sodium channels which are probably important in this are the tetrodotoxin-sensitive channels

Nav1.3 and Nav1.7 and the tetrodotoxin resistant channels Nav1.8 and Nav1.9 which are particularly present in small-diameter nociceptive afferent neurons. However, Nav1.7 to Nav1.9 are downregulated in injured afferent neurons, whereas Nav1.3 is upregulated (this channel is almost absent in adult dorsal root ganglion cells). Thus, it is unclear which type of sodium channel or which combination of sodium channels is important in the generation of ectopic ongoing activity in injured afferent neurons (Amir et al 2006, Cummins et al 2007, Kinnman and Levine 1995, Rogers 2006).

Mechanosensitivity: The molecular transduction mechanisms underlying the mechanosensitivity of injured (and intact) muscle afferents have to date not been investigated and are poorly understood (Hu et al 2006, Julius and McCleskey 2006, Lewin and Moshourab 2004, Marshall and Lumpkin 2011, Delmas et al 2011). However, in view of the wide range of mechanical thresholds of injured afferents showing distinct groups of high- and low-threshold afferents, it is not far-fetched to assume that several mechanisms contribute to the mechanosensitivity. The mechanical threshold of sensory neurons may not be related to the cellular organization of the mechanoreceptor but may lie in the properties of mechanotransducer apparatus (Delmas et al 2011). It is not yet clear whether rapidly and slowly adapting mechanosensitive currents identified with *in vitro* techniques relate to low threshold mechanoreceptors and high threshold mechanoreceptors *in vivo* (Delmas et al 2011).

From the studies on cutaneous mechanoreceptors it is known that mechanosensitive currents evoked in sensory neurons have a relatively short latency (0.4-0.8 ms; Hu and Lewin 2006), which argues against activation of a second messenger cascade and favours direct activation of mechanosensitive channels. Whether mechanosensitive currents are activated by stress in the lipid bilayer close to the transduction channels or through a tethering mechanism anchoring the channels to the cytoskeleton or the extracellular matrix is still unclear (Sachs 2010).

Candidate channels for mechanotransducers are:

- *Acid-sensing ion channels (ASICs)*, which are densely present in muscle afferent neurons. ASICs have a modulatory role in visceral and cutaneous mechanoreceptor functions, but do not seem to play a direct role in mechanotransduction (Delmas et al 2011).
- *The TRP channel superfamily*. In mammalian sensory neurons, TRP channels are best known for sensing thermal information. However, three TRP channels, TRPV2, TRPV4 and TRPA1, have been implicated in mechanical responsiveness. The TRPV4

channel contributes to osmosensation in mammals, to mechanical sensitivity (Brierley et al 2008) and possibly to baroreception. TRPA1 is suggested to play a role in mechanical nociception (Story et al 2003) and mechanical hyperalgesia (Bautista et al 2006, Kwan et al 2006). At the cellular level, it was shown that TRPA1 mediates slowly adapting mechanosensitive currents (in C-fibers) (Drew and Wood 2007, Vilceanu and Stucky 2010). However, rapidly adapting currents (in A-fibers) are also attenuated in TRPA1 knock-out mice. It remains to be determined whether TRPA1 channels serve as mechanotransducer channels or play an indirect part in mechanotransduction by amplifying or modulating the signal from the transduction channel.

- *Piezo proteins.* An elegant study has recently identified a novel class of proteins, the piezo protein family, as promising candidates for mechanosensing proteins (Coste et al 2010). Piezo 2 is abundant in DRGs, whereas piezo 1 is barely detectable. Using *in situ* hybridization in adult mouse DRGs, piezo 2 mRNA has been shown to be present in about 20% of DRG neurons, which were tentatively classified as both mechanosensitive and nociceptive. However, mRNA distribution argues for a role of piezo 2 protein in both low- and highthreshold mechanosensitive afferents triggering touch and pain sensation, respectively. Further experiments aiming at localizing piezo 2 proteins on mechanosensory nerve endings in the skin, deep somatic tissues and viscera, together with the study of piezo 2 protein knock out mice, are required to solve these important issues.

Thus, TRP and ASIC family channels are implicated in mechanical hypersensitivities under pathological conditions but do not seem to have a fundamental role in normal mechanotransduction. There is no clear evidence indicating that TRP channels and ASICs are mechanically gated. In addition, none of these ion channels, expressed heterologously, recapitulates the electrical signature of sensory mechanosensitive currents observed in their native environment. This does not rule out the possibility that ASICs and TRPs are mechanotransducers. Piezo proteins are undoubtedly mechanosensing proteins and must play an important role in the formation of rapidly adapting mechanosensitive currents in sensory neurons. Although their molecular structure remains to be determined, this novel family of mechanosensitive proteins is a promising subject for future research (Delmas et al 2011).

Other candidate channels for mechanotransduction are:

- *Stomatin* – one of the additional modulatory proteins that influence touch sensitivity through a modulatory role in sensory neurons (Delmas et al 2011).

- *Stretch-sensitive ion channels* which have been found widely in mammalian cell types. TREK-1, one of the stretch-sensitive two-pore domain potassium channels (K2P), plays an important role in the mechanosensitivity of nociceptors, as deletion of TREK-1 causes allodynia, an increase in sensitivity to nonpainful mechanical stimuli (Marshall and Lumpkin 2011). Intriguingly, mice lacking TREK-1 show decreased sensitivity to mechanical and thermal hyperalgesia, which indicates that this channel plays a role in sensitizing nociceptors (Alloui et al 2006). Taken together, TREK-1 and K2P-related members of ion channels regulate mechanical afferent messages, however, it is not yet clear whether K2P channels function as direct transducers of mechanical stimuli or as regulators of neuronal excitability (Delmas et al 2011).

Possible mechanisms of the mechano-sensation disorders:

A peripheral nerve inflammation can cause intact axons passing through the inflamed site to become mechanically sensitive, responding now to both pressure and stretch (Eliav et al 2001; Bove et al 2003; Dilley et al 2005; Dilley and Bove, 2008). Both central and peripheral mechanisms may contribute to altered mechanical sensation. The peripheral alteration may result from changes in transduction mechanisms and/or in the membrane stability of sensory fibres. One reason for the axonal mechanosensitivity in these conditions could be that inflammation can disrupt axoplasmic transport (Armstrong et al 2004). Compelling evidence indicates that mechanical hypersensitivity results from an abnormal expression or activity of the transducer molecules (Michaelis et al 1999, Sato 1991). For example, it has been shown that chronic compression of sensory fibres is sufficient to alter the distribution of the transducers in the peripheral membrane (Ma et al 2006). The increase in myelinated nociceptor sensitivity coincided with altered expression of NGF in the reinnervated target and ASIC3 and TRPV2 in the DRGs (Jankowski et al 2009). The rapidly increased expression of these channels in the DRG could be attributed to the injury itself or changes in the neurotrophic factors or other signalling molecules at the injury site. Thus, a combination of enhanced signalling through NGF/trkA and the changes in mechanically and thermally sensitive ion channels such as ASIC3 and TRPV2 could lead to the documented changes in A-fiber sensitivity (Table 3.2).

