

**Thermo- and mechanosensitivity of intact
and injured cutaneous afferent neurons and
their responsiveness to the transient
receptor potential agonist menthol**

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Summary

Introduction and goals. After a nerve lesion, injured afferent neurons can develop ectopic spontaneous activity, thermosensitivity and mechanosensitivity. This ectopic sensitivity may trigger in patients with nerve lesion burning spontaneous pain and mechanical and/or thermal (cold or heat) hyperalgesia and/or allodynia, cold allodynia being a frequent clinical finding. The mechanisms underlying this enhanced sensitivity to innocuous cold stimuli (allodynia) are poorly investigated. The ectopic activity of the injured afferent axons developing at the lesion site could be explained by the fact that the anterograde axonal transport (from the cell bodies to periphery) of sensory transduction molecules for thermal or mechanical stimuli is interrupted leading to accumulation of these molecules at the injury site. Recently it was reported that the transduction molecules for heat stimuli are also present in the membrane of the intact axons. The axonal cold- and mechano-sensitivity has up to date not been systematically investigated.

This experimental work on adult rats has two goals: **1.** to work out the axonal thermo- and mechano-sensitivity of intact cutaneous afferent neurons; **2.** to test, in intact and injured cutaneous afferent nerve fibres, if non-nociceptive (type 1) cold-sensitive C-fibres are sensitive to the transient receptor potential M8 (TRPM8) agonist menthol, whereas the nociceptive (type 2) cold-sensitive C-fibres and the cold-insensitive C-fibres are menthol-insensitive. Additionally it was tested in the intact afferent fibres whether menthol can activate type 1 cold-sensitive neurons when applied to their axons. The thermo- and mechano-sensitivity were investigated on afferent nerve fibres of the sural nerve in anaesthetized Wistar rats with neurophysiological methods *in vivo*.

Results. 1. In 32 experiments, 118 A-fibres and 109 C-fibres physiologically characterized by their responses to cold, heat and/or mechanical stimulation of the skin were tested for their axonal thermo- and mechano-sensitivity. Almost all non-nociceptive (type 1) cold-sensitive C-fibres could be activated by cold stimuli applied to their axons in the nerve. Axonal cold-sensitivity was present in 36% of the nociceptive cold-sensitive afferent fibres and axonal heat-sensitivity in 34% of the heat-sensitive afferent C-fibres. Responses to mechanical stimulation of A-fibres and C-fibres could be elicited from the skin only and not from the axons. **2.** In 33 experiments, 77 functionally identified afferent C-fibres (30 intact fibres, 47 injured fibres) and 34 functionally characterized A-fibres (11 intact fibres, 23 injured fibres) were tested for their responses to menthol applied to their receptive fields either in the skin or in the nerve. Almost all (32/34) non-nociceptive (type 1) cold-sensitive C-fibres (20 injured fibres, 12 intact fibres) were activated by menthol and practically all (N=18) nociceptive (type 2) cold-sensitive C-fibres, with one exception, were menthol-insensitive. All heat- and/or mechanosensitive C-fibres tested (intact or injured; N=25) were not excited by menthol. With two exceptions (1 injured type 2 cold-sensitive and 1 injured heat-sensitive fibre), all mechano- or heat-sensitive intact or injured A-fibres were menthol-insensitive. Only one out of 20 type 1 cold-sensitive C-axons tested was activated by menthol.

Conclusions. 1. Cold and heat sensitivity of cutaneous afferent neurons is not restricted to the afferent terminals in the skin but can principally extend over the axons in the nerve. Mechanosensitivity is restricted to the afferent endings in the skin. **2.** The intact

and injured cold-sensitive afferents innervating the rat hindlimb skin are low-threshold and can be activated by menthol, or high-threshold, which are menthol-insensitive. **3.** Identification of the mechanism underlying the cold allodynia in patients with nerve injury could lead to discovery of new therapeutic tools for treatment.

Zusammenfassung

Einleitung und Ziele. Nach einer Nervenläsion können geschädigte afferente Neurone ektope Spontanaktivität, Thermosensibilität und Mechanosensibilität entwickeln. Diese ektope Sensibilität kann bei Patienten mit Nervenläsionen brennende Spontanschmerzen und mechanische und/oder thermische (Kälte oder Hitze) Hyperalgesie und/oder Allodynie auslösen. Kälteallodynie ist ein häufiger klinischer Befund bei Patienten mit neuropathischen Schmerzen. Die Mechanismen, die dieser erhöhten Empfindlichkeit auf unschädliche Kältereizung (Allodynie) zugrunde liegen, sind wenig erforscht. Die ektope Aktivität der verletzten afferenten Axone, die sich am Ort der Läsion entwickeln kann, entsteht vermutlich durch die Akkumulation der Transduktionsmoleküle für thermische oder mechanische Reize, die durch den axonalen anterograden Transport vom Zellkörper in die Peripherie transportiert werden, am Verletzungsort. In jüngster Zeit wurde berichtet, dass die Transduktionsmoleküle für Hitze Reize auch in der Membran der intakten Axone vorhanden sind. Die axonale Kalt- und Mechanosensibilität ist bisher nicht systematisch untersucht worden.

Die vorliegende experimentelle Arbeit an erwachsenen Ratten hat zwei Ziele: **1.** Untersuchung der axonalen Thermo- und Mechanosensibilität von intakten kutanen afferenten Neuronen. **2.** Untersuchung an intakten und verletzten afferenten Nervenfasern der Haut, ob nicht-nozizeptive (Typ 1) kältesensible C-Fasern durch den transienten Rezeptorpotenzial M8 (TRPM8)-Agonisten Menthol aktiviert werden können, während die nozizeptiven (Typ 2) kältesensiblen C-Fasern und die nicht kältesensiblen C-Fasern gegenüber Menthol unempfindlich sind. Zusätzlich wurde untersucht, ob Menthol Typ-1 kältesensible Neurone aktivieren kann, wenn es an ihren Axonen appliziert wird. Die Thermo- und Mechanosensibilität wurden an afferenten Nervenfasern des Nervus suralis in narkotisierten Wistar-Ratten mit neurophysiologischen Methoden *in vivo* untersucht.

Ergebnisse. 1. In 32 Experimenten wurden die axonale Mechano- und Thermosensibilität an 118 A-Fasern und 109 C-Fasern, die durch ihre Reaktionen auf Kälte, Hitze und/oder mechanische Stimulation der Haut physiologisch charakterisiert worden sind, untersucht. Fast alle nicht-nozizeptiven (Typ 1) kältesensiblen C-Fasern konnten durch Kältereizung ihrer Axone aktiviert werden. Axonale Kältesensibilität war in 36% der nozizeptiven kältesensiblen afferenten Fasern vorhanden und axonale Hitzesensibilität in 34% der hitzesensiblen afferenten C-Fasern. Erregung von A-Fasern und C-Fasern auf mechanische Reizung konnte praktisch nur von der Haut ausgelöst werden, nicht aber von deren Axonen. **2.** In 33 Experimenten wurden 77 funktionell identifizierte afferente C-Fasern (30 intakte Fasern, 47 verletzte Fasern) und 34 funktionell charakterisierte A-Fasern (11 intakte Fasern, 23 verletzte Fasern) auf Menthol getestet, das an den rezeptiven Feldern in der Haut oder im Nerv appliziert wurde. Fast alle (32/34) nicht-nozizeptiven (Typ 1) kältesensiblen C-Fasern (20 verletzte Fasern, 12 intakte Fasern) wurden von Menthol aktiviert, während praktisch alle (N=18) nozizeptiven (Typ 2) kältesensiblen C-Fasern, mit einer Ausnahme, unempfindlich gegenüber Menthol waren. Alle getesteten hitze- und/oder mechanosensiblen C-Fasern (intakt oder verletzt; N=25) wurden nicht durch Menthol beeinflusst. Mit zwei Ausnahmen (eine verletzte Typ-2 kältesensible und eine verletzte hitzesensible Faser) konnten alle mechano- oder hitzesensiblen intakten oder

verletzten A-Fasern nicht durch Menthol beeinflusst werden. Nur eines von 20 getesteten Typ 1 kältesensiblen C-Axonon wurde von Menthol aktiviert.

Schlussfolgerungen. **1.** Kälte- und Hitzesensibilität kutaner afferente Neurone ist nicht auf die afferenten Endungen in der Haut beschränkt, sondern kann sich auch über die Axone im Nerven erstrecken. Mechanosensibilität ist auf die afferenten Endungen in der Haut beschränkt. **2.** Die intakten und verletzten kältesensiblen Afferenzen zur Haut der Rattenpfote sind niederschwellig und können durch Menthol aktiviert werden, oder hochschwellig, die nicht durch Menthol aktiviert werden können. **3.** Die Identifizierung der Mechanismus, die der Kälteallodynie zugrunde liegt, könnte zur Entdeckung neuer therapeutischer Mittel führen.

List of Abbreviations

ADC	Analog-to Digital Convertor
ADM	A δ -mechanical nociceptive fibres
ADMC	A δ -mechano-cold nociceptive fibres
ADMH	A δ -mechano-heat nociceptive fibres
ASIC	Acid Sensing Ion Channel
ATP	Adenosine Triphosphate
BK	Bradykinin
CCI	Chronic Constriction Injury
CCK	Cholecystokinin
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin Gene Related Peptide
CV	Conduction Velocity
DRG	Dorsal Root Ganglia
ECG	Electrocardiography
ENaC	Epithelial Na channel
GDNF	Glial Cell Line Derived Neurotrophic Factor
IB4	Isolectin B4
ip	intraperitoneal
iv	intravenous
NGF	Nerve Growth Factor
NPY	Neuropeptide Y

P2X3	Purino (ATP) receptor
PGE2	Prostaglandin E2
PI (4, 5) P2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase C
RA	Rapidly Adapting (mechanoreceptors)
RTX	Resiniferatoxin
SEM	Standard Error of the Mean
SP	Substance P
ST	Skin Temperature
TG	Trigeminal Ganglia
TM	Transmembrane
TP1	Testing Period 1
TP2	Testing Period 2
TRP	Transient Receptor Potential
TRPA	Ankyrin Receptor
TRPA1	Ankyrin Receptor type 1
TRPC	Canonical Receptor
TRPM	Melastatin Receptor
TRPM8	Melastatin Receptor type 8
TRPML	Mucolipin Receptor
TRPP	Polycystin Receptor
TRPV	Vanilloid Receptor
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)
VIP	Vasoactive Intestinal Peptide

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Chapter 1

Introduction

Neuropathic pain is caused by a lesion or disease of the peripheral or central nervous system. The causative factors might be metabolic, viral or mechanical nerve lesion. Specific to neuropathic pain is its burning, stinging, shooting character as well as a mechanical and/or thermal (heat and cold) allodynia (pain response to normally innocuous stimuli) and/or hyperalgesia (aggravated pain response to normally painful stimuli). Cold allodynia is a common complaint in patients with peripheral neuropathies and also was frequently observed in animal models of neuropathic pain (Zimmermann, 2001). However, cold sensitivity of the different types of sensory afferents in injured nerves is poorly investigated. Previous studies (Matzner and Devor, 1987; Blenk et al., 1996; Gorodetskaya et al., 2003; Grossmann et al., 2009a,b; Jänig et al., 2009) have demonstrated the presence of cold-sensitive fibres in experimental neuromas whose activity may contribute to the abnormal cold sensations that follow nerve injury. The molecular transduction mechanisms and the types of ion channels underlying the cold sensitivity are at this moment still poorly understood.

1.1 Neuropathic pain

In our laboratory, the conditions of neuropathic pain are simulated on animal models that underwent peripheral nerve lesions. Following peripheral nerve lesion sensory neurones become disconnected from their targets and the normal retrograde supply of trophic factors (e.g., NGF and GDNF) from the periphery is disrupted. The receptor molecules and ion channels follow the anterograde axonal transport to the sprouts of the regenerating nerve fibres. Here they accumulate and are incorporated into the cell membrane, thus causing ectopic excitability to mechanical/thermal stimulation (Gorodetskaya et al., 2003;

Grossmann et al., 2009a,b; Jänig et al., 2009). This leads to morphological, biochemical and physiological changes in injured afferent neurons (both peripherally and in the spinal cord) and secondary in the central neurons. These changes may become irreversible especially when the regeneration of their axons to the peripheral target cells is impaired.

1.1.1 Morphological changes

Transection of a nerve is followed first by morphological changes of the neurons projecting in the nerve and secondary by central morphological changes. The axons and cell bodies shrink (Jänig and McLachlan, 1984), therefore the conduction velocity of the axons will decrease (Blumberg and Jänig, 1982). Between 30-60% of the postganglionic neurones and afferent unmyelinated neurones innervating the skin may finally degenerate (Jänig and McLachlan, 1984; Hu and McLachlan, 2003) and this results in a partial central deafferentiation. The partial denervation of the second-order neurones in the superficial dorsal horn of the spinal cord, in particular lamina I and II, following the death of afferent neurones with unmyelinated fibres, triggers collateral sprouting of medium diameter (A δ) fibres from the lamina I into lamina II that normally has synaptic input exclusively from unmyelinated primary afferents (Köerber et al., 2001). The interconnection of myelinated primary afferents with the second-order neurones of nociceptors may lead to the development of central sensitization and neuropathic pain.

1.1.2 Biochemical changes

After a nerve lesion, the content and the distribution of transducer molecules, channel proteins and neuropeptides change in primary sensory neurones. The expression of transducer molecules which are involved in the transduction of thermal and/or mechanical stimuli in the peripheral nerve endings changed after a nerve lesion. The heat sensitive Vanilloid receptor type 1 (TRPV1) is down-regulated (Michael and Priestley, 1999), while the cold sensitive Melastatin receptor type 8 (TRPM8) is up-regulated after peripheral nerve lesion (Proudfoot et al., 2006). Nerve lesion leads also to down-regulation of the purinergic P2X3 receptors, which are probably involved in the transduction of mechanical stimuli (Nakamura and Strittmatter, 1996; Bradbury et al., 1998; Tsuzuki et al., 2001). The neuropeptides calcitonin gene related peptide (CGRP) and substance P (SP) which are expressed in peptidergic primary afferent neurons with C-fibres are down-regulated after a peripheral trauma. Both peptides are important neurotransmitters and modulators in the spinal cord (Snijdelaar et al., 2000). On the other hand, other peptides such

as vasoactive intestinal peptide (VIP), cholecystokinin (CCK), neuropeptide Y (NPY) or galanin which are synthesised at low levels in normal conditions are up-regulated following axotomy (Hökfelt et al., 1994). This up-regulation may have an important impact on spinal processing of the sensory information in the dorsal horn.

1.1.3 Physiological changes

Following nerve lesion, both myelinated and unmyelinated afferent nerve fibres may develop spontaneous activity, ectopic mechanical excitability and ectopic thermal excitability. The ectopic spontaneous activity may develop either in the injured nerve fibres or in the intact nerve fibres adjacent to the nerves which underwent partial nerve lesion (Matzner and Devor, 1994; Liu et al., 2000; Wu et al., 2001). The spontaneous activity may originate at the lesion site or in the dorsal root ganglion (DRG) (Wall and Devor, 1983). Ectopic ongoing activity is the result of changes in membrane excitability due to modulation or changes in expression of voltage dependent Na^+ , K^+ , Ca^{2+} channels in the neuronal membrane (McCleskey and Gold, 1999). Based on their sensitivity to tetrodotoxin (TTX) two types of Na^+ channels were identified: TTX-sensitive (TTX-S) channels with fast inactivation and slow recovery from inactivation and TTX-resistant (TTX-R) channels with slow activation and inactivation and rapid recovery from inactivation. The expression of TTX-R Na^+ channels is regulated by nerve growth factor (NGF). The under-supply of the nerve with NGF after injury is one reason why the expression of TTX-R Na^+ channels is down-regulated and the expression of TTX-S Na^+ channels is up-regulated (Cummins and Waxman, 1997; Cummins et al., 2000). This change leads to a shortening of the refractory period in the injured nerve fibres, which can lead to a pathological discharge frequency.