The abnormal response is not restricted to the point of compression or lesion, as axotomy also results in increased mechanical sensitivity in the sensory neuron somata, suggesting transcriptional regulation or altered targeting of transducer channels. The consequences of these alterations are the emergence of mechanical allodynia and

hyperalgesia. Recent studies of nerve inflammation, or neuritis, have shown that both myelinated (A-) and unmyelinated (C-) axons can develop mid-axonal ectopic mechanosensitivity (Eliav et al 2001; Bove et al 2003; Dilley et al 2005). This ectopic mechanosensitivity occurs largely in axons innervating deep somatic tissues and is a possible cause of radiating pain that often accompanies conditions such as back pain, repetitive strain injury or compressive neuropathies (Bove et al 2003; Dilley et al 2005; Dilley and Bove 2008). In these patients, symptoms are often triggered by movements that stretch the affected peripheral nerve. Symptoms often persist in the absence of a clinically detectable peripheral nerve injury. It is suggested that these patients may have a minor inflammatory insult of a nerve sufficient to cause the disruption of axoplasmic transport with subsequent axonal mechanosensitivity. With the identification of mechanotransducer candidates, these functional alterations in nociception could be addressed at the molecular level. Future research should therefore focus on the identification of the molecular basis of the mechanosensation of living cells, both in physiological and pathophysiological conditions.

4.2. Responses of injured muscle afferent fibers to TRP channels agonists (group B)

The main goal of this experimental study was to work out the molecular transduction mechanisms underlying heat- and cold-sensitivity of injured muscle afferents. To investigate this question we have studied for the first time the responsiveness of myelinated and unmyelinated injured muscle afferents to capsaicin (specific agonist of the transient receptor potential (TRP)V1 ion channel), menthol (specific agonist of the TRPM8 ion channel) and mustard oil (agonist of the TRPA1 ion channel) applied to their receptive fields in the nerve 4-7 days after crush lesion of the muscle nerve.

4.2.1. Possible transduction mechanisms of heat-sensitivity of injured muscle afferents

Up to date the molecular transduction mechanisms underlying the thermosensitivity of injured (and intact) muscle afferents have almost not been investigated. Most of the data has been obtained in studies on cutaneous afferents. According the literature, there are four main

candidates for the transduction of the heat sensitivity: TRPV1, TRPV2, TRPV3 and TRPV4 ion channels (Table 4.2; adapted from Belmonte and Viana 2008).

Channel	Thermal	Mechanical	Chemical	Blockers
TRPV1	T>42°C	hyper-osmolarity (N-terminal variant), modulates mechanosensitivity	protons, capsaicin, resiniferatoxin, camphor, eugeneol, anandamide, lipoxygenases, 2-APB, clotrimazole, hydroxy-alpha-sanshool	Ruthenium Red (RR), capsazepine, BCTC, DD01050, iodo-resiniferatoxin, SB-452533, Cu:phenantroline
TRPV2	T>52°C	hypo-osmolarity	2-APB, Δ9-THC, probenecid	RR, SKF96365
TRPV3	T>30–39°C		camphor, 6-tert-butyl-m-cresol, carvacrol, eugenol, dihydrocarveol, thymol, carveol and (+)-borneol, 2-APB, arachidonic acid (AA); diphenylboronic anhydride	diphenyltetrahydrofuran, RR,
TRPV4	T>25–35°C	mechanical (shear stress), hypo-osmolarity	4α-PDD, anandamide, epoxyeicosatrienoic acids, bisandrographolide A	RR
TRPM8	T<23–28°C	membrane tension	menthol, eucalyptol, icilin, WS23, LPC	BCTC, SKF96365, clotrimazole, Cu:phenantroline, intracellular acidification, 2-APB
TRPA1	T<18°C	mechanical	icilin, cinnamaldehyde, mustard oil, allicin, BCTC, 2-APB, 4-hydroxynonenal, hydroxy-alpha-sanshool, acetaldehyde, Δ9-THC, formaldehyde, trinitrophenol, GsMTx-4, methyl p-hydroxybenzoate, menthol, Ca ²⁺	camphor, Gd ³⁺ , RR, gentamicin, amiloride, HC-030031, menthol, clorpromazine, AP18
TRPC1		mechanical		Gd ³⁺ , La ³⁺ , 2-APB
TRPC6		hypo-osmolarity	1-oleoyl-2-acetyl-sn-glycerol	2-APB
ASIC1	cold (positive modulator)	mechanical?	Protons	amiloride, aspirin, Psalmitoxin, A-317567
ASIC2		mechanical	Protons	amiloride, aspirin, A-317567
ASIC3	cold (positive modulator)	mechanical	protons, lactic acid	amiloride, aspirin, APETx2, A-317567
P2X2		mechanical (modulates ATP release)	ATP, UTP	suramin
P2X3		mechanical (modulates ATP release)	ATP, α,β-meATP	suramin, A-317491, TNP-ATP
TREK1	heat	mechanical	AA, LPC, protons, riluzole, inhalation anesthetics, flufenamic acid	clorpromazine, diltiazem, bupivacaine, fluoxetine, sipatrigine
TREK2	heat	mechanical	AA, LPC, protons, riluzole, inhalation anesthetics, Zn ²⁺ , flufenamic acid	Pb ²⁺ , diltiazem
TASK1			inhalation anesthetics	protons, Zn ²⁺ , hypoxia, anandamide, Gαq
TASK3			inhalation anesthetics	protons, Zn ²⁺ , Ba ²⁺ , RR, anandamide, Gαq

Table 4.2. Multimodal gating in mammalian somatosensory channels (adapted from Belmonte and Viana 2008). The list of agonist and antagonist cited is extensive but not comprehensive, merely illustrating the degree of polymodality observed in different channels. Agonists for TRPV2 show marked species differences (Neeper et al 2007). In case of TREK and TASK channels, blockers produce an augmentation of excitability while activators reduce it. Note that menthol being the TRPM8 channel agonist appears to activate and block the TRPA1 channel.

“Capsaicin or vanilloid receptor”, now named TRPV1 was the first identified transducing molecule in the mammalian somato-sensory system (Caterina et al 1997). This membrane receptor is a Ca²⁺-permeable non-selective cationic channel, and is activated by temperatures in the noxious range of >43 °C and by capsaicin, the pungent ingredient of chili pepper (Caterina et al, 1997; Jordt and Julius, 2002). TRPV1 was primarily expressed on small/medium diameter afferent neurons, identified as putative nociceptors. TRPV1 is also excited by low pH and can be potentiated by a number of factors known to participate in

inflammation, including nerve growth factor, bradykinin, lipids, prostaglandins and ATP (Tominaga & Caterina, 2004). Therefore TRPV1 was proposed as a molecular substrate for polymodality in nociceptor neurons.

Later, other channels of the TRP family were cloned and characterized. The next candidate for heat sensitivity transduction, TRPV2, is expressed in DRG neurons, as well as in some brain regions, and in some non-neuronal tissues including gastrointestinal tract, and smooth muscle cells (Beech et al 2004; Muraki et al 2003). This channel is activated by high temperatures ($>52^{\circ}\text{C}$), but it is unresponsive to typical activators of TRPV1 (e.g. low pH, capsaicin). TRPV2 has been also described as a stretch-activated channel, and was found to mediate the hypotonic swelling- and stretch-induced increase of Ca^{2+} concentration in vascular smooth muscle cells. TRPV2 is reported to be highly expressed in rat afferent cell bodies that are medium to large in diameter (Fang et al 2005). TRPV2 is connected with the capacity to develop heat-heat sensitization, often observed in heat-sensitive myelinated cutaneous fibers (Rau et al 2007, Park et al 2011). Thus, TRPV2 is the main candidate to mediate the heat-sensitivity of myelinated afferents; however it appears quite difficult to prove this hypothesis in vivo since TRPV2-specific agonists do not exist yet (Park et al 2011).

TRPV3 and TRPV4 are expressed in DRG neurons, trigeminal neurons, brain, keratinocytes, tongue, testis and multiple excitable and non-excitable peripheral cell types (Peier et al 2002, Chung et al 2003, Nilius et al 2004, Moqrich et al 2005). TRPV3 is activated by innocuous ($\geq 30\text{--}33^{\circ}\text{C}$) warm temperatures and by the natural compound camphor, which is known to sensitize skin for warm stimuli, and which does not activate any of the other TRPVs. TRPV4 can be activated by diverse stimuli, such as moderate heat (TRPV4 is constitutively active at normal body temperature), cell swelling, shear stress and polyunsaturated fatty acids (Nilius et al 2004). TRPV3 and TRPV4 likely make limited and strain-dependent contributions to innocuous warm temperature perception or noxious heat sensation; but its activation cannot be mimicked by a specific agonists (Huang et al 2011). The recently discovered TRPM3 is molecularly and functionally expressed in a large subset of small-diameter sensory DRG neurons and implicates to be involved in the detection of the noxious heat (Vriens et al 2011).

4.2.2. Capsaicin sensitivity of injured muscle afferents

C-FIBERS:

The main results of our study are:

- Most (31/34) heat-sensitive C-fibers were dose-dependently activated and desensitized by capsaicin.
- Only 3 fibers with high heat thresholds were not excited, but desensitized by it.
- Activation and desensitization of the heat response were dose-dependent.
- Cold- and mechano-sensitivity were not affected by capsaicin (in the doses used).

Thus, our study showed that injured and regenerating muscle C-fibres with heat-sensitivity were excited and/or desensitized by the TRPV1 agonist capsaicin, arguing that heat sensitivity of muscle unmyelinated afferents is dependent on the TRPV1 (transient receptor potential vanilloid 1) channel. This is in accordance with previous studies, showing that about 50% of intact muscle C-fibers is capsaicin-sensitive (Hoheisel et al 2004).

Capsaicin-sensitivity of afferent neurons is thought to be a marker of their nociceptive nature. All small- to medium-sized rat dorsal root ganglion (DRG) neurons that respond to temperatures in the noxious range (average threshold 45.3°C) were also capsaicin-sensitive (Caterina 2007). It was shown in the literature that pharmacological blockade of TRPV1 reduces exercise-induced muscle hyperalgesia in the rat (Fujii et al 2008). In our laboratory, it was showed that the intact and injured heat-sensitive cutaneous C-fibres were also excited by the TRPV1 agonist capsaicin (40/46, 87% Batsch 2012). But it should be emphasized that a much higher percentage of C-fibers innervating muscle stain positive for TRPV1, then those innervating skin (Christianson et al 2006).

After nerve injury the number of neurons excited by heat is significantly increased. This is correlated with increased expression of TRPV1. However, one should not exclude the possible role the other transduction molecules, the expression levels of which are also increased after nerve lesion. For example, both TRPV3 and TRPV4 expression is increased after nerve injury (Jankowski et al 2009). It has been suggested that they could be involved in the release of ATP in keratinocytes, which may activate purinergic channels contained in the C-fibers (Perier et al 2002, Chung et al 2003).

Another candidate for the molecular transduction of the heat sensitivity in injured muscle fibers is TRPV2. TRPV2 positive-stained cells were found in afferent neurons with myelinated and unmyelinated fibers and in sympathetic postganglionic neurons, which compose about half of the neurons with unmyelinated fibers projecting in the gastrocnemius soleus nerve (Gaudet et al 2004, Lawson et al 2008). Interestingly, this group found that approximately 20% of lumbar sympathetic neurons that project to the sciatic nerve are TRPV2-positive. After injury to the peripheral projections of sympathetic neurons by sciatic nerve transection, they found that there was a 50% increase in the proportion of sympathetic

neurons that were immunoreactive for TRPV2. Thus, one could conclude that TRPV2 may have a role in sympathetically maintained pain (SMP), and that the presence of TRPV2 in sympathetic neurons may be indicative of a potential afferent function. However, recent investigation in our laboratory shows that injured postganglionic axons are not activated by strong heat stimuli (Struck 2012).

A-FIBERS:

The overall results of our investigation are:

- About one third (10/35) of the heat-sensitive A-fibers were activated during local application of capsaicin. These fibers were spontaneously active (SA) and had low heat thresholds.
- Heat-sensitive A-fibers without SA had high thresholds and were only dose-dependently desensitized by capsaicin.
- Cold- and mechano-sensitivity were not affected by capsaicin.

Thus, the heat-sensitivity of only one third of the myelinated muscle injured fibers could be mediated by the TRPV1 (transient receptor potential V1) channel. Heat-sensitivity of the capsaicin-insensitive A-fibers might be mediated by the other transduction molecule. The same hypothesis was raised by the investigators, who studied large-diameter cutaneous heat-sensitive neurons with heat threshold of $\geq 51^{\circ}\text{C}$ and showed that they are insensitive to capsaicin. Thus, it was concluded, that these neurons possess a heat-sensitive mechanism distinct from TRPV1 (Nagy and Rang 1999, Ringkamp et al 2001).

TRPV2 seems to be the best candidate to mediate heat-sensitivity of the myelinated fibers, since it is present in the DRG mainly in medium- to large-diameter A δ or A β type primary afferent neurons that are not TRPV1-positive (Djoughri and Lawson 2004, Gaudet et al 2004, Fang et al 2005; Lawson et al 2008; Jankowski et al 2009). TRPV2 protein levels are up-regulated in rat dorsal root ganglia after intra-plantar injection of complete Freund's adjuvant to induce peripheral inflammation (Shimosato et al 2005). In addition, TRPV2 protein expression is also up-regulated in rat sympathetic postganglionic neurons following nerve injury (Gaudet et al 2004), but despite the presence of TRPV2 in the grey ramus, no TRPV2-IR was observed in sympathetic baskets surrounding DRG neuron cell bodies (which appear following spinal nerve lesion [McLachlan et al 1993]). Previous studies and our studies indicate that A-fibers are sensitized under injury and inflammatory conditions; the percentage of cutaneous and muscle A-fibers responding to heat is increased after inflammation or injury of the peripheral nerve (Andrew and Greenspan 1999, Kirillova et al

2011a). Taken together, these studies suggest that thermal hyperalgesia depends also on the sensitization of capsaicin insensitive A-fibers and that possibly TRPV2 may be involved in this pain sensation.

However, the role of TRPV2 in nociception is still debated (Neeper et al 2007, Lawson et al 2008). Some results of *ex vivo* studies of cutaneous afferents suggest that mechanical sensitivity, but not heat sensitivity is mediated by TRPV2. It was found that TRPV2-positive myelinated cutaneous nociceptors have lower mechanical thresholds than TRPV2-negative ones (Lawson et al 2008). The same authors found that only a small number of TRPV2-immunopositive cutaneous cells are activated by heat. Muscle sensory neurons have not been studied for TRPV2 immunostaining so far.