Also the expression pattern of K^+ channels, which are important by their hyperpolarizing effect on the stabilization of the membrane potential, is changing. A nerve injury reduces the expression of K^+ channels and this reduction causes a less stable membrane potential that can contribute to ectopic impulse firing (Everill and Kocsis, 1999).

The ectopic mechano- and thermo-sensitivity of injured myelinated and unmyelinated afferent nerve fibres may develop within 4-6 hours after axotomy (Michaelis et al., 1995; Blenk et al., 1996) and may be maintained for periods of months and years, regardless of whether the nerve fibres have the opportunity to regenerate to their targets or not, thus ending in a neuroma of the injured fibres (Blumberg and Jänig, 1984; Gorodetskaya et al., 2003, 2005). After a nerve lesion the receptor molecules and the ion channels are transported by anterograde axonal transport to the sprouts of the regenerating nerve fi-

bres. Here they are integrated into the cell membrane, thus causing ectopic excitability (Gorodetskaya et al., 2003; Grossmann et al., 2009a; Jänig et al., 2009). The ectopic activity may generate central hyperexcitability based on the plastic changes in the central sensory representations. Therefore, discharges in injured and possibly intact afferent nociceptive and non-nociceptive neurons can contribute to various signs of neuropathic pain [(e.g., ongoing pain, mechanical and/or thermal allodynia and/or hyperalgesia, paresthesia, dysesthesia) (Baron, 2008)].

The abnormal spontaneous and evoked activity developed by lesioned afferent neurons can change the excitability of nociceptive neurons in the spinal cord leading to generation of central hyperexcitability.

1.2 Responses of intact cutaneous afferent nerve fibres to physiological (thermal & mechanical) stimulation

In the rat sural nerve, myelinated fibres can be divided dependent on their diameter in $A\beta$ fibres (large diameter) and $A\delta$ fibres (medium diameter). Most $A\beta$ nerve fibres are mechanosensitive consisting of different types of mechanoreceptors: G-hair, field, rapidly adapting (RA), slowly adapting type I and type II (Leem et al., 1993). Very few of these fibres are additionally heat- or cold-sensitive (Jänig et al., 2009). The sural $A\delta$ fibres are nociceptive, low-threshold mechanosensitive (D-hair) or cold sensitive. The nociceptive $A\delta$ -fibres consist of three groups: 1) $A\delta$ -mechanical (ADM) nociceptive fibres, the dominant population, respond only to strong mechanical stimuli; 2) $A\delta$ -mechano-heat (ADMH) nociceptive fibres; 3) $A\delta$ -mechano-cold (ADMC) nociceptive fibres (Leem et al., 1993).

The population of unmyelinated C-fibres can be divided into three groups (Jänig et al., 2009):

1. Mechano- and/or heat-sensitive nerve fibres most of them being nociceptive; few of the mechanosensitive C-fibres are non-nociceptive, having a low-threshold.
2. Nociceptive high-threshold cold-sensitive C-fibres (called also type 2 cold-sensitive C-fibres). They have a high cold activation threshold ($\approx 12^\circ\text{C}$) and exhibit only a few impulses to cold stimuli. Most of them are additionally mechanosensitive (high threshold) and/or heat-sensitive (high threshold). These cold-sensitive C-fibres are silent.

3. Non-nociceptive low-threshold cold-sensitive C-fibres (called also type 1 cold-sensitive C-fibres). Almost all of these C-fibres are only activated by cold stimuli and inhibited by heat stimuli. They exhibit ongoing activity at a skin temperature of about 28-30°C, have a low threshold ($\approx 28^\circ\text{C}$) to cold stimuli and are activated in a graded manner by cooling the skin. They are mechano-insensitive and a few fibres are additionally activated by heat stimuli.

1.3 Responses of injured cutaneous afferent nerve fibres to physiological (thermal & mechanical) stimulation

After injury of a cutaneous nerve, many lesioned afferent A- and C-fibres develop spontaneous activity and ectopic sensitivity to mechanical and/or thermal stimuli applied either to the nerve end neuroma (in case of section and ligation [Blenk et al., 1996; Blumberg and Jänig, 1984; Michaelis et al., 1995, 1999]) or to the nerve distal to the lesion site from which the nerve fibres regenerate (in case of a crush or transection and resuturing of the nerve [Gorodetskaya et al., 2003, 2009; Constantin, 2004; Grossmann et al., 2009a,b; Jänig et al., 2009]). This ectopic excitability is believed to play a major role in the generation of neuropathic pain states.

In the time interval of 7-14 days after sural nerve crush (about 20 mm proximal from the ankle), the afferent nerve fibres may have regenerated into the peripheral sural nerve up to 14 mm or more distal to the lesion site assuming a rate of regeneration of 1 mm/day for C-fibres and a somewhat higher rate for A-fibres (Lisney, 1989; Shea and Perl, 1985; Sunderland, 1978). A third of the C-fibres and about 10% of the A-fibres can still be activated from the lesion site, but no fibre could be activated from the skin by physiological stimuli applied 7-14 days after crush lesion (Gorodetskaya et al., 2003; Grossmann et al., 2009b). Most injured A-fibres are only mechanosensitive, a few are additionally heat- or cold-sensitive. The thermo-sensitivity in A-fibres was always weaker than the mechanosensitivity.

Injured C-fibres exhibit mechano-, heat- or cold-sensitivity, or combinations of these sensitivities. They can be divided into three functional groups (Grossmann et al., 2009a,b; Jänig et al., 2009):

1. Heat- and/or mechano-sensitive C-fibres; the heat-sensitive C-fibres are divided into low-threshold (activation threshold $38.2 \pm 0.39^\circ\text{C}$, range 35-42°C, median 40°C) and

high-threshold fibres (activation threshold $\geq 43^{\circ}\text{C}$; $47.1 \pm 0.28^{\circ}\text{C}$, range $43\text{--}50^{\circ}\text{C}$, median 48°C). The high-threshold fibres are further divided into two subgroups, those having additionally type 1 cold-sensitivity and those lacking type 1 cold-sensitivity. Their activation threshold ranged from about 35 to 50°C , starting from a baseline temperature on the nerve surface of about 28°C ; the mechanosensitive C-fibres are also divided into low-threshold (≤ 10 mN, median = 2 mN) and high-threshold fibres (≥ 20 mN, median = 30 mN). Their mechanical threshold ranged from 0.45 to > 70 mN (median 7.5 mN).

2. Cold-sensitive C-fibres which behave like nociceptive cold fibres (type 2 cold-sensitive fibres): these fibres are silent or show a low rate of ongoing activity at 28°C , have a high threshold (median 5°C) and low maximal discharge rates (2.4 ± 0.4 impulses/second (imp/s)) to cooling. Most of them are also heat-sensitive and/or mechanosensitive. These C-fibres are, apart from their cold sensitivity, functionally indistinguishable from C-fibres with mechano- and/or heat sensitivity only.
3. Cold-sensitive C-fibres which behave like non-nociceptive cold fibres (type 1 cold-sensitive C-fibres). They have a high rate of activity at 28°C on the nerve surface and show graded responses to cooling with maximal discharge rates of 11.5 ± 1.1 imp/s. They show a low threshold ($17\text{--}22^{\circ}\text{C}$) and are inhibited on warming. Almost all of them are mechanoinsensitive and some 40% are excited by heating.

Thus, the three functional groups of intact cutaneous afferent C-fibres are qualitatively and quantitatively preserved in regenerating and regenerated nerve fibres up to 15 months after sural nerve injury. They are present in sural nerve fibres that have regenerated to the skin as well as in sural nerve fibres that can only be activated from the nerve but not from the skin by the physiological stimuli (Grossmann et al., 2009a,b; Jänig et al., 2009; Gorodetskaya et al., 2009).

1.4 Molecular mechanisms underlying the responsiveness to physiological stimuli (TRP channels)

In the last decade, some molecular mechanisms underlying the thermo- and mechanosensitivity of the afferent neurons have been described. Thermo- and mechano-sensitivity are essential sensory functions, which are subserved by a variety of transducer molecules, including those from the Transient Receptor Potential (TRP) ion channel superfamily.

The TRP cation channels, expressed in primary afferent neurons, were recently cloned

and described biochemically and functionally. These experimental investigations have revolutionized our understanding of cutaneous temperature detection. The best-characterized example is the capsaicin- and heat-sensitive TRPV1 receptor (Caterina et al., 1997). Caterina et al. first cloned and characterized TRPV1 as a cation channel directly gated by capsaicin and noxious heat ($\geq 42^\circ\text{C}$). Within the DRG and trigeminal ganglia, TRPV1 is plentifully expressed in peptidergic primary afferent neurons with C-fibres expressing CGRP and SP and in non-peptidergic afferent neurons with C-fibres expressing isolectin B4 (IB4). It is also found within cell bodies of sensory neurons that innervate the viscera, urinary bladder or airways (Birder et al., 2002; Dodt and Zotterman, 1952; Funakoshi et al., 2006; Kollarik et al., 2003; Michael and Priestley, 1999). In addition to noxious heat and capsaicin, TRPV1 is activated and/or modulated by a variety of stimuli involved in nociception and inflammation. The channel is directly gated by resiniferatoxin (RTX, ultra-potent capsaicin analog) and acidic pH (≤ 5.9) (Caterina et al., 1997; Tominaga et al., 1998). Many components of the "inflammatory soup" (mixture of inflammatory mediators such as bradykinin (BK), prostaglandin E2 (PGE2), histamine, serotonin, protons) sensitize TRPV1 so that it is active at body temperature, providing a plausible mechanism for heat hyperalgesia. In vitro studies show that TRPV1 is sensitized by a number of factors generated during inflammation including mild acidification (pH ≈ 6.5), bradykinin, prostaglandin E2, nerve growth factor and adenosine triphosphate (ATP) (Tominaga et al., 1998; Tominaga and Caterina, 2004).

Much less is known about cool-sensitive TRPs, but they are at present the target of intensive research (McKemy, 2005). The transient receptor potential melastatin type 8 (TRPM8) is activated at innocuous cool temperatures ($\leq 28^\circ\text{C}$) and by pharmacological agents such as menthol and icilin (McKemy et al., 2002; Peier et al., 2002), which act as selective (but not totally specific) activators of the channel (Story et al., 2003; Eccles, 1994). Activation of cold-sensitive afferent nerve fibres by menthol has been first shown in the cat tongue (Hensel and Zotterman, 1951). The threshold of TRPM8 is very flexible and regulated by activity of TRPM8 itself, so that it adapts to a range of steady state temperatures and responds to small increments of cooling. TRPM8 was originally detected in the prostate, but is widely expressed, with high levels in sensory neurons (Clapham, 2003). It is expressed in 5-20% of the cells of the dorsal root ganglia (DRG). The cell bodies are small and connected to A δ fibres or C-fibres, but not to large myelinated fibres (McKemy et al., 2002; Peier et al., 2002).

TRPM8 is a nonselective cation channel with relatively high Ca^{2+} permeability ($\text{PCa}/\text{PNa} = 3.3$) and it shows an outwardly rectifying current-voltage (I-V) relationship

like TRPV1. TRPM8 appears to function both as a plasmalemmal Ca^{2+} channel and as an intracellular Ca^{2+} release channel (Turner et al., 2003). The main physiological role of TRPM8 is thought to be a thermosensor in sensory neurons (Patapoutian et al., 2003). Channel agonists (cold, menthol, icilin) shift the voltage dependence of TRPM8 to more negative (physiologically relevant) potentials, similar to the shift in voltage dependence seen for TRPV1 (Nilius et al., 2005; Brauchi et al., 2004; Voets et al., 2004; Weil et al., 2005). Intracellular pH modulates activation of TRPM8 by cold and icilin, but not by menthol (Andersson et al., 2004). Efficient icilin-mediated activation of TRPM8 requires a concomitant increase in intracellular $[\text{Ca}^{2+}]$ (Chuang et al., 2004) and it recently has been shown that phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] is critically involved in the activation of TRPM8 by both cold and menthol (Rohacs et al., 2005). Subsequent to activation of TRPM8, Ca^{2+} influx through the channel was proposed to activate a Ca^{2+} -sensitive phospholipase C (PLC), leading to depletion of PI(4,5)P₂ and consequent decline of channel activity. Importantly, the sensitivity of TRPM8 to PI(4,5)P₂ is dependent on the highly conserved proximal C-terminal TRP domain.

The transient receptor potential type A1 (TRPA1) channel is also expressed in dorsal root and trigeminal ganglia (Story et al., 2003; Nagata et al., 2005). In vivo expression, analyses of DRG show that TRPA1 mRNA is localized within a small subset of nociceptive C-fibres (and possibly thinly myelinated A δ -fibres) marked by CGRP and SP (Story et al., 2003). Accordingly, the TRPA1 population of DRG neurones does not overlap with TRPM8.

TRPA1 is activated by cooling temperatures beginning at 5°C-6°C that are considerably lower than those for TRPM8 (Story et al., 2003; Bandell et al., 2004) and by noxious chemicals such as cinnamaldehyde and mustard oil (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004). However, the role of TRPA1 in physiological cold sensation is currently debated (McKemy, 2005), with some reports of TRPA1 not being activated by cold (Nagata et al., 2005; Jordt et al., 2004) and of normal cold sensitivity in TRPA1 knockout mice (Bautista et al., 2006). In contrast, another study of TRPA1 knockout mice reported attenuated responses to noxious cold (Kwan et al., 2006).

Activation of the TRPA1 channel is Ca^{2+} -dependent and also shows some voltage-dependent properties (Nagata et al., 2005). Surprisingly, TRPA1 exhibits intriguing gating promiscuity, and might be involved in nociception, temperature sensing, as well as mechanical transduction, e.g. hearing (Voets et al., 2004; McKemy, 2005).

Some studies clearly establish that TRPA1 contributes to the pathogenesis of cold hyperalgesia in vivo, a condition common in patients suffering from neuropathic pain

(Story et al., 2003).

1.5 Experimental aims

A. Axonal mechano- and thermosensitivity of afferent neurones

During the first hours after nerve transection afferent axons of peripheral nerves have been shown to develop ectopic discharge properties such as spontaneous activity and evoked thermo- and mechano-sensitivity at the proximal stump (Michaelis et al., 1995; Blenk et al., 1996). This ectopic excitability is believed to play a major role in the generation of neuropathic pain states. The hypothesis explaining the generation of ectopic activity is that after a peripheral lesion, the anterograde axonal transport of sensory transducer molecules is interrupted leading to an accumulation of these proteins at the injured site. The question arises whether the sensory properties observed in injured axons represent new properties or whether they are already present in the axonal membrane. In vivo or in vitro studies (Zimmermann and Sanders, 1982; Blenk et al., 1996; Sauer et al., 2001) have reported axonal heat-sensitivity in afferent C-fibres. Hoffmann et al. (2008) reported that axonal heat-sensitivity of intact cutaneous afferents may become a source of ectopic activity and pain if axonal heat threshold decreases to body temperature, e.g. during tissue inflammation. Until now axonal sensory properties of intact cutaneous afferent neurons have not been systematically studied.