There are also obvious species-specific differences in TRPV2 activation by heat. Thus, in rodents, but not in humans, TRPV2-positive afferent neurons respond to heat and to the non-selective agonist 2-aminoethoxydiphenyl borate (2-APB). The explanations for this lack of response by human TRPV2 will require further *in vivo* investigations, which would greatly benefit from the future development of specific pharmacological tools. In the meantime, it would be interesting to check in *in vivo* preparations whether heat-sensitive injured myelinated muscle afferents respond to the local application of the non-selective agonist 2-APB to prove that TRPV2 is a potential transduction molecule for heat in these fibers.

4.2.3. Possible transduction mechanisms of cold-sensitivity of injured muscle afferents

Up to now there was no report about possible transduction mechanisms of the cold-sensitive injured muscle C- and A-fibers. The main candidates for the transduction of the cold-sensitivity in injured muscle afferent fibers are: the menthol-sensitive TRPM8 channel (Bandell et al 2004, Bautista et al 2007, Belmonte et al 2009, Madrid et al 2006, McKemy et al 2002, Story et al 2003) or the TRPA1 channel (Bandell et al 2004, Jordt et al 2004, Karashima et al 2009, Story et al 2003), or potassium channels (Babes 2009).

TRPM8 is a non-selective cation channel of the TRPM (melastatine) family responding to non-noxious cold stimuli, to substances causing a sensation of coolness such as menthol, icilin or eucalyptol and with a modest permeability to calcium (McKemy et al 2002, Peier et al 2002, Story et al 2003, Bandell et al 2004, Madrid et al 2006, Bautista et al 2007, Dhaka et al 2007). This channel may underlie the type 1 (low-threshold) cold-sensitivity of cutaneous

afferents, since it was shown that the intact or injured afferent fibres with type 1 cold sensitivity are specifically excited by menthol applied to their receptive fields in the skin or nerve (Teliban 2012).

The physiological function of TRPM8 must play a critical role in cold transduction and perception; it provides the ability to discriminate warm from cool temperatures (Bautista et al 2007, Dhaka et al 2007). TRPM8-deficient mice show no clear thermal preference in the range between 15 and 35°C but develop aversion to cold temperatures at or below 10°C (Bautista et al 2007, Dhaka et al 2007).

After nerve lesion TRPM8 expression is increased in the ipsilateral DRG neurons (Proudfoot et al 2006). It has been shown that topical or intrathecal application of TRPM8 activators (cold, menthol or icilin), attenuates both thermal and mechanical hypersensitivity in a rodent chronic constriction injury model of neuropathic pain (CCI), in the Complete Freund's Adjuvant (CFA) model of inflammatory pain and in the cinnamaldehyde-induced hypersensitivity (Proudfoot et al 2006). While these studies clearly show the ability of TRPM8 agonists to reverse the hypersensitivity observed across a wide spectrum of pain models, other data suggest that TRPM8 blockade may lead to an analgesic effect as well (Colburn et al 2007). Thus, although TRPM8 is acting in the range of non-noxious cold stimuli there is some evidence suggesting that TRPM8 may play an important role in nociception.

Another potential cold transducer, the TRPA1 channel, expressed in DRG neurons and in the inner ear is a prime candidate for transducing noxious cold (Corey et al 2004, Story et al 2003, Story and Gereau 2006). TRPA1 is activated by temperatures <18°C (about 5°C lower than for TRPM8), and is suggested to mediate cold-sensitivity in cutaneous high-threshold type 2 cold-sensitive C-fibres (Teliban 2012). It was shown, that TRPA1 is also activated by mustard oil or cinnamaldehyde (Story et al 2003, Bandell et al 2004, Jordt et al 2004 and Karashima et al 2009); however, these agonists are not selective. Interestingly, this channel was found to be activated by menthol at low concentrations, but blocked at high concentrations (Karashima et al 2007). TRPA1 expression, similar to TRPV1, is increased after peripheral inflammation in the complete Freund's adjuvant (CFA) model or after nerve injury (Obata et al 2005).

However, it is debatable whether the TRPA1 channel is specifically responsible for the transduction of noxious cold stimuli (Bautista et al 2006, Kwan et al 2006, Zurborg et al 2007, Belmonte and Viana 2008, Belmonte et al 2009). In fact, many afferent cold-sensitive neurons do neither express the TRPM8 nor the TRPA1 protein, and it is suggested that several other

channels, in particular potassium channels, are involved in the responses of afferent neurons to cold stimuli (Munns et al 2007, Babes 2009).

4.2.4. Menthol and mustard oil sensitivity of injured muscle afferents

C-FIBERS:

The main results of our study are:

- Menthol dose-dependently activated all cold-sensitive C-fibers and sensitized them for cold response (15/15).
- Mechano-sensitivity was not affected by menthol.
- Most non-cold-sensitive C-fibers were unresponsive to menthol (15/21).
- Most unmyelinated injured muscle fibers were unresponsive to mustard oil (17/19).

Thus, cold-sensitivity of injured unmyelinated muscle afferents appears to be mediated by the TRPM8 channel. Many afferent fibers recorded in our study exhibited not a single discharge modality, but were polymodal neurons. The location of transduction is normally the sensory ending which, in many cases, co-expresses different transduction channels with different activation profiles (Belmonte and Viana 2008). Thus, in our investigation injured muscle cold- and heat-sensitive unmyelinated fibers were activated by both menthol and capsaicin applied to the injury site of the nerve, giving evidence of co-expression of both of these channels in these muscle afferents. This fact is supported by several authors, who have shown using functional assays that at least in rat and mouse DRGs, a significant fraction of cold- and menthol-sensitive (and thus very likely TRPM8-expressing) neurons are also activated by the TRPV1-specific agonist capsaicin (Reid et al 2002, McKemy et al 2002). Similarly, other authors (Okazawa et al 2004; Takashima et al 2007) reporting about a 30% co-expression of TRPM8 and TRPV1 in the cold-/menthol-sensitive spinal and trigeminal afferent neurons also in vitro studies, show a significantly higher percentage of neurons co-expressing TRPM8 and TRPV1. The functional consequence of this overlap between TRPM8 and TRPV1 is unclear.

A-FIBERS:

- Most cold-sensitive A-fibers were depressed by menthol (17/28).
- Activation was observed only in cold-sensitive A-fibers without or with a low rate of spontaneous activity (SA); this activation was always followed by depression of SA.

- All cold-sensitive A-fibers were desensitized for cold stimuli by menthol.
- Mechano- and heat-sensitivity was not affected by menthol.
- Many injured cold-sensitive muscle A-fibers were activated by mustard oil.