In the first part of this experimental work, the axonal functional properties of intact cutaneous afferent A-fibres and C-fibres dissected from the sural nerve of the Wistar rat are systematically studied. For this purpose, the axonal cold, heat and mechanosensitivity of the intact functionally identified cutaneous neurons are investigated.

B. Responses of cutaneous afferent neurons to menthol

In the rat sural nerve, the regenerating and intact cold-sensitive C-fibres consist of two types (Grossmann et al., 2009b; Jänig et al., 2009): (1) non-nociceptive low-threshold (called type 1) cold-sensitive fibres; (2) nociceptive high-threshold (called type 2) cold-sensitive fibres. Type 1 cold-sensitivity in cutaneous afferent nerve fibres is suggested to be mediated by the transient receptor potential M8 (TRPM8). This channel is also activated by the agonist menthol, known to produce the sensation of cold in humans (Hensel and Zotterman, 1951; McKemy et al., 2002; Peier et al., 2002; Viana et al., 2002; Story et al., 2003; Jordt et al., 2004; de la Peña et al., 2005; Madrid et al., 2006;

Dhaka et al., 2007, 2008). Type 2 cold-sensitivity of afferent fibres is probably mediated by the transient receptor potential A1 (TRPA1) that is also activated by mustard oil or cinnamaldehyde (Story et al., 2003; Story, 2006; Bandell et al., 2004; Jordt et al., 2004; Karashima et al., 2007, 2009). However, the role of TRPA1 in sensing noxious cold needs further clarification. Some other channels such as potassium channels are suggested to be involved in the transduction of noxious cold stimuli, too (Munns et al., 2007). The molecular transduction mechanisms underlying the cold sensitivity are still poorly understood.

The second part of this work is connected to the transduction channels for cold (TRPM8, TRPA1) expressed in the membranes of primary afferent neurons. Using the agonist menthol for activating the TRPM8 channel in the membranes of the afferent fibres, it should be possible, (1), to classify better and more precisely the two groups of cold-sensitive fibres, (2), to find out which type of cold-induced responses are related to true cool-sensitive afferent neurons and to cold-nociceptive afferent neurons and (3), to understand peripheral mechanisms underlying the cold allodynia. For this purpose, the responsiveness of intact and regenerating afferent neurons (6-12 days following crush of the sural nerve) to local application of menthol is investigated. Additionally, the axonal responsiveness of the intact cutaneous neurons to menthol is tested. This experimental work focuses on the following investigations:

1. the responses of intact and regenerating cutaneous afferent neurons with type 1 cold-sensitivity to local application of menthol (TRPM8 agonist);
2. the responses of intact and regenerating afferent neurons with type 2 cold-sensitivity or heat- and/or mechano-sensitivity to menthol;
3. the axonal responses of intact cutaneous neurons to local application of menthol.

Chapter 2

Methods

Neurophysiological experiments were performed on 65 male Wistar rats weighting between 300–520 grams. The animals were housed singularly in plastic cages with sawdust as bedding material and were provided with water and food ad libidum. The experimental protocols were approved by the local Animal Care Committee.

2.1 Nerve lesions and groups of animals

Animals were assigned to the following experimental groups:

1. Group A – Rats with sural nerve lesion

Sural nerve lesion was performed on 23 rats (body weight, 300–450 g) 6–12 days before the final experiments. The animals were anesthetized with pentobarbital sodium (Narcoren, 60 mg/kg, intraperitoneal (i.p.)). Under aseptic conditions, the left sural nerve was exposed 20–25 mm proximal from the ankle. Under visual control through a stereomicroscope, the nerve was crushed with fine watch maker's forceps three times for 10 s each (width of lesion, 1–1.5 mm). In order to recognize later the lesion site, a mark was made with unabsorbent thread at 2–3 mm distance from nerve lesion on the surface of gastrocnemius-soleus muscle. The wound was closed in layers and recovery was uneventful.

2. Group B – Control group

A group of 10 rats without nerve lesions were used as controls. In these rats, physiological (mechanical and thermal) stimuli were applied only on the skin surface (innervation territory of the sural nerve).

3. Group C – Axonal group

Neurophysiological experiments were performed on 32 rats without nerve lesion. In these experiments the physiological stimuli were applied both to the skin and to the sural nerve in order to investigate axonal mechano- and thermosensitivity.

2.2 Anaesthesia and animal maintenance during the experiments

On the day of the final experiments the rats were re-anaesthetised with pentobarbital sodium (Narcofen, 60 mg/kg, i.p). A catheter was inserted into the jugular vein for the administration of fluids and drugs (Fig. 2.1). Additional doses of pentobarbital were given regularly (10–20 mg/kg/h, i.v.) in order to maintain a sufficient level of anaesthesia judged from the absence of spontaneous blood pressure fluctuations. Arterial blood pressure was measured continuously via a catheter inserted into the tail artery. For this purpose a transducer LM-22 provided by List, Darmstadt was used. A controlled administration of drugs and fluids was maintained during the experiment. In this way, the mean arterial blood pressure was always kept higher than 70 mmHg. Blood gases were also measured regularly throughout the experiment (ABL30, Radiometer Copenhagen); an arterial $pO_2 > 100$ mmHg and a pH of 7.4 were considered as a standard for a sufficient respiration. Acid-base status was regularly measured, too.

The trachea was cannulated, the rats were paralysed with Pancuronium (Organon, initially 1 mg/kg, i.v; maintenance with 0.4 mg/kg/h) and artificially ventilated. The ventilation was performed with 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 servo-controlled heating blanket under the rat.

At the end of the experiments, the animals were killed under deep anaesthesia by an intravenous injection of a saturated potassium chloride solution.

2.3 Surgical procedure

The left sural nerve was exposed about 10 mm from the ankle to its junction with the sciatic nerve. The sciatic nerve, containing the sural nerve of the left hindlimb, was isolated from its surrounding tissue over about 10–15 mm at its proximal end. A pool

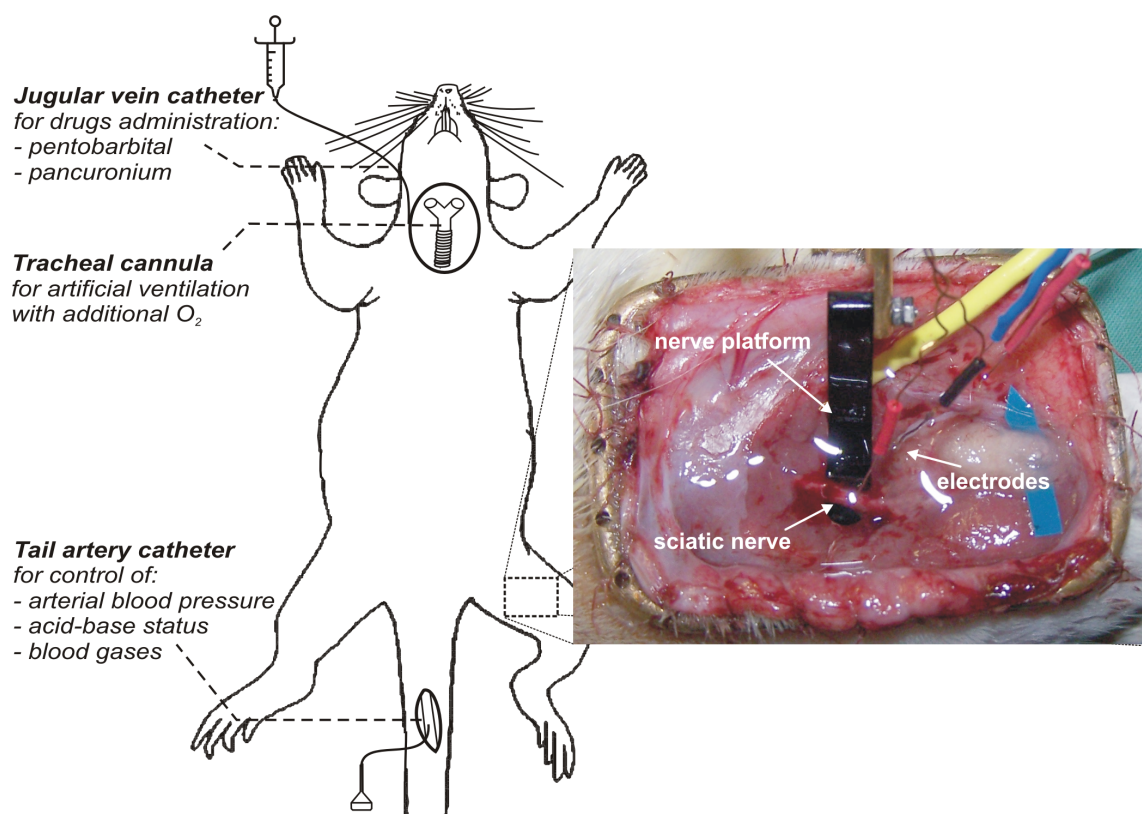


Figure 2.1. Preparation of the animal. The catheter inserted into the jugular vein was used for regular administration of the anaesthetic (Pentobarbital) and the relaxant (Pancuronium). Over a catheter inserted into the tail artery the arterial blood pressure was measured as well as blood samples were 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. The zoom-out picture of the left hind paw shows the original set-up of the experiment. The pool made from the surrounding skin flaps, the sciatic nerve placed on the black platform and electrodes for recording and for electrical stimulation are presented in this picture.

was formed from the surrounding skin flaps and the nerve was covered with 28°C warm paraffin oil. The most proximal site of the sciatic nerve was placed on a rigidly fixed black perspex platform of 5×8 mm² and its epineurium was removed (Fig. 2.1).

2.4 Recording and electrical stimulation

Under a stereomicroscope fine filaments were dissected with a pair of sharpened watch maker forceps from the sural nerve and put on the recording electrode. About 15 to 25 mm distal to the recording site the sural nerve was isolated over a distance of about 6

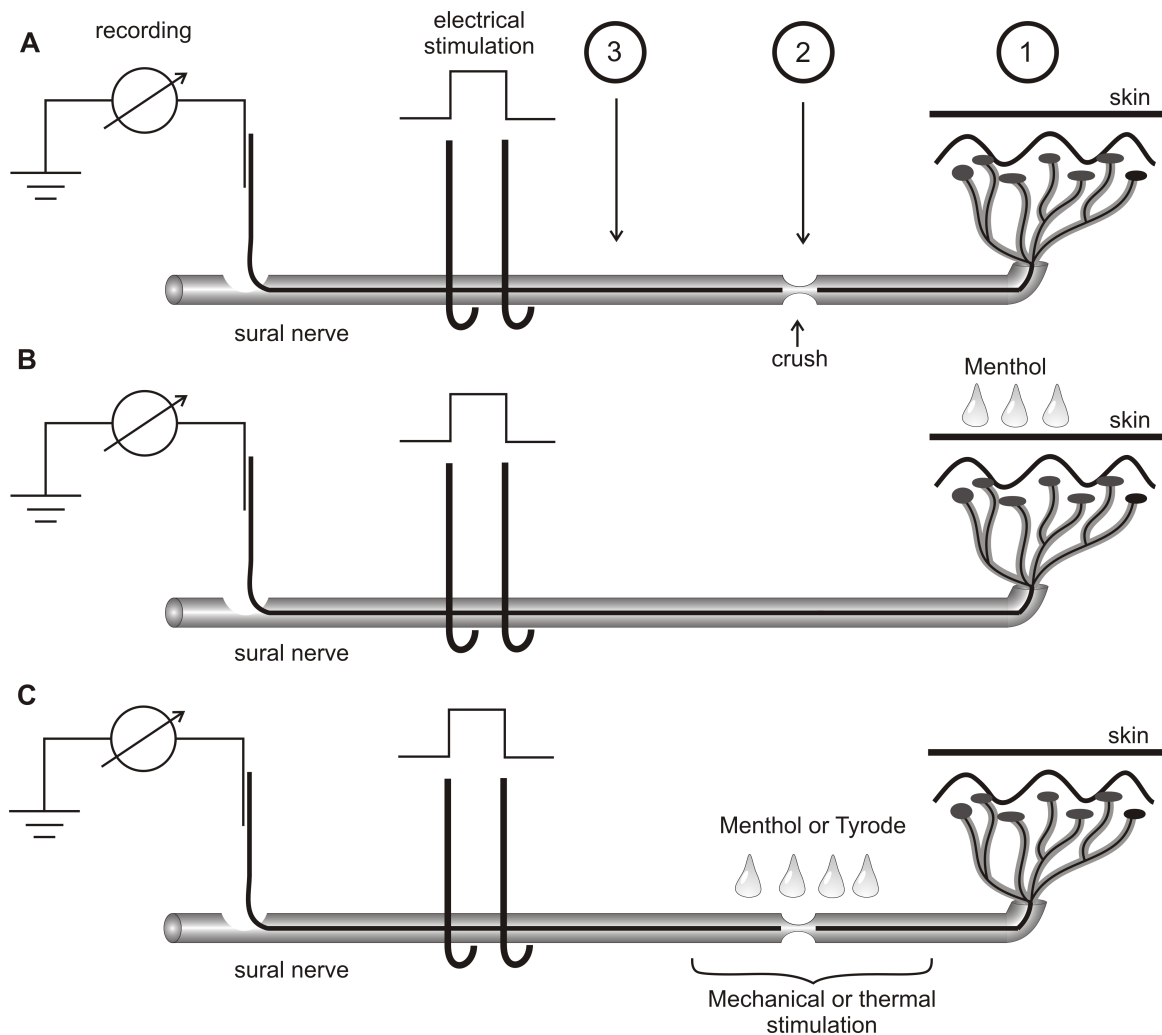


Figure 2.2. Schematic representation of the sural nerve fibre preparation. Fine filaments teased from the sural nerve were put on a platinum recording electrode to measure the fibres activity; the reference electrode was connected to the nearby tissue. The sural nerve was isolated from the surrounding tissue about 15 to 25 mm distal to the recording electrode and put on a pair of electrodes for electrical stimulation and identification of the nerve fibres as C- and A- fibres. **A.** Afferent A- and C-fibers isolated and recorded from the sural nerve were physiologically characterized by their responses to mechanical and thermal (cold, heat) stimuli applied to the skin (site 1) and tested for their responses to the same stimuli applied directly to the sural nerve between sites 2 and 3 on a length of about 14–15 mm. The nerve was stimulated successively on 5 locations being about 2–3 mm apart. After this testing procedure the sural nerve was crushed at site 2 in 20 experiments. After nerve crush the responses of the same nerve fibres to mechanical and thermal stimulation of the nerve proximal to the crush site between sites 2 and 3 were tested again. **B.** Mechanical and thermal stimuli were applied to the sural nerve innervation territory of the hind paw. After the afferent fibres were physiologically characterized, menthol was applied to their cutaneous receptive fields. **C.** In the rats which underwent a chronic nerve lesion, the physiological (thermal and mechanical) stimuli were applied along the lesioned sural nerve at the area of sprouting of the nerve fibres (lesion site) and both proximal and distal to the lesion site. In the same area, after the physiological characterization of the nerve fibres, Menthol's or Tyrode's solution was applied.

mm from the surrounding tissue and put on a pair of electrodes for electrical stimulation (Fig. 2.2). The sural nerve was electrically stimulated with square wave pulses of 0.1 ms (A-fibres) or 0.5 ms (C-fibres) duration at a frequency of 0.3, respectively 3 Hz and with variable intensities up to 40 V. Recorded fibres were identified as A- or C-fibres by electrical stimulation of the sural nerve (Fig. 2.3). Some fibres could not be clearly identified by this method and they were classified based on their size, shape and duration of the action potential as A- or C-fibres. The last procedure turned out to be reliable with a probability of 90 to 95% (Michaelis et al., 1994; Blenk et al., 1996). Sometimes the afferent fibres were classified as A- or C-fibres on the basis of the size of the action potential elicited by physiological stimulation in relation to the size of the action potentials elicited by electrical stimulation of the sural nerve. Thus, when a fibre generates action potentials induced by natural stimulation larger than the C-fibre action potentials induced by electrical stimulation the fibre was classified as an A-fibre. Or when a fibre generated action potentials induced by natural stimulation that were smaller than the A-fibre action potentials induced by electrical stimulation the fibre was classified as a C-fibre. Conduction velocity (CV) of the myelinated and unmyelinated fibres was calculated from the latency of the response to single pulse electrical stimulation and the distance between recording and proximal stimulation electrode.

Action potentials were recorded unipolarly using a pair of platinum wire electrodes with the reference electrode connected to the nearby tissue. The signals were 10000 times amplified using an AC and a differential amplifier (10 M Ω input impedance). Further on, the signals were filtered by a bandpass filter between 120 Hz to 1–1.2 kHz for unmyelinated fibres and between 120 Hz to 40 kHz for myelinated fibres. The network interferences (50 Hz) were filtered by a noise reduction system (Hum Bug, AutoMate Scientific[®]). Action potentials were separated from other activity by means of a window discriminator. The output of the window discriminator was also fed into a loud-speaker, so that the researcher can "listen" to the electrical activity picked up by the electrode. The neural signals were analog-digital converted using the System Spike II (Cambridge Instruments) and recorded by a computer. Action potentials were displayed in real time on an oscilloscope (Tektronix, Oregon, USA) (Fig. 2.4). The arterial blood pressure, electrocardiography (ECG), intratracheal pressure and thermal stimuli recorded simultaneously with the neural activity were fed into a computer and stored on disc for off-line analysis.

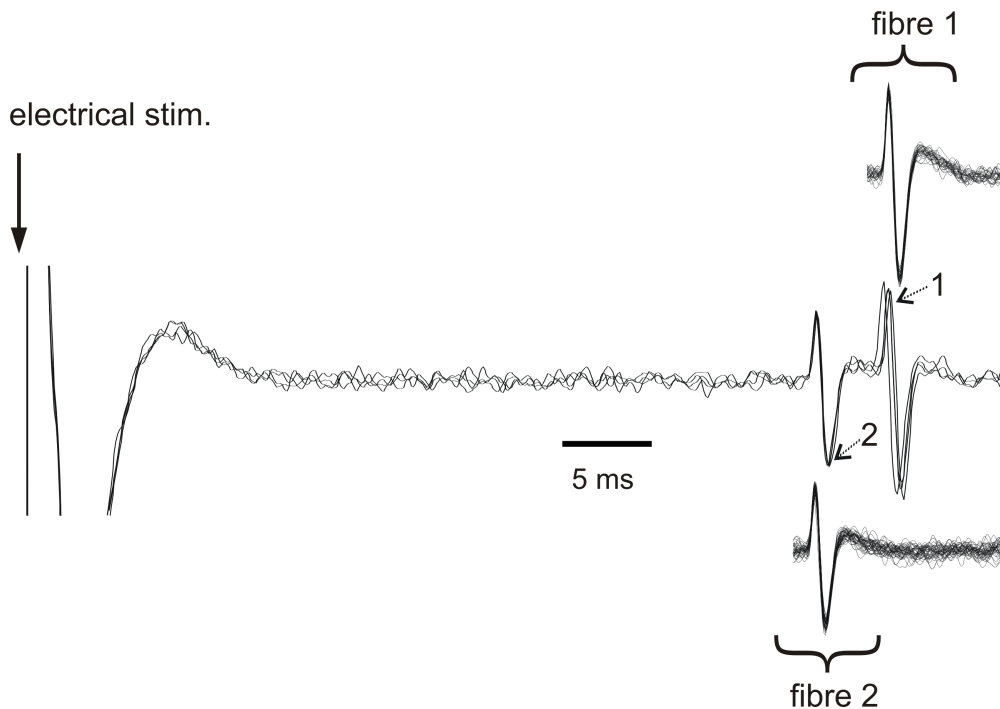


Figure 2.3. Identification of two C-fibres to electrical stimulation of the sural nerve. In the middle record the response of the fibres to nerve stimulation is shown. In the upper and lower records the response of fibre 1 (upper record) and fibre 2 (lower record) generated by cold stimulation of the skin was depicted. Note that the shapes of the electrically and physiologically evoked action potentials are identical. Several action potentials are superimposed.

2.5 Physiological stimulation of afferent fibres

In the animals with sural nerve injury, mechanical and thermal stimuli were applied to the nerve at or distal to the lesion site. In the control animals without sural nerve injury physiological stimuli were applied only to the hind paw area innervated by the sural nerve. In 32 experiments, in which the axonal sensitivity was tested, physiological stimuli were applied first to the innervation territory of the sural nerve and after that along the sural nerve.

2.5.1 Mechanical stimulation

Mechanosensitivity of injured and intact sural nerve fibres (axonal group) was tested with a fine-tipped blunt glass rod applied to the nerve at or distal to the lesion site for lesioned fibres and along the nerve for intact fibres under visual control using a stereomicroscope. Phasic mechanical stimuli were applied to the sural nerve in order to identify the loca-

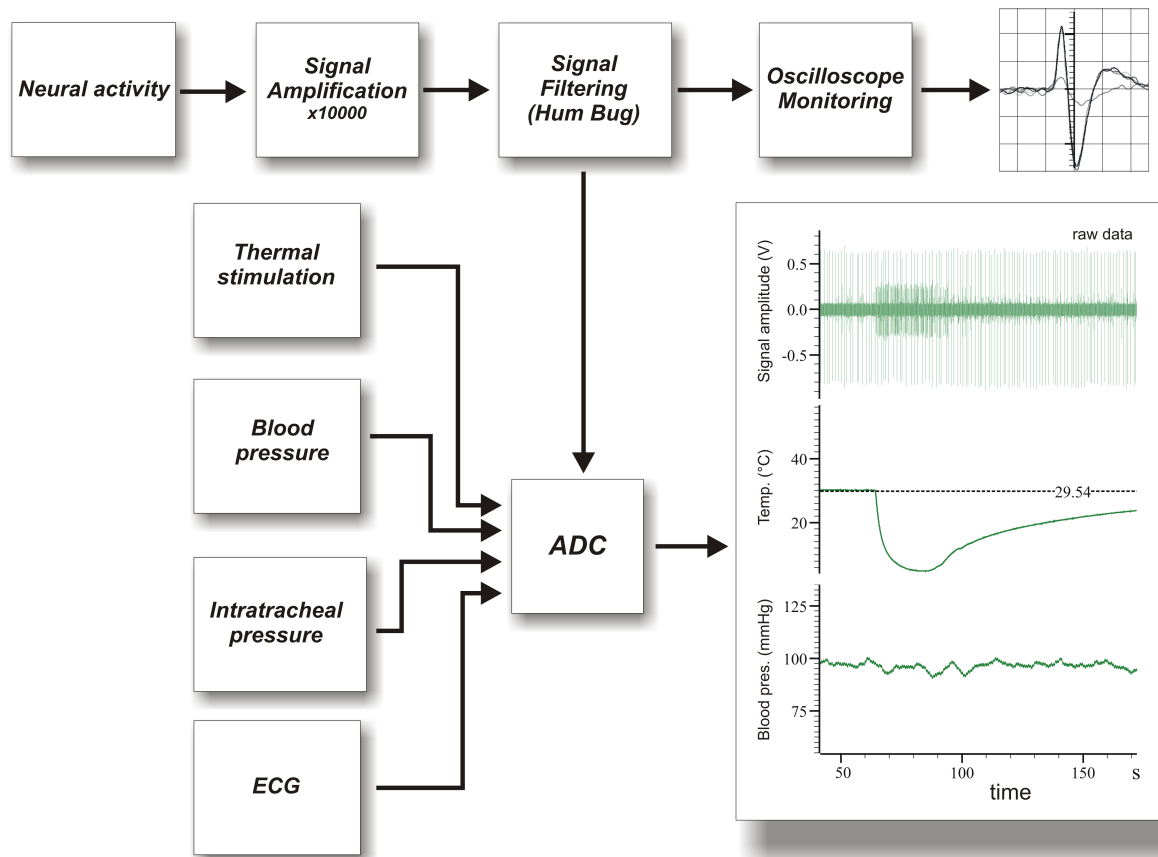


Figure 2.4. Set-up for recording of neuronal activity. Neural activity (action potentials) recorded from the afferent nerve fibres was amplified, filtered and fed into a computer using the Spike II System. Action potentials, temperature of the thermal stimuli, the marker for stimuli, arterial blood pressure, intra-tracheal pressure and electrocardiography (ECG) were fed into the computer. The signals were also monitored on an oscilloscope. By ADC, analog-to-digital convertor is denoted.

tion of receptive field(s) of individual afferent fibres (Fig. 2.2.C). After identification of the receptive field, the mechanical threshold of the afferent fibres was determined using calibrated von Frey glass-fibre filaments with circular tips of 0.5 mm diameter and forces between 0.45 and 100 mN (MARSTOCKnervtest, Fruhstorfer, Marburg, Germany). Under visual control and by means of a Metronome, the mechanical stimuli were applied with a frequency of 70 strokes per minute over a period of 20–30 seconds. The first stimulus was a von Frey filament with a force of 7.5 mN. In case of activation, the next weaker filament was tested, in case of no response, the next stronger filament was used.

An activation threshold was defined when: 1) a silent fibre or a nerve fibre with rate of ongoing activity below 0.5 imp/s showed at least three additional action potentials during

10 seconds of a mechanical stimulation; 2) a nerve fibre with rate of ongoing activity of > 0.5 imp/s increased the ongoing activity by $\geq 20\%$ during the mechanical stimulation. The strength of the finest filament, at which a nerve fibre was activated, was defined as activation threshold.

In the controls and experiments where mechanical axonal sensitivity was tested, the mechanical stimuli were applied to the skin surface by a fine-tipped glass rod (Fig. 2.2.A, 2.2.B). Light mechanical stimuli were applied first to the skin to excite low-threshold mechanoreceptors, such as hair follicle, field or slowly adapting receptors (Kress et al., 1992; Leem et al., 1993). For identification of the high-threshold mechanoreceptors, strong mechanical stimuli were applied to the skin using a fine-tipped glass rod (strong pressure stimuli) or a forceps (pinch stimuli).

2.5.2 Thermal stimulation

Thermosensitivity of the injured and intact sural nerve fibres (axonal group) was tested first qualitatively using copper rods at temperatures of about $50\text{--}53^\circ\text{C}$ or $3\text{--}5^\circ\text{C}$. The blunt tips of the copper rods had a diameter of 1.5 mm. After identification of the receptive field, the thermosensitive afferent fibres were tested using a U-shaped water-perfused thermode for quantitative testing (Fig. 2.5.A). The surface of the thermode tube contacting the nerve was 2 mm long. Due to thermal conduction the stimulus covered a distance of 4 mm along the nerve. The thermal stimuli were applied at two different sites (the lesion site; distal to the lesion site) on lesioned nerve and at five sites on the intact nerve. For qualitative identification of cold sensitive fibres the thermode was initially perfused with ice water, so that on the nerve a temperature of $3\text{--}5^\circ\text{C}$ was reached. In case of activation, a series of cold stimuli at higher temperatures (10, 15, 18, 20, 25°C) was applied, until a temperature was reached, by which no activation of the afferent fibre was measured. For identification of heat sensitive fibres strong heat stimuli of $50\text{--}53^\circ\text{C}$ were applied first. In case of activation, a series of heat stimuli at lower temperatures (45, 40, 35°C) was applied, until a temperature was reached, by which no more activation was measurable. The duration of the temperature stimuli was 20–25 seconds and the baseline temperature on the nerve was about $28\text{--}30^\circ\text{C}$. A fibre was regarded as thermosensitive when: 1) At least three action potentials were generated during thermal stimulation in silent fibres or fibres with rates of ongoing activity below 0.5 imp/s. 2) The ongoing activity increased by $\geq 20\%$ during stimulation in fibres with rates of ongoing activity > 0.5 imp/s. The lowest/highest temperature, that could activate a nerve fibre using these criteria, was defined as its activation threshold.

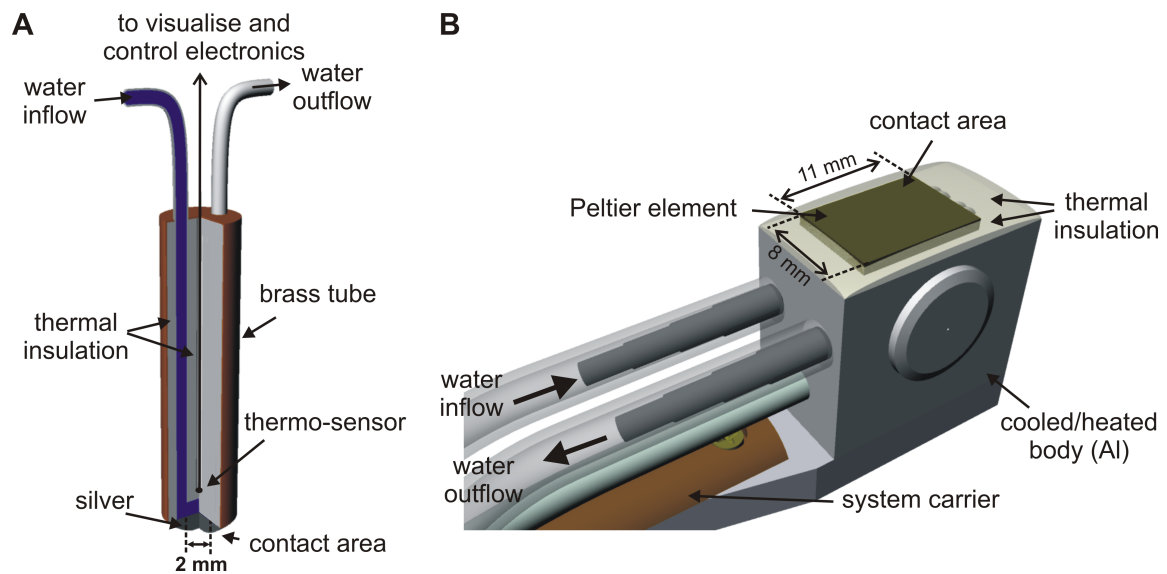


Figure 2.5. Instruments used for thermal stimulation of the nerve and of the skin. **A.** Technical sketch of the thermode used for thermal stimulation of the nerve. The thermode consisted of a U-shaped tube which is inserted in a brass tube of approx. 75 mm in length and 5 mm in diameter. The outer walls of the U-tube are thermally insulated. The thermal insulation in the tube was interrupted at its contact area with the nerve for 2 mm^2 to allow optimal thermal conduction to the nerve. In order to control the temperature at the contact area, water of different temperatures was perfused through the tube. A thermo-sensor of about 1 mm diameter (KTY 21-7, Siemens, Germany) placed in the inner concavity of the U-tube, opposite to the nerve, measured continuously the temperature on the surface of the contact area. **B.** The thermode used for thermal stimulation of the skin. Thermosensitivity of the intact sural nerve fibres was tested using a "home made" thermode based on a Peltier element. An Aluminium body, water perfused, was designed as the cold/heat sink of the Peltier element. In this way cooling and heating of the contact area can be provided with high precision rates. It can deliver temperature ramp stimuli at rates of 0.5°C/s , 1°C/s or 1.5°C/s .