Thus, cold-sensitivity of injured muscle A-fibers must not be mediated by the menthol-sensitive TRPM8 channel. However, the mustard oil (MO) sensitive TRPA1 channel seems to be the most suitable candidate for this role. The work of Karashima et al (2007) gives strong evidence, that menthol has two independent effects on the TRPA1 channel: activation at low (micromolar) concentrations and blockade at higher ones (Table 4.2). First, menthol forces the channel to operate in a burst mode and shifts the voltage dependence of activation toward negative values, which leads to an overall increase of TRPA1 current at lower menthol concentrations. Second, menthol induces a rapid channel block, as evidenced by significantly reduced open times during periods of burst activity. This blocking effect becomes predominant at high micromolar concentrations and eventually fully shuts the channel off at a concentration of 1mM. Our study has shown that low concentrations of menthol applied to the injury site could activate some of the injured cold-sensitive muscle A-fibers. High menthol concentrations elicited either short activation of the fibers followed by the depression of the activity or depression only. Reversible activation of these fibers by the application of MO (Fig. 3.51) argues for the presence of the TRPA1 channel in the injured myelinated muscle fibers.

Early reports argue that TRPM8 and TRPA1 are not expressed in primary afferent neurons marked by neurofilament (NF), suggesting that these two channels are only expressed in C-fiber neurons (Story et al 2003). However, recently it was shown, that both populations of afferent neurons with unmyelinated and small myelinated fibers were positively stained for TRPA1 indicating that both C- and A δ -fibers express TRPA1 (Ji et al 2008). Localization of TRPA1 in C and A δ -fibers is consistent with the single fiber recordings shown in the cutaneous hairless skin preparation by the same group. There was a significant increase in the percentage of cold-sensitive A δ -fibers and a concomitant decrease of the mechanoreceptive A δ -fibers in rats with spinal nerve ligation compared with naive rats. Taking in account, that intact muscle A-fibers are predominantly mechanosensitive only (Fig. 4.1A), our study on injured muscle afferents shows a similar phenotypic shift (Fig. 4.4C) following nerve lesion. The most likely explanation for the change in the combinations of discharge characteristics is that after nerve injury many muscle mechanoreceptive A-fibers start to express ion channels/receptors activated by cooling (i.e. TRPA1) and/or by heating (i.e. TRPV2). This hypothesis is supported by Ca²⁺-imaging studies which demonstrated a significant increase in

the proportion of MO-sensitive L4 DRG cells after SNL compared with sham and naïve (Ji et al 2008). The MO-sensitive cell size corresponded to that population which would give rise to small myelinated axons (A δ -fibers). Our cold-sensitive injured muscle myelinated fibers were equally distributed along A β - and A δ -fibers conduction velocity range. This discrepancy has to be cleared using separate muscle myelinated neurons staining for TRPA1 in the DRGs.

One more evidence for the action of TRPA1 in muscle afferents was given by other research groups, which have shown that injection of MO into the masseteric muscle in the rat evokes nocifensive responses in a concentration dependent manner (Ro et al 2009). Thus, it is likely that TRPA1, along with TRPV1, contributes to pathological muscle nociceptive responses under injury or inflammation conditions. Both TRPV1 and TRPA1 channels may be recruited and sensitised via distinct intracellular pathways by various inflammatory mediators, which could manifest as overall muscle hyperalgesia. Understanding the mechanisms by which various pre-nociceptive receptors or channels engage TRP channels in producing mechanical or thermal hypersensitivity in muscle tissue is, therefore, of great clinical significance.

Other thermo-sensitive ion channels that may participate in cold sensing of injured muscle myelinated afferents are potassium channels (Viana et al 2002). Two pore domain (K2P) potassium channels (discussed above as activated by membrane stretch or free fatty acids, such as TREK-1 and TRAAK) have features of a cold transducer. These channels are active at resting membrane potentials and are strongly temperature-dependent (sensitized by warming and inhibited by cooling) (Kang et al 2005). Closing of their leakage activates sensory nerve endings (Viana et al 2002). Accumulating evidence indicates that K2P channels are subject to modulation by inflammatory mediators (for details see next subchapter 4.2.5), which may contribute in an increased excitability and inflammatory sensitisation of peripheral nerve endings (Fig. 4.5, modified from Babes 2009; Alloui et al 2006, Cohen et al 2009).

4.2.5. Possible mechanisms of the thermo-sensation disorders

Damage of the peripheral nerves can lead to mechanical and thermal hyperalgesia and other chronic pain states. Some silent nociceptors are activated after injury and become responsive to heat, cold and mechanical stimulation. According to the hypothesis of the excitation of the nociceptors after nerve injury by thermal sensitization (Reeh and Pethö 2000), inflammatory mediators, including bradykinin (possibly PGE2 and histamine), low pH, certain lipids and altered neurotrophic factors act on nociceptors, at least partly, by enhanced signalling

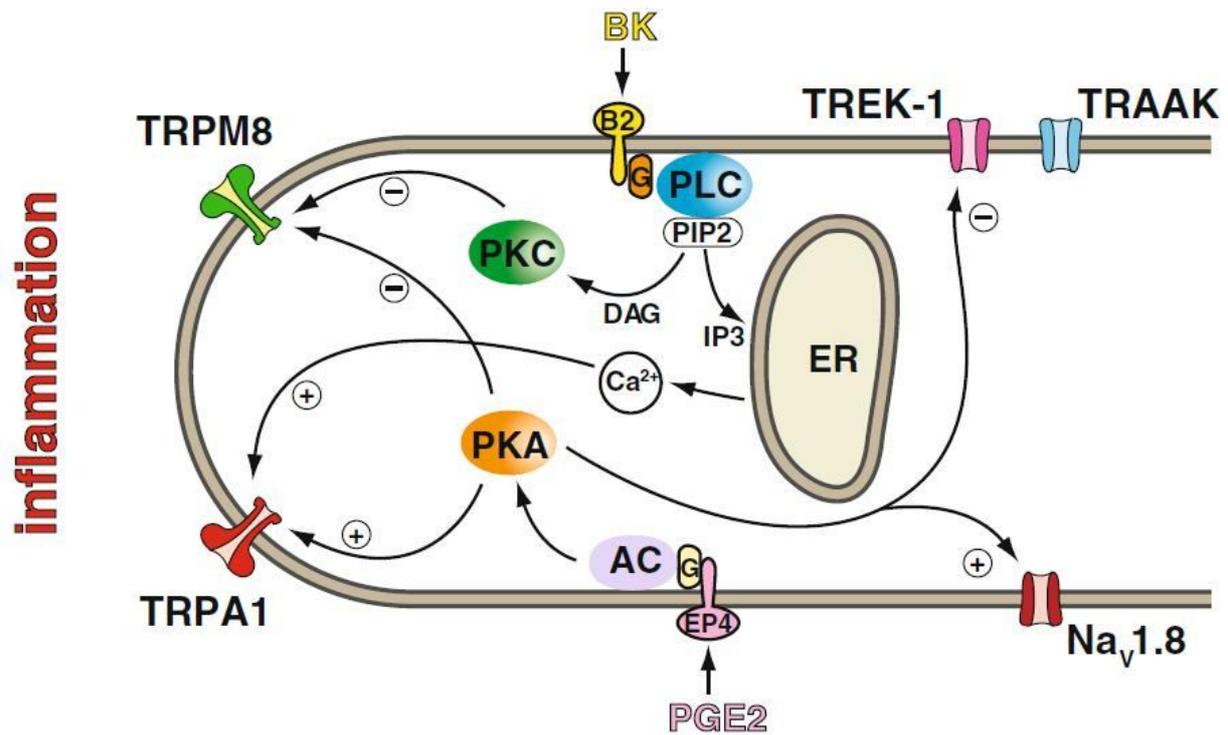


Fig. 4.5. Modulation of cold-sensitive nerve endings by inflammatory mediators (taken from Babes 2009) is illustrated using bradykinin (BK) and prostaglandin E2 (PGE2) as examples. Stimulation of BK receptors (B2) leads to activation of phospholipase C (PLC), depletion of phosphatidylinositol 4,5-bisphosphate (PIP2), and generation of diacyl glycerol (DAG) and inositol tri-phosphate (IP3). Calcium is released from stores (ER) and directly activates TRPA1. Protein kinase C (PKC) is activated and, together with PIP2 depletion, drives desensitization of TRPM8. PLC sensitizes TRPA1 via protein kinase A (PKA). PGE2 signalling leads to activation of adenylate cyclase (AC) (or PLC, depending on the receptor type) and PKA-mediated phosphorylation, which in turn inhibits TRPM8 and TREK-1 and positively modulates Nav1.8