Thermosensitivity of the intact sural nerve fibres (control and axonal experiments) was tested first qualitatively using hot or cold copper rods of about $50\text{--}52^\circ\text{C}$ and $3\text{--}5^\circ\text{C}$, respectively which were applied on the skin. When the receptive field was identified and accessible for the thermode, the thermosensitive afferent fibres were stimulated by temperature ramp stimuli using a Peltier thermode applied to the skin surface (Fig. 2.5.B). The ramp stimuli were delivered at a rate of 1°C/s starting from an adaptation temperature of about 30°C and increasing either to $50\text{--}55^\circ\text{C}$ (plateau of 15 seconds) or decreasing to about 0°C (plateau of 15 seconds).

2.6 Experimental protocols

Menthol ($C_{10}H_{20}O$) is an organic compound that can be extracted from mint plants (peppermint) or made synthetically. Menthol has local anaesthetic and counterirritant qualities, and it is widely used to relieve minor throat irritation. Menthol activates the cold-sensitive TRPM8 channel in primary afferent neurons. It provokes the well-known cooling sensation when it is inhaled, eaten or applied to the skin (Eccles, 1994).

2.6.1 Group A-rats with sural nerve lesion

In the rats with sural nerve lesion, the final experiments were performed 6–12 days after the crush lesion. First it was tested whether a filament isolated from the sural nerve contained fibres with at least one of the following functional properties: cold sensitivity or/and heat or/and mechanosensitivity. The filaments which contained a small number of fibres (3–4 fibres/filament) with sufficient signal-to-noise ratio and at least one of the above-mentioned functional properties were further analysed. In case of a filament which contained a spontaneously active fibre, first the ongoing activity was recorded for 3–5 minutes in order to determine its rate and pattern. This was followed by the first testing period which included a series of thermal and mechanical stimuli applied at the lesion site and 10–15 mm distal to the lesion site (Fig. 2.6). In this way, the individual nerve fibres were characterized within a filament in relation to their ectopic excitability, the respective receptive fields and the activation thresholds. The first testing period lasted between 30–60 minutes.

After the nerve fibres in a filament were fully characterized (first testing period), menthol dissolved in ethanol (0.2% or 0.4% in tyrode) in concentrations of 4 mM or 8 mM was applied to the receptive fields of the afferent nerve fibres, followed by a washout with tyrode solution (physiologic electrolyte solution, pH=7.4). For this purpose, small pieces of filter paper ($4 \times 4 \text{ mm}^2$) soaked in menthol or tyrode solutions were placed on the nerve in 23 experiments with nerve lesion. Menthol was applied for periods of 5 minutes and Tyrode for periods of 4 minutes. In some experiments ethanol solutions of 0.2% or 0.4% were applied to the nerve before menthol application in order to show that ethanol has no influence on the activity of the fibres.

The spontaneous activity was recorded before, during and for 3–5 minutes after application of menthol. Subsequently, in a second test period, the responsiveness to physiological stimulation of the same afferent fibres was examined in the same way as in the first test period (Fig. 2.6). The thermal sensitivity was tested first and then the mechanosensitivity,

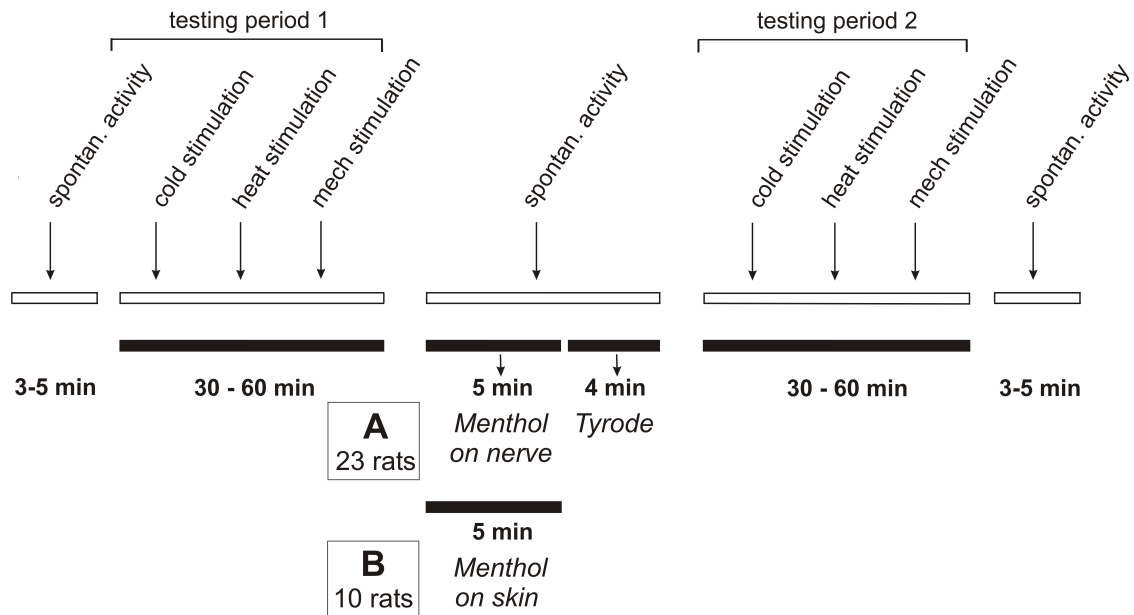


Figure 2.6. Experimental procedure. First the spontaneous activity (if the fibre was spontaneous active) was recorded for 3–5 min. After that a series of cold, heat and mechanical stimuli was applied either to the nerve or to the skin surface to determine the number of activated fibres, their receptive fields and their activation threshold. After the first testing period, menthol was applied either to the nerve (in Group A 23 rats with nerve lesion) or to the skin (in Group B 10 rats without nerve lesion) for five minutes. The application of menthol to the nerve was followed by a washout with tyrode’s solution for 4 min. In testing period 2 a similar series of thermal and mechanical stimuli was repeated as in testing period 1. After that the ongoing activity was measured again for 3–5 minutes to determine any changes in its rate and pattern.

the whole period of testing lasting 30–60 minutes.

When more than one concentration of menthol was applied, the responses of the nerve fibres to the physiological stimuli were tested after every application of menthol. The same series of stimuli as after the first lowest concentration of menthol was applied to the nerve.

The quantitative responses of the afferent nerve fibres to thermal and mechanical stimuli were analyzed offline.

2.6.2 Group B-control group

In the rats without nerve lesion used as controls, firstly the cutaneous innervation field of the sural nerve was explored to locate the receptive fields of the myelinated and unmyelinated fibres using cold, heat and mechanical stimuli. The filament which contained fibres with cold- and/or heat-sensitivity or mechano-sensitivity was analyzed further. For fibres with ongoing activity, first this activity was recorded for about 3–5 minutes. Then, the

cutaneous afferent A- and C- fibres were tested for their responses by cold, heat and mechanical stimuli applied to the skin for about 30 minutes in the first test period (Fig. 2.6). Details about application of the stimuli to the skin can be found under subchapter 2.5. After the afferent nerve fibres were characterized (testing period 1), menthol was applied to their receptive fields in the skin. Small pieces of cotton soaked in menthol which was solved in concentrations of 10%, 20%, 30% or 40% in ethanol (96%) were applied to the surface of the skin in 10 experiments (Fig. 2.2.B, 2.6). Menthol was applied in ascending concentrations. Ethanol (96%) was applied as vehicle in seven experiments (before application of menthol). A radiant lamp for heating the skin in order to suppress the ongoing activity of the low-threshold cold-sensitive fibres was used. The rate of ongoing activity of low-threshold cold-sensitive fibres at 30°C was high. It would have been impossible to observe the effect of menthol on the afferent fibres without suppressing this ongoing activity (see section 3.3.1.1 in Results). To prevent the solution of menthol from evaporation (due to heating by the radiant lamp) the cotton soaked with the menthol solution was covered by a piece of parafilm. Menthol was applied for periods of 5 min. The activity of the afferent fibres was recorded before, during and for 3–5 minutes after application of menthol. The application of menthol was followed by the testing period 2, in which the same afferent fibres were tested again for their responses to thermal and mechanical stimuli applied to the skin. This period lasted for about 30 minutes.

A fibre was regarded as menthol-sensitive if the activity during application of menthol increased by $\geq 20\%$ in fibres which exhibit ongoing activity.

The criteria for sensitization of an intact or injured activated fibre after application of menthol were:

1. The cold answer to the same stimulus was stronger after the application of menthol $\geq 100\%$ than before application of menthol.
2. The cold activation threshold increased by $\geq 4^\circ\text{C}$ after application of menthol compared to the period before application of menthol.

2.6.3 Group C-axonal group

In thirty-two experiments the axonal sensitivity of the afferent fibres to mechanical or thermal stimuli was tested. Firstly, the cutaneous innervation field of the sural nerve was explored in order to locate the receptive fields of the A- and C-fibres using mechanical, heat or cold stimuli (Fig. 2.2.A). The application of these stimuli was described in detail in the previous subchapter (2.5). In case of fibres which exhibited spontaneous activity,

first the spontaneous activity was recorded for 3–5 minutes in order to determine its rate and pattern. Then, the myelinated and unmyelinated fibres were tested for their responsiveness to thermal and mechanical stimuli applied to the skin in order to identify their functional properties.

After the fibres were fully characterized from the skin, a series of thermal and mechanical stimuli were applied to the sural nerve at five approximately equidistant sites (between locations 2 and 3 in Fig. 2.2.A) until a response was elicited or not. In this way, the axonal sensitivity of the nerve fibres to mechanical or thermal stimuli was tested over a length of 14–15 mm of the nerve. Testing of the nerve lasted about 30–35 min.

After this testing procedure, in order to abolish the ongoing activity in non-nociceptive cold-sensitive nerve fibres originating at the nerve terminals in the skin, the sural nerve was crushed at site 2 in 20 experiments while continuing to record from the nerve fibres (Fig.2.2.A). After the (rarely occurring) postinjury discharge in C-fibres, lasting maximally for 15 minutes (Blenk et al., 1996) the afferent fibres were tested again for their responses to mechanical and thermal stimuli applied to the axons. This testing protocol was repeated 1-3 times over periods of 30 min to maximally 4.5 h.

In some experiments, the afferent fibres identified functionally from skin and axon or only from axon after acute crushing of the nerve were tested for their responses to menthol applied to their axons in the nerve. Small pieces of filter paper soaked in menthol-ethanol-solutions in concentrations of 4 mM (0.2% ethanol) and 8 mM (0.4% ethanol) were applied to the axons. After removal of menthol, the nerve was washed out with tyrode applied for 4 minutes. In the experiments where the nerve was acutely crushed, the menthol was applied 4-16 mm proximal from the lesion site.

The C-fibres identified as type 1 cold-sensitive (innocuous cold-sensitive) from the skin and showing axonal sensitivity exhibited a high rate of ongoing activity originating from the skin. The effect of menthol applied under these conditions it will be impossible to be observed. In order to depress this ongoing activity, we either crushed acutely the sural nerve or applied warm stimuli of 35–40°C on the cutaneous receptive fields of these fibres (fibres inhibited by heating of the skin).

2.7 Data analysis

Neural activity, temperature of the Peltier thermode, temperature of the thermode for stimulation of the sural nerve, arterial blood pressure, electrocardiography (ECG) and endotracheal pressure were simultaneously fed into a computer using the Spike II System

(Fig. 2.4). Data analysis was performed off-line using the general purpose capture and analysis package Spike II (Cambridge Electronic Design Ltd, 4 Science Park, Milton Road, Cambridge, UK).

The offline analysis performed with the Spike II System enables the identification and discrimination of individual nerve action potentials using the shape and size of the action potentials (Fig. 2.7). For multi-unit recordings, Spike II contains tools for sorting spikes based on the spike waveform shape. All events crossing a threshold are captured. For this purpose a combination of template matching and cluster cutting based on Principal Component Analysis (PCA), user-defined measurements correlations or errors is used to sort spikes into different units. Spikes can also be sorted interactively by simply drawing a line through overdrawn spikes and classifying any which intersect the line. After the spikes were sorted into different units, we can display and analyse every unit separately. The activity of every neuron can be displayed in different modes such as: rate, mean frequency, instantaneous frequency, raster, waveform, etc.

The neural activity during the various stimuli was evaluated quantitatively as follows:

- *spontaneous activity*: the initial rate of spontaneous activity, which was recorded before the first test period was taken as the baseline (Fig. 2.6). Before application of the menthol solution, the spontaneous activity was measured again for about 2 min. After application of menthol and tyrode and before starting with the second test period, the spontaneous activity was recorded for 3–5 min. The spontaneous activity at 10, 20 and 40 min after application of menthol was measured over 1 min.
- *heat-evoked responses*: for every fibre, the activity during the entire duration of heat stimuli (20s) was calculated.
- *cold-evoked responses*: the activity of every fibre was counted for a duration of 20 seconds following the start of the cold stimuli.

The results are expressed as means \pm Standard Error of the Mean (S.E.M.). The paired t-test, the chi²-test, the Wilcoxon matched pairs signed-rank test, the Mann-Whitney U-test and the ANOVA were used for statistical analyses. The level of statistical significance was set at $p < 0.05$.

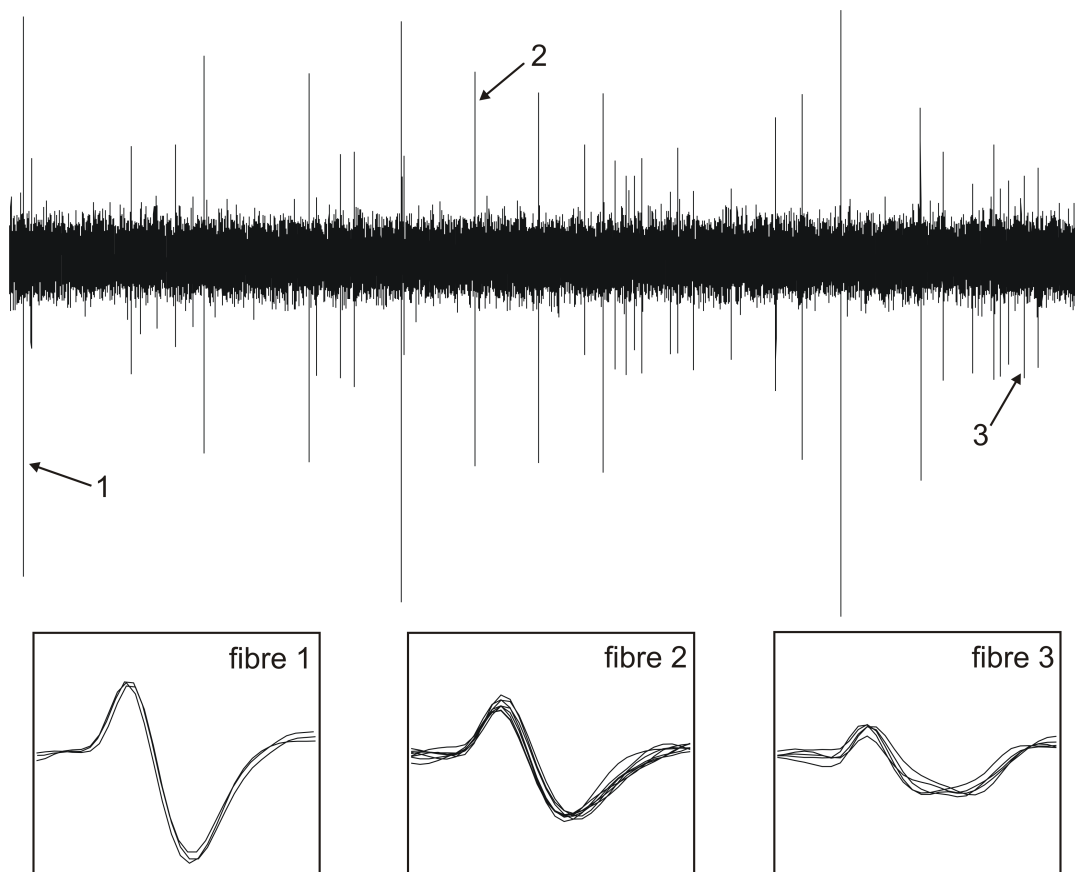


Figure 2.7. Ongoing activity of three C-fibres. The single fibres were discriminated from the row data. The criteria of discrimination in single units were: the size and the shape of the action potentials.

Chapter 3

Results

3.1 Responses of cutaneous afferent nerve fibres to thermal and mechanical stimulation

This work is based on the investigation of 193 afferent C-fibres and 152 A-fibres identified functionally from the skin or injured sural nerve (Table 3.1).

3.1.1 Functional properties of intact nerve fibres

One hundred thirty-nine intact afferent C-fibres and 129 intact A-fibres activated by physiological stimuli applied to the skin were studied (Table 3.1). The quantitative distribution of the response characteristics for thermal and mechanical stimulation of the skin does not represent the real quantitative distribution in the rat sural nerve (Jänig et al., 2009), since in 18 experiments we focused on cold-sensitive fibres and in 24 experiments on heat-sensitive fibres.

3.1.1.1 C-fibres

The population of cutaneous afferent C-fibres analysed in this study was functionally heterogeneous, as has been reported for the intact sural nerve by some groups (Kress et al., 1992; Leem et al., 1993; Jänig et al., 2009). The population of intact C-fibres was divided into three large groups: (1) Innocuous cold-sensitive C-fibres (called type 1 cold-sensitive fibres); (2) Noxious cold-sensitive C-fibres (called type 2 cold-sensitive fibres); (3) Heat- or mechano-sensitive C-fibres (cold-insensitive fibres). The cold sensitivity of the intact C-fibres was divided into type 1 and type 2 cold sensitivity on the basis of the following criteria: threshold response to cooling, rate of ongoing activity at 30°C,

Table 3.1. Numbers of intact and injured A- and C-fibres activated from skin or nerve.

Number of fibres activated from skin or injured nerve		A-FIBRES		C-FIBRES	
		152		193	
		intact	injured	intact	injured
		129	23	139	48
INDIVIDUAL PROPERTIES	Type 1 cold-sensitive	1	0	42	22
	Type 2 cold-sensitive	1	1	35	11
	Heat-sensitive	3	8	70	22
	Mechanosensitive	124	18	36	5
	Spontaneous activity	2	3	45	34
COMBINATIONS OF PROPERTIES	Type 1 cold-sensitive/SA*	1/1	0/0	36/35	17/17
	Type 1 cold- & heat - sensitive/SA*	0/0	0/0	6/5	5/4
	Type 2 cold-sensitive/SA*	1/0	0/0	12/2	8/3
	Type 2 cold- & heat - sensitive/SA*	0/0	1/0	12/0	3/1
	Type 2 cold- & mechano-sensitive/SA*	0/0	0/0	6/0	0/0
	Type 2 cold, heat - & mechano-sensitive/SA*	0/0	0/0	5/0	0/0
	Heat-sensitive/SA*	3/0	4/2	37/3	9/5
	Heat - & mechano-sensitive/SA*	0/0	3/1	10/0	5/3
	Mechanosensitive	124/1	15/0	15/0	0/0
	Spontaneous activity	0	0	0	1

* SA—Spontaneous activity. Numbers behind slash indicate the numbers of fibres with spontaneous activity at 30°C; these fibres are included in the numbers before the slash.

maximal rate of activity to cooling, response to mechanical stimulation and depression of activity to heat stimuli. These two types of cold-sensitivities were described recently

in details for injured regenerating and regenerated C-fibres (Grossmann et al., 2009a,b; Jänig et al., 2009; Gorodetskaya et al., 2009) and for intact C-fibres (Jänig et al., 2009).

Type 1 cold-sensitive C-fibres

In all, forty-two intact C-fibres showed type 1 cold-sensitivity (Table 3.1). Almost all of these fibres were only activated by cold stimuli and inhibited by heat stimuli. They had a low cold activation threshold and a high rate of discharge during cooling of the skin (Fig. 3.1A). With two exceptions, all type 1 cold-sensitive C-fibres showed ongoing activity at a skin temperature of 30°C (Table 3.1). All these fibres were mechano-insensitive. Figure 3.1A, B shows a typical example of an intact type 1 cold-sensitive C-fibre activated by cooling and inhibited by heating.

Type 2 cold-sensitive C-fibres

Thirty-five intact C-fibres were activated by strong cold stimuli applied to their cutaneous receptive fields. Most of these fibres were also heat-sensitive (high threshold) and/or mechanosensitive (high threshold) (Table 3.1). They had a high cold threshold and exhibited only a few impulses to cold stimuli (Fig.3.1C). All type 2 cold-sensitive C-fibres were with two exceptions silent (Table 3.1). Figure 3.1.C, D illustrates the responses of a typical intact C-fibre with type 2 cold-sensitivity activated during cold and heat stimulation of the skin.

Cold-insensitive C-fibres

This category includes fibres which were heat- and/or mechano-sensitive. In all, forty-seven C-fibres were heat-sensitive only or heat- and mechano-sensitive (Table 3.1). They had a high threshold to heat stimuli and with a few exceptions were silent. Figure 3.1F shows the response of an intact C-fibre to heat stimulation of the skin. Twenty-five C-fibres responded to mechanical stimuli only or mechanical and heat stimuli applied to the skin (Table 3.1). Their activation threshold to mechanical stimuli was either high (pressure or pinch stimulation, N=22) or low (hair follicle stimulation, N=3).

3.1.1.2 A-fibres

One-hundred twenty-nine intact A-fibres functionally identified from the skin were analysed (Table 3.1). Almost all intact A-fibres were mechanosensitive only (N=124). These fibres were not further differentiated: (i) in A β -fibres and A δ -fibres; (ii) into different

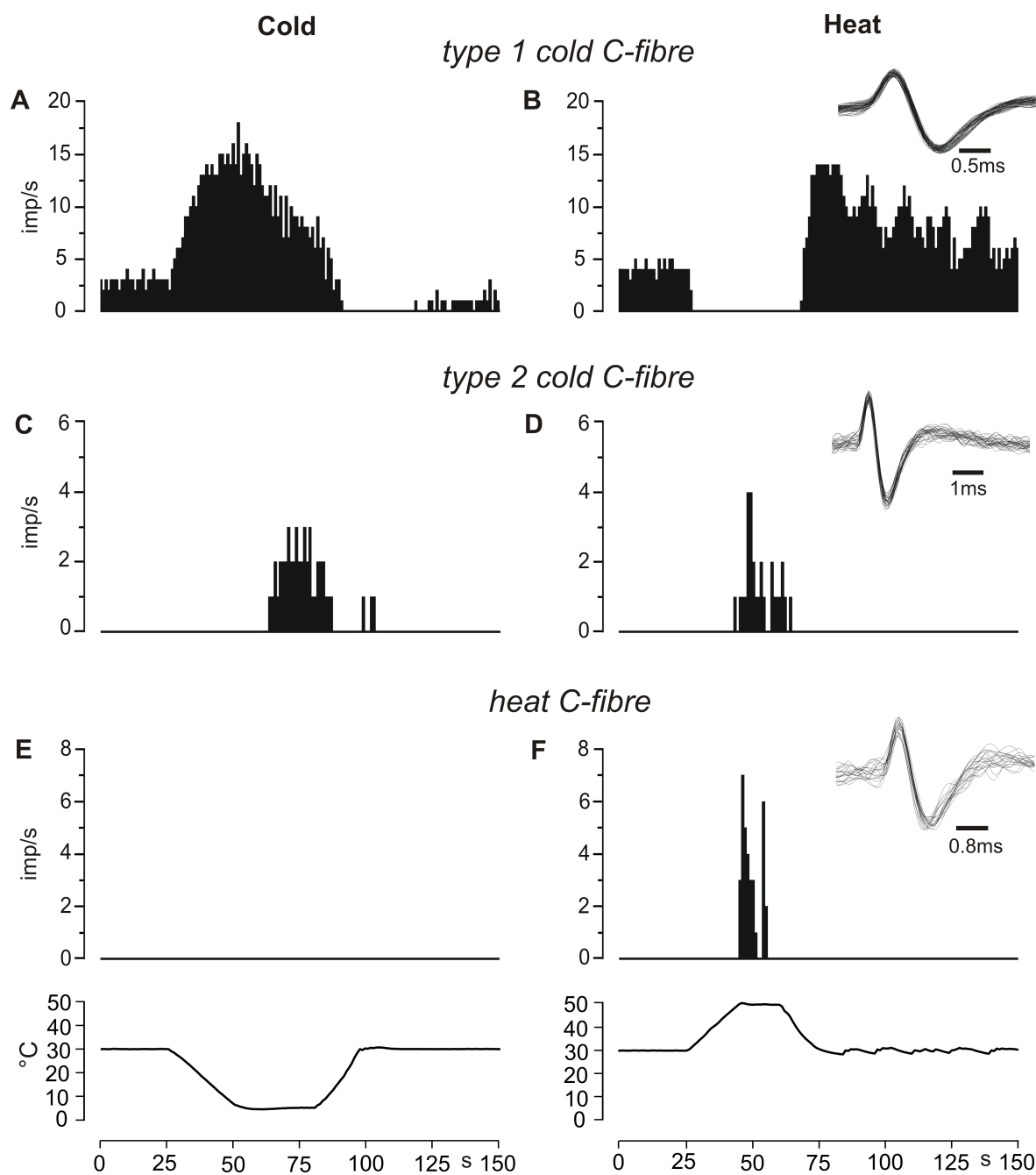


Figure 3.1. Responses of three intact afferent C-fibres to cold and heat stimuli applied to their receptive fields in the skin. The thermal stimuli were delivered by a Peltier thermode at a rate of $1^{\circ}\text{C}/\text{s}$, starting from an adaptation temperature of 30°C and going to 5°C and 50°C , respectively. **A**, **B**. Responses of an intact C-fibre with type 1 cold-sensitivity to cooling and heating of the skin. **C**, **D**. Activation of an intact C-fibre with type 2 cold-sensitivity to cold and heat stimulation of the skin. **E**, **F**. Responses of an intact C-fibre with heat-sensitivity to a heat and cold stimulus applied to the skin. Insets in **B**, **D**, **F**: action potentials recorded from the C-fibres superimposed several times.

types (RA, SA, G-hair). Five fibres were thermosensitive only, 3 were heat-sensitive and 2 cold-sensitive (one type 1 cold-sensitive fibre and one type 2 cold-sensitive fibre). All intact afferent A-fibres were with two exceptions silent.

3.1.2 Functional properties of injured nerve fibres

In total, forty-eight injured C-fibres and twenty-three injured A-fibres activated by physiological stimuli applied to the injured sural nerve were studied. The individual response characteristics of these fibres and their patterns of discharge correspond to our previous results (see Table 3.1; Gorodetskaya et al., 2003; Grossmann et al., 2009a,b; Jänig et al., 2009). The quantitative distribution of these response characteristics for mechanical and thermal stimulation does not represent the real quantitative distribution in the rat injured sural nerve (Grossmann et al., 2009a,b; Jänig et al., 2009), since we concentrated in most experiments on cold-sensitive nerve fibres.

3.1.2.1 C-fibres

Forty-eight injured C-fibres activated by physiological stimuli applied to the injured nerve were analysed in this study (Table 3.1). The functional characteristics as seen in the intact afferent C-fibres are qualitatively and quantitatively preserved in regenerating and regenerated nerve fibres up to 15 months after sural nerve injury (Grossmann et al 2009a, b; Jänig et al 2009; Gorodetskaya et al 2009).

Type 1 cold-sensitive C-fibres

Twenty-two of the ectopically activated C-fibres exhibited type 1 cold-sensitivity (Table 3.1). These fibres were strongly activated by cold stimuli and inhibited by heat stimuli applied to the lesion site of the sural nerve or distally to the injury site (Fig. 3.2A, B). Almost all injured type 1 cold-sensitive C-fibres showed ongoing activity at a nerve temperature of about 30°C. Five out of 22 injured C-fibres with type 1 cold-sensitivity were also activated by heat stimuli. These fibres had a low threshold to cold stimuli and were mechano-insensitive. The responses of an injured afferent C-fibre with type 1 cold-sensitivity to cold and heat stimuli applied to the nerve are illustrated in Figure 3.2A, B.

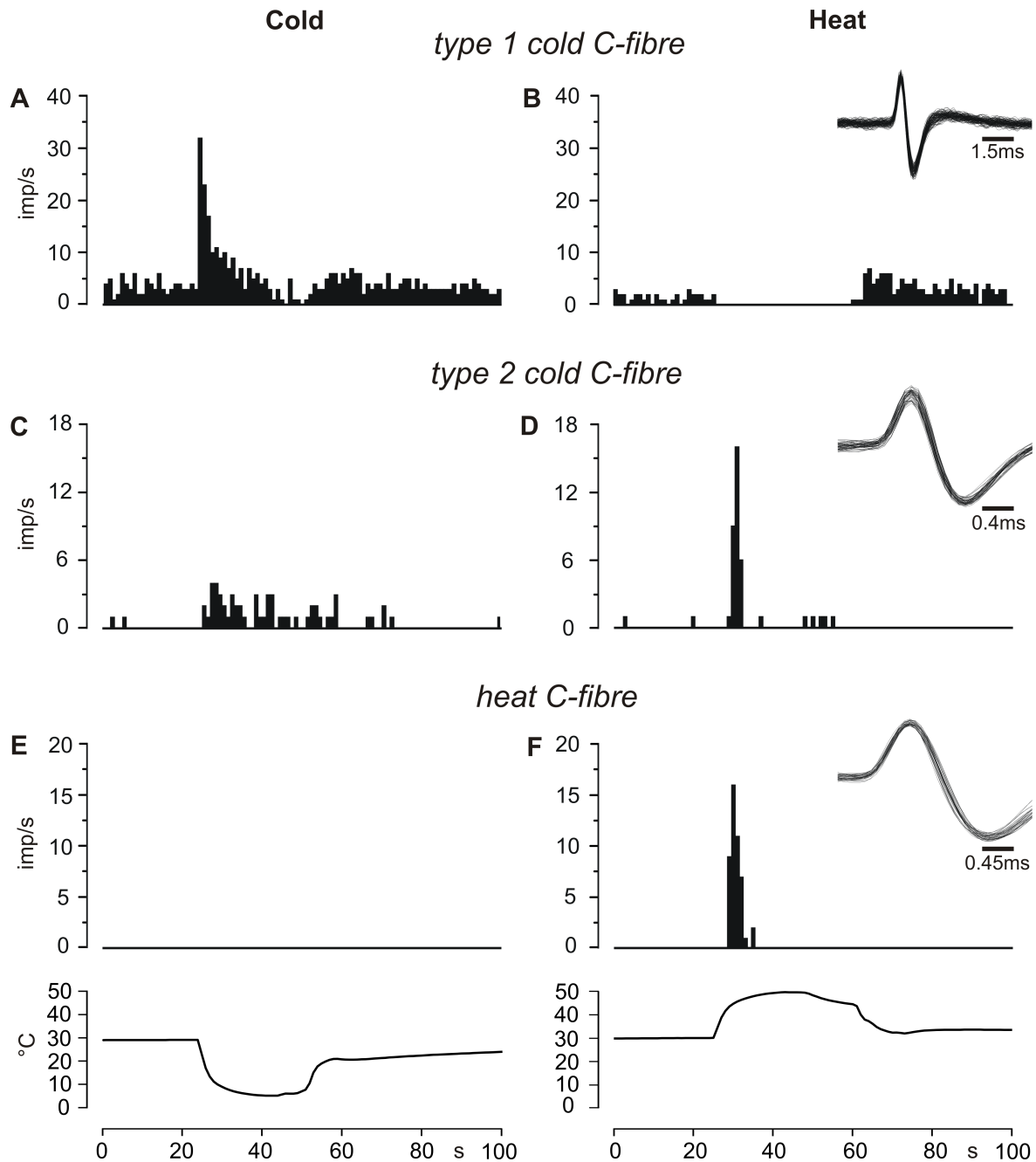


Figure 3.2. Responses of three injured C-fibres to cold and heat stimuli applied to their receptive fields in the nerve. The cold and heat stimuli were applied by a thermode, starting from a baseline temperature of about 30°C and going to 5°C or 50°C, respectively. **A, B.** Responses of a type 1 cold-sensitive C-fibre to cold and heat stimulation of the nerve. **C, D.** Activation of a type 2 cold-sensitive C-fibre to cold and heat stimuli applied to the nerve. The rate of discharge during cold stimuli is lower than the rate of discharge of type 1 cold-sensitive C-fibre. **E, F.** Responses of an injured C-fibre with heat-sensitivity to a heat and cold stimulus applied to the nerve. Insets in **B, D, F:** action potentials recorded from three C-fibres several times superimposed.