through the phospholipase C (PLC), phospholipase A (PLA), NGF/trkA, GDNF/GRF α 1 and other pathways. These leads to the sensitization of the afferents - lowering the threshold of their thermal transduction mechanisms so profoundly that body or lower tissue temperatures become a driving force of excitation of nociceptors (Fig. 4.5; Babes 2009, Jankowski et al 2009).

Our study showed that 4-7 days after lesion of the nerve (when the inflammation process already started to act) increased incidence of ongoing activity, increased proportion of mechano-, heat- and cold-sensitive unmyelinated and myelinated fibers were observed (Kirillova et al 2011a). It is possible, that intact muscle C-fibers and A-fibers already possess thermo-transduction molecules similar to those reported or predicted in our study (TRPM8 and TRPV1 in C-fibers, and probably TRPA1 and TRPV2 in A-fibers). TRPV2 and TRPA1 channels after nerve injury could lower their activation threshold and thus be already excited by stimuli in non-noxious temperature range. Thus, a combination of enhanced signalling (Fig. 4.5) followed by the changes in mechanically and thermally sensitive ion channels such

as ASIC3, TREK-1, TRAAK, TRPA1, TRPM8, TRPV1 and TRPV2 could lead to the documented changes in A- and C-fiber sensitivities (Table 3.2, 3.3).

Overall this hypothesis is attractive from the pharmacological point of view and awaits future basic research of the mechano- and thermo-activated ion channels.

4.3 Possible explanations of the lack of the responses of injured muscle afferents to electrical stimulation of the sympathetic supply (group C)

Here we tried to investigate in a chronic GS nerve preparation (4-7 days after nerve injury) the injured afferent muscle fibers for their responses to activation of postganglionic axons by electrical stimulation of the sympathetic chain (SCS). The overall results were negative. The spontaneous activity, mechano- and thermo-sensitivity of the injured muscle fibers were almost unchanged by repetitive activation of the sympathetic postganglionic neurons. Activity of about one third of the A-fibers could be modified by SCS, but it required high-frequency stimulation of the sympathetic neurons in the non-physiological range (5-10Hz). Thus we failed to prove the presence of the sympathetic-afferent coupling to injured A- and C-fibers 4-7 days after the lesion of the muscle nerve.

In general our investigation brought the same results as previous works in this field, showing that in the first 2–3 weeks after nerve lesion, some A-fiber neurons and very few C-fibers are excited by SCS (for review see Baron and Jänig 2010). Activation of the afferent neurons required high-frequency unphysiological stimulation of the sympathetic neurons at 5 to 20 Hz. Afferent neurons in the DRG may be activated indirectly by ischemia generated by decrease of blood flow during vasoconstriction in the DRG following electrical stimulation of the sympathetic trunk and not directly via adrenoceptors expressed in the DRG cells in the spinal ligation model (SNL) (Häbler et al 2000).

The most probable reason of the negative results in our study could be in the time period chosen in the nerve injury experiments. 4-7 days could be too early for the development of the sympathetic-afferent coupling. Unfortunately in the gastrocnemius soleus (GS) nerve crush lesion model this was the maximum possible time frame, otherwise most myelinated fibers would have regenerated to and reinnervated the GS muscle. In a few experiments we tried to use a long-term model, which allows to test the regenerating muscle afferent fibers. For this aim we made a cross-union between the proximal stump of the GS

nerve and the distal stump of the sural nerve (Tode 2012). Unfortunately this experimental set up together with the procedure of the implantation of the electrodes in the lumbar sympathetic chain was too severe to be able to keep animal in a good state for the nerve fiber recording (the preparation before fiber registration needed up to 10 hours).

Sympathetic-afferent coupling must exist and it is very important to develop the appropriate model to prove its role. In previous studies of the sympathetic-afferent interaction longer time periods after nerve lesion have been used. 14-27 days after partial nerve lesions, electrical stimulation of the sympathetic supply (1ms 20Hz 30s) or close-arterial injections of epinephrine [EPI] or norepinephrine [NE] (50ng in 0.2ml of 0.9% NaCl) excited some polymodal C-fibers, which are not affected by NE in intact animals (Bossut et al 1996, Sato and Perl 1991, O'Halloran 1997). These excitatory effects persisted for months, with maximal effects 2-3 weeks after nerve injury. Note that in this study as well as in previous ones (Blumberg and Jänig 1984, Sato and Perl 1991), activation of afferent fibers required rather high (unphysiological) frequencies of stimulation of the sympathetic supply (20-50Hz). Arterial occlusion didn't activate responsive units, suggesting, that the excitation was not caused by vascular or temperature changes, or ischemia (Jänig 2009). This suggests that circulating EPI, acting via α_2 -adrenoceptor subtype, can play a role in the development and/or maintenance of sympathetically maintained pain (SMP). Furthermore, unmyelinated fibers terminating in old neuromata or regenerated C-fiber 1 year after complete section and reanastomosis of the peripheral nerves may be excited by stimulation of postganglionic fibers at a low frequency of 0.5-4Hz, but this activation was mediated by a nonadrenergic mechanism (Jänig 1990).

Another less likely explanation of the negative results could be using the inappropriate nerve injury model. The nature of the nerve lesion and experimental design could determine that only distinct components of neuropathic pain behaviour are reduced after surgical or pharmacological sympathetic block. Thus, in the *partial lesion of the sciatic nerve* model chemical sympathectomy by guanethidine injected intraperitoneally prevented thermal hyperalgesia but caused very little change in mechanical hyperalgesia (Shir and Seltzer 1991). When performed months after nerve lesion, sympathectomy by guanethidine alleviated all sensory disorders. Surgical sympathectomy performed 1 week after partial sciatic nerve lesion had no significant effects on the mechanical and cold allodynia or ongoing pain (Kim et al 1997). In the *chronic constriction injury* of the sciatic nerve, both sympathectomy and depletion of sympathetic transmitters by guanethidine result in a reduction of thermal hyperalgesia, with a little or no effect on mechanical hyperalgesia and spontaneous pain

behaviour (Desmeules et al 1995). The model with *ligation and transection of the spinal nerves* L5 and L6 or L5 alone is subject to considerable differences between rat's strains and substrains in the development of signs of mechanical allodynic behaviour or adrenoceptors (Yoon et al 1999). More importantly, the dependence of mechanical allodynic behaviour on the sympathetic supply, postulated by one research group (Kim et al 1993), could not be reproduced in an extensive experimental study conducted by two other independent research groups using two different strains of rat and two different testing procedures (Ringkamp et al 1999a, 1999b). Later it was shown in the L5 spinal injury model, that sympathetic-sensory coupling does not occur during physiological activity in sympathetic neurons (Häbler et al 2000).