Type 2 cold-sensitive C-fibres

Eleven C-fibres were activated by strong cold stimuli applied to the injured nerve (Table 3.1). They had a high cold activation threshold and a low rate of discharge during cold stimuli. Some of these fibres were polymodal fibres being responsive to heat and/or mechanical stimulation of the nerve injury site and showed ongoing activity at a nerve temperature of about 30°C. Figure 3.2C, D shows the responses of a typical injured type 2 cold-sensitive C-fibre to cold and heat stimulation of the nerve. This fibre exhibited some low-rate of ongoing activity.

Cold-insensitive C-fibres

This group includes 14 C-fibres which were heat- and/or mechano-sensitive (Table 3.1). They had a high threshold to heat stimuli and most of them showed ongoing activity. Responses of an injured C-fibre with heat-sensitivity to a cold and a heat stimulus applied to the nerve are depicted in Figure 3.2E, F.

3.1.2.2 A-fibres

Twenty-three injured A-fibres with ectopic activity were investigated in this study (Table 3.1). Eighteen of them were activated to mechanical stimuli applied to the injured nerve. Three of the mechanosensitive fibres were additionally heat-sensitive. All mechanosensitive A-fibres were with one exception silent. Eight injured A-fibres were activated to thermal stimuli applied to the nerve. Seven of them were heat-sensitive or heat- and mechano-sensitive and one was a type 2 cold-sensitive. The thermosensitivity in intact afferent A-fibres and in injured afferent A-fibres was always weaker than the mechanosensitivity.

3.2 Axonal thermo- and mechano-sensitivity of cutaneous afferent neurons

In 32 experiments it was tested if cutaneous afferent myelinated fibres (A-fibres) and afferent unmyelinated fibres (C-fibres) respond to the same natural stimuli applied to their endings in the skin as well as to their axons. The afferent fibres were tested first for their responses to mechanical, cold and heat stimuli applied to the skin and then, these fibres were tested for their responses to the same stimuli applied to the nerve. The stimuli were applied to the nerve at five locations over a length of 15 mm. In some cases, the

nerve was distally crushed after the nerve fibres were characterized by their responses to natural stimulation of the skin and the nerve. After crushing, the fibres were tested again for their responses to physiological stimuli applied to the nerve proximal to the crush lesion.

Table 3.2. Response characteristics of afferent nerve fibres activated from skin or from nerve only.

	A-Fibres		C-Fibres	
	Skin	Nerve only	Skin	Nerve only
Number of fibres	118	3	109	34
Cold type 1 (C1) only	1/1	0	25/24	1/1
C1 and heat	0	0	5/4	1/1
Cold type 2 (C2) only	1	0	9/1	6
Heat only	3	1	31/3	24
Mech only	113/1	2	11	1
C2 and heat	0	0	11	1
C2 and mech	0	0	3	0
Heat and mech	0	0	9	0
C2, heat and mech	0	0	5	0

Numbers behind slash indicate the numbers of fibres with ongoing activity; these fibres are also included in the numbers before the slash. Nerve only: these fibres were activated from the nerve while testing the responsiveness of other afferent fibres that could be activated from the skin. Their receptive fields in the skin were not found.

In 63 filaments, 118 A-fibres and 109 C-fibres activated by physiological stimulation of the skin were investigated. These fibres were characterized by their conduction velocity and/or the shape of their action potentials and by their responses to physiological stimulation of the skin.

The functional properties of the studied afferent fibres are shown in Table 3.2. This table displays the numbers of fibres activated from the skin and from the nerve only by physiological stimulation. The quantitative distribution of the functional characteristics for cold and heat stimulation does not represent the real quantitative distribution in the rat sural nerve (Jänig et al., 2009): in 10 experiments we focused particularly on cold-

sensitive nerve fibres and in 22 experiments on heat-sensitive nerve fibres. Thirty-four C-fibres and 3 A-fibres were only activated from the nerve when we were searching for the axonal responses of fibres identified from the skin. For these fibres we could not find a receptive field in the skin. This observation can be explained by the following reasons: (i) small branches of the sural nerve innervating more proximal skin have been transected during preparation of the nerve; (ii) 11.5% of the sural nerve fibres innervate deep somatic tissues (Gorodetskaya et al., 2009); (iii) we probably missed the cutaneous receptive fields of some of these afferents because they were hidden in inaccessible places for the testing device or because we did not systematically apply strong mechanical pinch stimuli to the skin.

Table 3.3 shows the number of A-fibres and C-fibres activated only from skin or both from skin and nerve by cold, heat and mechanical stimulation. In this table the

Table 3.3. Numbers of afferent A- and C- fibres activated from skin only or from skin and nerve.

	A-Fibres			C-Fibres		
	Total	Skin only	Skin & nerve	Total	Skin only	Skin & nerve
Cold type 1 sensitivity	1	1	0	30	1	29
Cold type 2 sensitivity	1	1	0	28	17	11
Heat sensitivity	3	3	0	61 ^a	40	21
Mechano-sensitivity	113	111	2	28	27	1

^aThis number includes five type 1 cold-sensitive fibres which were also heat-sensitive from the skin, three of them activated from the skin only and two of them from both skin and nerve. Five type 1 cold-sensitive fibres were not activated by heat stimulation of the skin but by heat stimulation of the nerve after crush injury. These fibres are not included under heat sensitivity.

total number of C-fibres is higher than in the table 3.2 because thirty-three C-fibres were activated by more than one type of physiological stimulus.

3.2.1 C-fibres

In total, 109 C-fibres were investigated in this study (Table 3.2). The distribution of cold- (type 1 and type 2), heat- and mechano-sensitivity and the combinations of these functional characteristics in the population of cutaneous afferent C-fibres are shown in tables 3.2 and 3.3.

3.2.1.1 Type 1 cold-sensitive C-fibres

In all, 30 C-fibres have shown type 1 cold sensitivity (Tables 3.2, 3.3). All type 1 cold-sensitive C-fibres were activated by non-noxious cold stimuli and inhibited by heat stimuli applied to the skin. The mean activation threshold to cold stimuli was low: $28.5 \pm 0.17^\circ\text{C}$ (N=25; in five fibres the threshold was not measured). A typical example of a type 1 cold-sensitive C-fibre activated by cold stimulation and inhibited by heat stimulation of the skin is shown in Figure 3.3A, B.

Five of 30 type 1 cold-sensitive C-fibres were activated additionally by heat stimuli applied to the skin following the depression of the ongoing activity. Figure 3.4A, B shows a C-fibre with type 1 cold-sensitivity activated by both cooling and heating stimulation of the skin. The heat activation was weak in three afferent C-fibres (Fig. 3.4B) and strong in two afferents. Almost all fibres with type 1 cold-sensitivity showed ongoing activity at a skin temperature of 30°C (rate, 5.1 ± 0.63 imp/s, N=28). The ongoing activity was completely depressed by heat stimuli of 30.5 - 38°C (Fig. 3.3B, 3.4B). Thus at a skin temperature of about $\geq 30.5^\circ\text{C}$ most of these fibres would probably have been silent. All type 1 C-fibres were not activated by mechanical stimulation of the skin.

Almost all type 1 cold-sensitive C-fibres (97%, 29/30 fibres) were also activated by cold stimuli applied to the nerve (Table 3.3; Fig. 3.3A, B, D). This cold-activation from the nerve could be generated from four to five of the five tested sites. Thus, the axonal cold type 1 sensitivity was distributed evenly along the nerve. The cold-sensitivity from the nerve could be detected only after the ongoing activity (probably generated by the fibre terminals in the skin) was suppressed by warm stimuli applied to the skin (Fig. 3.3B) or after the sural nerve was crushed (Fig. 3.3C, D; Fig. 3.4C). Those fibres activated additionally by heat stimuli applied to the skin (Fig. 3.4B) could also be activated by heat stimuli applied to the nerve (Fig. 3.4D). Five fibres which were not activated by heat stimulation of the skin were activated by heat stimulation of the nerve after the sural nerve was crushed.

A comparison between the mean responses of 12 type 1 cold-sensitive fibres to cold

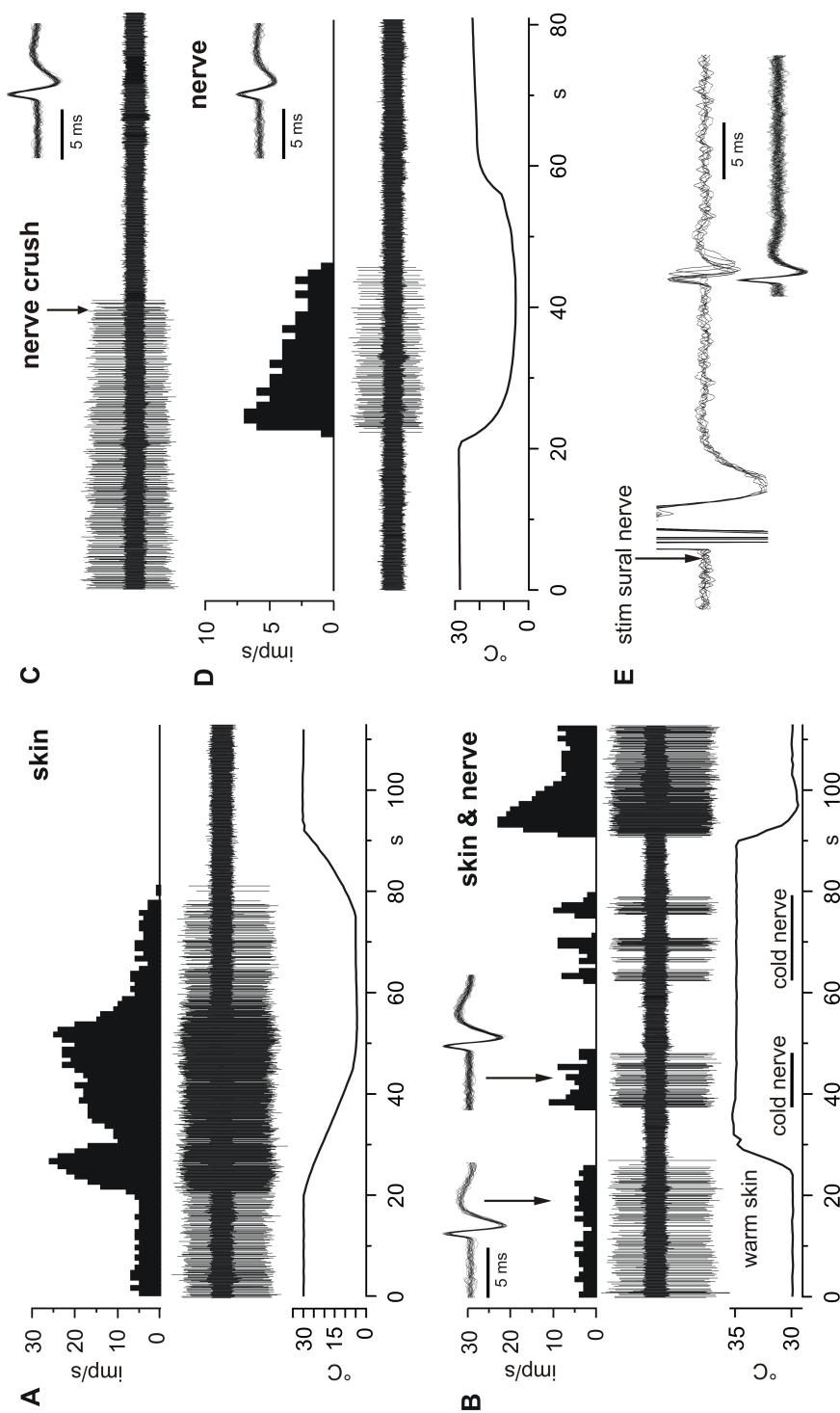


Figure 3.3. Responses of a C-fibre with type 1 cold-sensitivity to cold stimuli applied to the skin or the nerve before and after crushing of the nerve. **A.** Response of the C-fibre to cold stimuli applied to its cutaneous receptive field. The cold stimuli were delivered at a rate of 1°C/s, starting from an adaptation temperature of 30°C and going to a plateau of 5°C. **B.** A heat stimulus was applied on the skin for depression of the ongoing activity. The depression of the activity was followed by rebound activation after the heat stimulus. During the depression of the activity, a cold stimulus was applied on the surface of the sural nerve with a copper metal rod. The afferent fibre was activated by the cold stimulation of the nerve. Insets: action potential elicited from the sural nerve. **C.** The crushing of the nerve was followed by disappearance of the ongoing activity. Inset: action potential elicited from the sural nerve. **D.** Response of the fibre to cold stimulation of the skin is similar with the shape of the action potential elicited from the sural nerve. **E.** Identification of the fibre to electrical stimulation of the nerve at a stimulus strength of 9 volt (pulse duration 0.5 ms). Upper record: activation of the fibre to electrical stimulation of the nerve several times superimposed. Lower record: action potential evoked by cooling the nerve several times superimposed.