These experiments demonstrate that the nature of the nerve lesion determines that only distinct components of neuropathic pain behaviour are reduced after surgical sympathectomy. Furthermore it is possible that in a neuropathic pain condition one symptom, for example heat hyperalgesia, is sympathetically maintained whereas another symptom, for example mechanical hyperalgesia or spontaneous pain, is sympathetically independent.

All deep tissues are richly innervated by sympathetic as well as afferent fibers. Measurements on patients with sympathetically maintained pain (Baron et al 2002, Schattschneider et al 2006) argue that the sympathetic-afferent coupling takes place in the deep somatic tissues of the affected extremity. Especially in acute phases months after the injury, the pain component that is dependent on the sympathetic innervation of deep somatic structures is more important than the cutaneous SMP component (Schattschneider et al 2006). However, the possible mechanisms underlying sympathetic-afferent interaction in the deep somatic tissues are entirely unknown. Taking our negative results into account, one could attempt in a future series of experiments on 1-12 month old neuroma of a muscle nerve (similar experiments on the skin nerve were largely negative [Blumberg and Jänig 1984]) to test the responsiveness of the chronically injured muscle fibers for both – lumbar sympathetic chain stimulation and for the local application of the phenethylamines to the injury site of the nerve.

4.4. Afterdischarge of the muscle A-fibers

Devor and co-workers were the first reporting of the existence of the phenomenon “afterdischarge” (AD) in myelinated fibers (Devor 1984, Lisney and Devor 1987, Devor and

Wall 1990). They recorded from the sciatic nerve neuroma fibers 4-14 days after ligation and section of the nerve and found, that single pulses of stimulation occasionally (in about 10% of all microfilaments examined) evoked a few seconds of repetitive AD. Firing always began just at the threshold for directly driving the unit was reached. Further increasing the stimulus strength had no additional effect. Silent units sometimes showed such direct AD, but it was more common in spontaneously active units. In the late 90s the phenomenon of the AD in response to the suprathreshold stimulation (STS) of the muscle GS nerve was recorded in Prof. Jänig's labor (Liu, unpublished observation). Contrary to the Devor's recordings the response of the myelinated fibers was shown to be strength-dependent. These afterdischarges were absent in myelinated and unmyelinated cutaneous afferents (Gorodetskaya, Teliban, personal communication). Therefore, Liu's preliminary data indicated that the AD was a distinctive feature of muscle myelinated fibers.

The main results of our study are:

- AD is a property of many intact (about 25%) muscle A-fibers (but not C-fibers), probably originating in the muscle spindles.
- The threshold of activation ranged from 4 to 14 Volts, and the response was strength-dependent.
- Lidocaine in doses not causing conduction block or affecting the rate of spontaneous activity, dose-dependently decreased the strength of the response to STS.
- After nerve injury the ability of A-fibers to exhibit AD is decreased. Thus, the strength of the response to STS decreased with time after nerve injury.
- Most chronically injured muscle A-fibers with AD had irregular spontaneous activity, unstable responses to the thermal stimuli and some of them exhibited uncontrolled bursts of activity.

Thus, AD is a specific property of the myelinated muscle afferents. It may be dependent either on the abundant presence of sodium channels or on the presence of a special type of sodium channel in the myelinated muscle afferents, which are obviously absent in cutaneous myelinated fibers and in afferent muscle C-fibers. Note, that both intact and injured cutaneous myelinated fibers very seldom exhibit spontaneous activity, which is also believed to be mediated by sodium channels (Fig 4.1, dark part of the columns; chapter 4.1.7, Kirillova et al 2011b).

Biophysical analysis and modelling spike initiation and propagation (Coggan et al 2011) argues that the ability to exhibit afterdischarges in intact myelinated muscle fibers could be triggered only if a irregular bursting spontaneous activity occurs. The modelling

shows, that the discharge terminates itself due to ion accumulation. After nerve injury the ability of A-fibers to exhibit the AD decreases, possibly since the changes in multiple ion channels occurring following demyelination (Waxman 2006, Costigan et al 2009). As immediate consequence of demyelination, Na⁺ channel density in this newly exposed membrane is low (Waxman et al 1995), which results in failed spike propagation. As the ratio of Na⁺ and leak channels is re-adjusted, propagation recovers but is nonetheless slowed.

Conclusions

- Four to 13 hours after injury of a muscle nerve ectopic activity is almost absent in A-fibers but already present in many C-fibers.
- Four to 7 days after injury of a muscle nerve almost all injured afferent A- and C-fibers develop ongoing and/or evoked activity originating at the injury site.
- Mechanosensitivity is prominent in the majority of A- and C-fibers.
- Heat and cold sensitivity in injured A-fibers and cold sensitivity in injured C-fibers appear to be novel functional properties.
- In the first two weeks after a nerve injury the proportion of A- and C-fibers exhibiting ectopic activity are double as high in a muscle nerve than in a skin nerve.
- Capsaicin dose-dependently activates most heat-sensitive C- and some heat-sensitive A-fibers, desensitizes almost all heat-sensitive muscle afferents for heat stimuli without affecting cold- or mechanosensitivity. Thus heat-sensitivity of most C- and some A-muscle afferents may be dependent on the TRPV1 channel.
- Heat-sensitivity of the capsaicin-insensitive A-fibers might be mediated by other channels (e.g., TRPV2), which are reported to be present mainly in medium- to large-diameter primary afferent neurons.
- Menthol dose-dependently activates all cold-sensitive C-fibers and sensitizes their cold response. At the same time menthol depresses activity in most cold-sensitive A-fibers and desensitizes their cold response.
- Cold-sensitivity of injured unmyelinated muscle afferents is probably mediated by the TRPM8 channel. However, other channels (e.g., the TRPA1 channel) may be involved in the novel cold- sensitivity of injured muscle A- afferents.

-
- Sympathetic-afferent coupling is probably absent 4-7 days after the lesion of the muscle nerve. Longer regenerating periods are probably required to show that afferent-efferent cross-talk exists.
 - Strength-dependent activation (afterdischarge) in response to the suprathreshold electrical stimulation of the nerve is a special property of many muscle myelinated fibers (but not C-fibers).
 - Lidocaine without causing conduction block or affecting the rate of spontaneous activity, dose-dependently decreases the strength of the afterdischarge. Thus, afterdischarge is probably mediated by sodium channels.

Whether and to what degree the discharge properties of injured muscle afferents contribute to deep neuropathic pain, dysesthesias and paresthesias as well as other functional changes in patients with nerve injury has to be explored.

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1. Y. A. Altman, M.Y. Agaeva, **I. Y. Kirillova**. The influence of sound source movement in vertical plane on postural reaction. *Russian Journal of Physiology*, vol. 91, №7, 810-820, 2005;
2. M. Y. Agaeva, Y. A. Altman, **I. Y. Kirillova**. Effects of a sound source moving in a vertical plane on postural responses in humans. *Neuroscience and Behavioral Physiology*, 36(7), 773-780, 2006;
3. **I. Kirillova**, V. H. Rausch, J. Tode, R. Baron and W. Jänig. Mechano and thermosensitivity of injured muscle afferents. *J Neurophysiol*, V. 105, 2058–2073, 2011a;
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5. W. Jänig, **I. Kirillova**. Chronic nerve injury models. In: Arendt-Nielsen, L., Handwerker, H.O. (eds.) *Models for Translation Pain Research*. IASP Press Seattle, 2012;
6. J. Tode, **I. Kirillova**, V. Rausch, R. Baron, W. Jänig. Mechano- and thermosensitivity of lesioned muscle afferents 20 to 80 days after injury. Manuscript in preparation, 2012;
7. **I. Kirillova**, W. Jänig. Responses of injured muscle afferents to the TRP agonists capsaicin, menthol and mustard oil. Manuscript in preparation, 2012;
8. **I. Kirillova**, W. Jänig. Afterdischarge of myelinated muscle afferents to low frequency electrical stimulation and its inhibition blockade sodium channels. Manuscript in preparation, 2012.

Conference contributions:

1. M. V. Soboleva, **I.Y. Kirillova**. Social adaptation of two years old children depending on time of first nursing. Conference: «Mother. Child. Family: Modern problems», SPb, 2003, 365-367 p;
2. M. V. Soboleva, **I.Y. Kirillova**. Social adaptation and their emotional development. Conference: «The child's health as a cultural value. Child in a modern world. The childhood and a culture», SPb, 2003, 24-27 p;

3. **I. Kirillova**, V. Rausch, J. Tode, N. Gorodetskaya, R. Baron, W. Jänig. Ectopic discharges of lesioned muscle afferent fibers: an electrophysiological study. *Der Schmerz*, Band 21. Supplement. Oktober 2007, P6.6, p. 95;
4. **I. Kirillova**, N. Gorodetskaya, R. Baron, W. Jänig. Ectopic activity in muscle regenerating afferent nerve fibers following nerve crush lesion in the rat. *Acta Physiologica*, Vol 192, Supplement 663, March 2008, PT12-07, p. 165;
5. **I. Kirillova**, V. Rausch, J. Tode, R. Baron, W. Jänig. Ectopic activity in muscle regenerating afferent nerve fibers following nerve crush lesion in the rat. *NeuPSIG Satellite on Neuropathic Pain to the Glasgow 2008 World Congress on Pain. Programme & Abstract book*. Abstract 13, p. 15;
6. **I. Kirillova**, V. Rausch, J. Tode, R. Baron, W. Jänig. Patterns of ongoing activity in chronically injured muscle afferents. *Third International Congress on Neuropathic Pain, May 2010, Program, Workshop Summaries and Plenary Abstracts*, Abstract 23, p. 23;
7. J. Tode, **I. Kirillova**, V. Rausch, R. Baron, W. Jänig. Discharge characteristics of regenerating muscle afferents in a chronically lesioned nerve model. *Third International Congress on Neuropathic Pain, May 2010, Program, Workshop Summaries and Plenary Abstracts*, Abstract 55, p. 28;
8. **I. Kirillova**, W. Jänig. Responses of cold- or heat-sensitive injured muscle afferent fibers to the TRPV1 and TRPM8 ion channel agonists. *European Journal of Pain. Supplements*, Vol 5, Num 1, September 2011, Abstract T262, p.54.

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Curriculum Vitae

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Erklärung

Erklärung gem. §9 Promotionsordnung

Ich erkläre hiermit,

- a) dass die Abhandlung, abgesehen von der Beratung durch meinen wissenschaftlichen Lehrer und Verwendung der angegebenen Hilfsmittel, nach Inhalt und Form meine eigene Arbeit ist.
- b) dass die Arbeit nicht schon einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat, veröffentlicht worden ist oder zur Veröffentlichung eingereicht wurde.
- c) dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

Teile dieser Arbeit sind bereits veröffentlicht, wurden zur Veröffentlichung in Fachzeitschriften eingereicht oder sind in Vorbereitung, eingereicht zu werden:

I. Kirillova, V. H. Rausch, J. Tode, R. Baron and W. Jänig. Mechano and thermosensitivity of injured muscle afferents. *J Neurophysiol*, V. 105, 2058–2073, 2011a. *WJ und IK (40%) haben die Studie entworfen; IK (70%), VHR und JT haben die Daten erhoben; IK (100%) hat die Daten analysiert und (100%) die Abbildungen hergestellt; WJ und IK (20%) haben das Manuskript geschrieben; WJ und IK (50%) haben weiterhin beim Überarbeiten des Manuskripts mitgewirkt.*

I. Kirillova, A. Teliban, N. Gorodetskaya, L. Grossmann, F. Bartsch, V. H. Rausch, M. Struck, J. Tode, R. Baron, W. Jänig. Effect of local and intravenous lidocaine on ongoing activity in injured afferent nerve fibers. *Pain*, 152 (7), 1562–1571, 2011b. *WJ, NG und IK (20%) haben die Studie entworfen; IK (50%), AT, NG, LG, FB, VHR, MS und JT haben die Daten erhoben; IK (50%) und AT haben die Daten analysiert; IK (50%), VHR und AT haben die Abbildungen hergestellt; WJ und IK (20%) haben das Manuskript geschrieben; WJ und IK (50%) haben weiterhin beim Überarbeiten des Manuskripts mitgewirkt.*

W. Jänig, I. Kirillova. Chronic nerve injury models. In: Arendt-Nielsen, L., Handwerker, H.O. (eds.) *Models for Translation Pain Research*. IASP Press Seattle, 2012 in press. *IK (50%) hat die Daten erhoben; IK (50%) hat die Daten analysiert und (20%) die Abbildungen hergestellt; WJ und IK (20%) haben das Manuskript geschrieben; WJ und IK (50%) haben weiterhin beim Überarbeiten des Manuskripts mitgewirkt.*

I. Kirillova, W. Jänig. Responses of injured muscle afferents to the TRP agonists capsaicin, menthol and mustard oil. Manuscript in preparation, 2012. *WJ und IK (60%) haben die Studie entworfen; IK (100%) hat die Daten erhoben; IK (100%) hat die Daten analysiert und die Abbildungen hergestellt; IK (70%) und WJ haben das Manuskript geschrieben.*

I. Kirillova, W. Jänig. Afterdischarge of myelinated muscle afferents to low frequency electrical stimulation and its inhibition blockade sodium channels. Manuscript in preparation, 2012. *WJ und IK (40%) haben die Studie entworfen; IK (100%) hat die Daten erhoben; IK (100%) hat die Daten analysiert und die Abbildungen hergestellt; IK (70%) und WJ haben das Manuskript geschrieben.*

J. Tode, I. Kirillova, V. Rausch, R. Baron, W. Jänig. Mechano- and thermosensitivity of lesioned muscle afferents 20 to 80 days after injury. Manuscript in preparation, 2012. *WJ und IK (40%) haben die Studie entworfen; IK (50%), JT and VHR haben die Daten erhoben; JT, IK (30%) und VHR haben die Daten analysiert, JT und IK (20%) haben die Abbildungen hergestellt; WJ, JT und IK (20%) haben das Manuskript geschrieben.*

Kiel, den.....