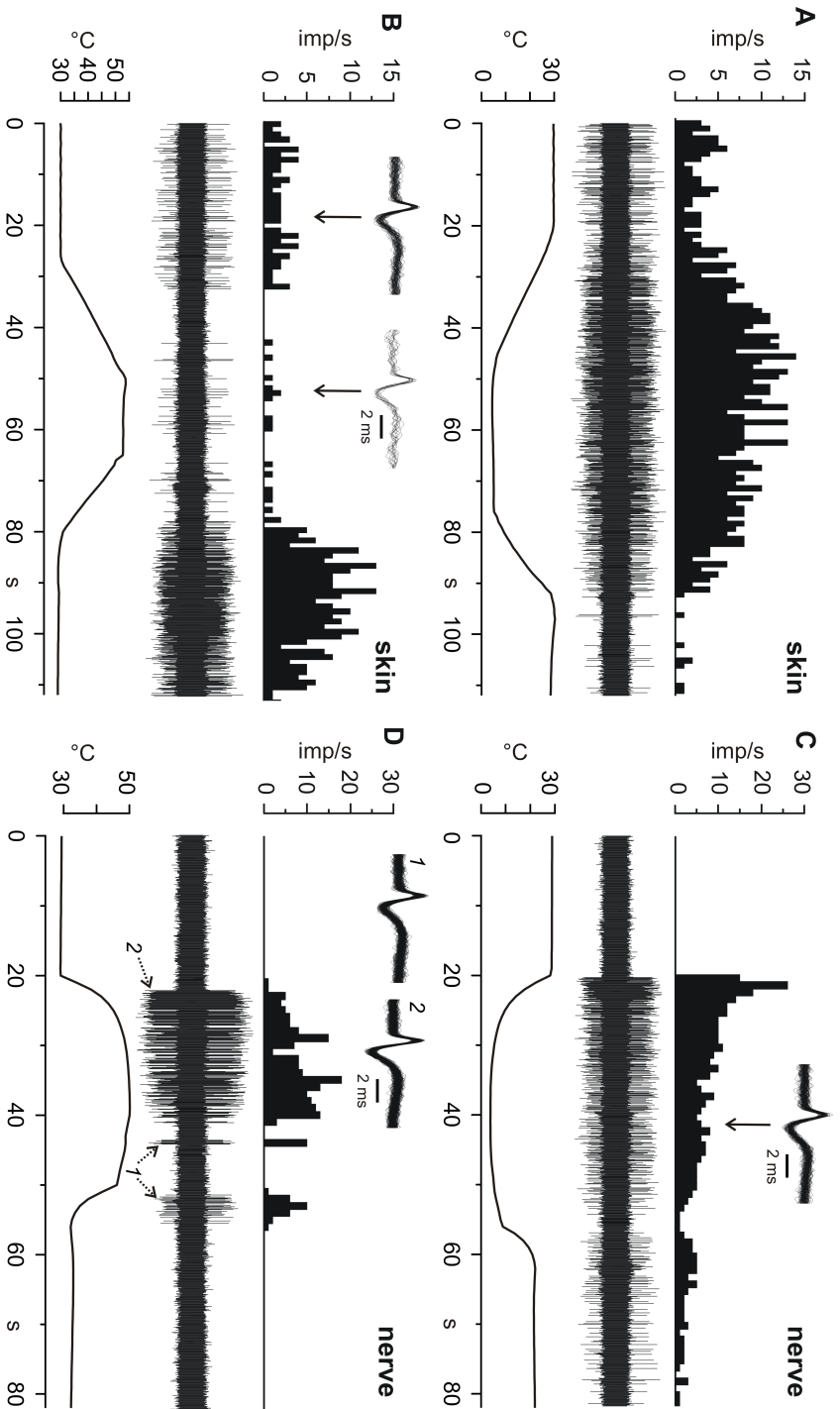


Figure 3.4. Responses of a C-fibre with type 1 cold-sensitivity to cold stimuli applied to the skin or the nerve and to heat stimuli applied to the nerve. **A.** Response of the fibre to cold stimulation of the skin. The cold ramp stimulus was delivered at a rate of $1^{\circ}\text{C}/\text{s}$, starting from a baseline temperature of 30°C and going to a static temperature of 5°C . Activation of the fibre was followed by depression of the activity. **B.** Response of the fibre to heat stimulation of the skin. The heat ramp stimulus started from a preset temperature of 30°C and increased to 55°C . The depression of the fibre during the heat stimulation of the skin was followed by a weak activation during the heat stimulus and rebound activation after the stimulus. Inset: action potential several times superimposed. **C.** Response of the fibre to cold stimulation of the nerve after nerve crush. The cold stimulus was applied by a thermode starting from a baseline temperature of about 30°C and going to 5°C . The response of the fibre to cold stimuli was followed by after-discharge, probably because the nerve temperature was still at about 20°C . **D.** Response of the fibre with type 1 cold-sensitivity (fibre 1) and a new silent fibre (fibre 2) to heat stimulation of the nerve. The heat stimulus started from an adaptation temperature of about 30°C and increased to about 50°C . Insets: action potential several times superimposed recorded from two fibres. The cutaneous receptive field of fibre 2 was not found.

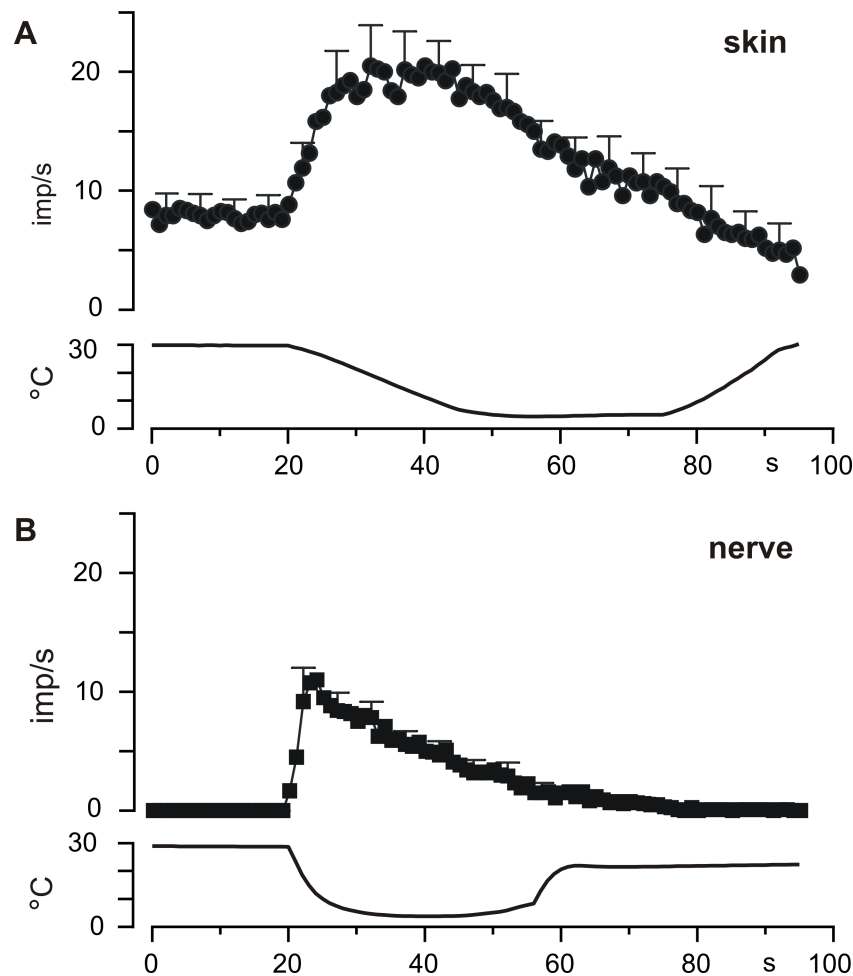


Figure 3.5. Mean responses of 12 type 1 cold-sensitive C-fibres to a cold stimulus of $\sim 5^{\circ}\text{C}$ applied to the skin or the nerve. **A.** Responses (mean+1 SEM) to cold stimulation of the skin. The cold ramp stimulus was delivered at $1^{\circ}\text{C}/\text{s}$ starting from an adaptation temperature of 30°C and going to a plateau temperature of 5°C . The fibres showed ongoing activity at a skin temperature of 30°C and the cold activation started within a few seconds after start of the cold ramp. **B.** Responses (mean+1 SEM) to cold stimulation of the nerve. The cold stimulus was applied by a thermode, starting from an adaptation temperature of about 30°C and going to a plateau temperature of about 5°C . The activation of the fibres was recorded after the nerve was crushed (after crushing of the nerve the ongoing activity disappeared).

stimulation of the skin and of the nerve after the nerve was crushed is shown in Figure 3.5.

The mean peak response over 5s to cold stimulation of the skin (after subtracting the ongoing activity) was not significantly larger than the mean peak response to stimulation of the nerve (12.3 ± 2.1 imp/s vs 9.9 ± 1.8 imp/s, $p=0.5$, Wilcoxon paired test). The response to nerve stimulation showed a fast adaptation.

The rate of maximal response to cold stimuli of 5°C applied to the skin was in the

range of 8.5 to 29.8 imp/s. The maximal response rates to cold stimuli of about 5°C applied to the nerve ranged from 3.5 to 19.8 imp/s. The maximal response rates over 5 s to cold stimulation of skin and nerve did not correlate with each other.

3.2.1.2 Type 2 cold-sensitive C-fibres

Twenty-eight afferent C-fibres exhibited type 2 cold-sensitivity (Table 3.2, 3.3). These fibres were activated by strong cold stimuli applied to the receptive fields in the skin. An example of a C-fibre with type 2 cold-sensitivity activated by cold stimulation of the skin is shown in Figure 3.6A.

The activation threshold of type 2 cold-sensitive C-fibres to cold stimuli applied to skin was significantly higher ($15.8 \pm 7.4^\circ\text{C}$, $N=27$) than the cold activation threshold of type 1 cold-sensitive C-fibres ($28.5 \pm 0.17^\circ\text{C}$, $N=25$; $p < 0.001$, Mann-Whitney U-test). The magnitude of the cold response evoked from the skin of the type 2 cold-sensitive C-fibres was lower than the magnitude of the response evoked from the skin of the type 1 cold-sensitive C-fibres (Fig. 3.3A, 3.4A, 3.6A). These type 2 cold-sensitive C-fibres exhibited, with one exception (0.14 imp/s), no ongoing activity at a skin temperature of about 30°C (Fig. 3.6A). Nineteen of them were polymodal fibres, being also heat- and/or mechano-sensitive (Table 3.2). The maximal response rates to cold stimuli (5°C) applied to the skin were with two exceptions in the range of 0.75 to 6 imp/s. The two exceptions showed rates of maximal discharge of 15 and 22 imp/s (Fig. 3.7B) similar to those of type 1 cold-sensitive C-fibres.

Eleven of 28 type 2 cold-sensitive C-fibres (39%) activated by cold stimuli applied to the skin were also activated by cold stimuli applied to the nerve (Table 3.3; Fig. 3.6). These fibres could be activated from the nerve from two to four of the five tested locations. The maximal responses rates to cold stimuli of 5°C applied to the skin ranged from 0.5 imp/s to 22 imp/s.

The rate of maximal response to cold stimuli of about 5°C applied to the nerve was in the range of 2.7 to 7.3 imp/s (Fig. 3.7C). There was no correlation between the maximal response rates (over 5 s) to cold stimuli applied to the skin and the maximal response rates (over 5 s) to cold stimuli applied to the nerve.

For example, the two type 2 cold-sensitive C-fibres with rates of maximal response to cold stimulation of the skin of 15 and 22 imp/s exhibited rates of maximal response to cold stimulation of the nerve of 2.5 and 4 imp/s.

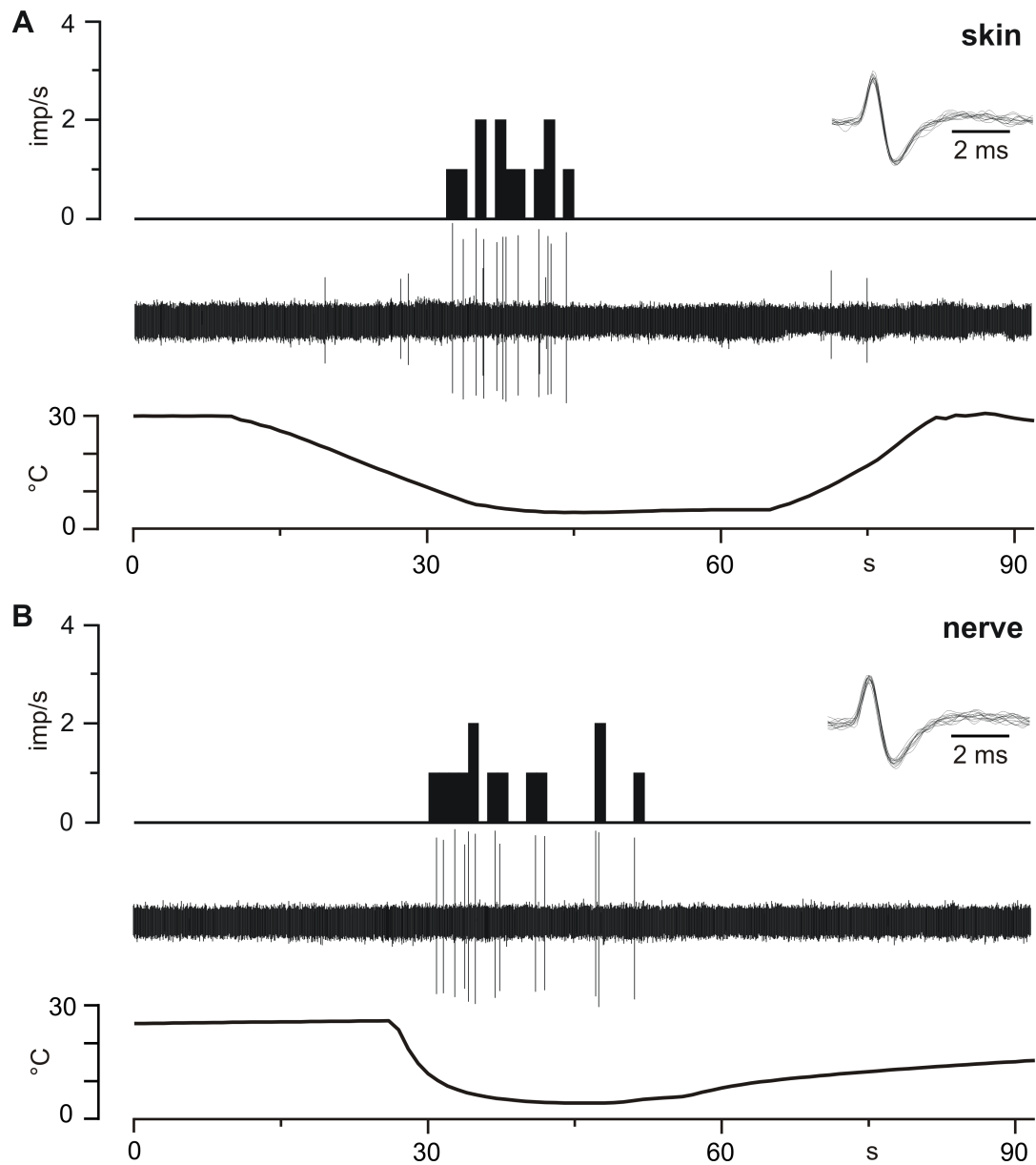


Figure 3.6. Responses of a C-fiber with type 2 cold-sensitivity to cold stimuli applied to the skin (**A**: ramp stimulus delivered at $1^{\circ}\text{C}/\text{s}$ starting from a preset temperature of 30°C and going to a plateau temperature of 5°C) or the nerve (**B**: stimulus starting from an adaptation temperature of about 30°C and decreasing to a temperature of about 5°C). Insets in **A** and **B**: action potential several times superimposed. Note that the shapes of the action potentials elicited during cold stimulation of the skin or during cold stimulation of the sural nerve are identical.

3.2.1.3 Heat-sensitive C-fibres

In all, 61 C-fibres were activated by heat stimuli applied to the skin (Table 3.3). In 22 of them only the responsiveness to heat stimuli was tested. The other 39 heat-sensitive

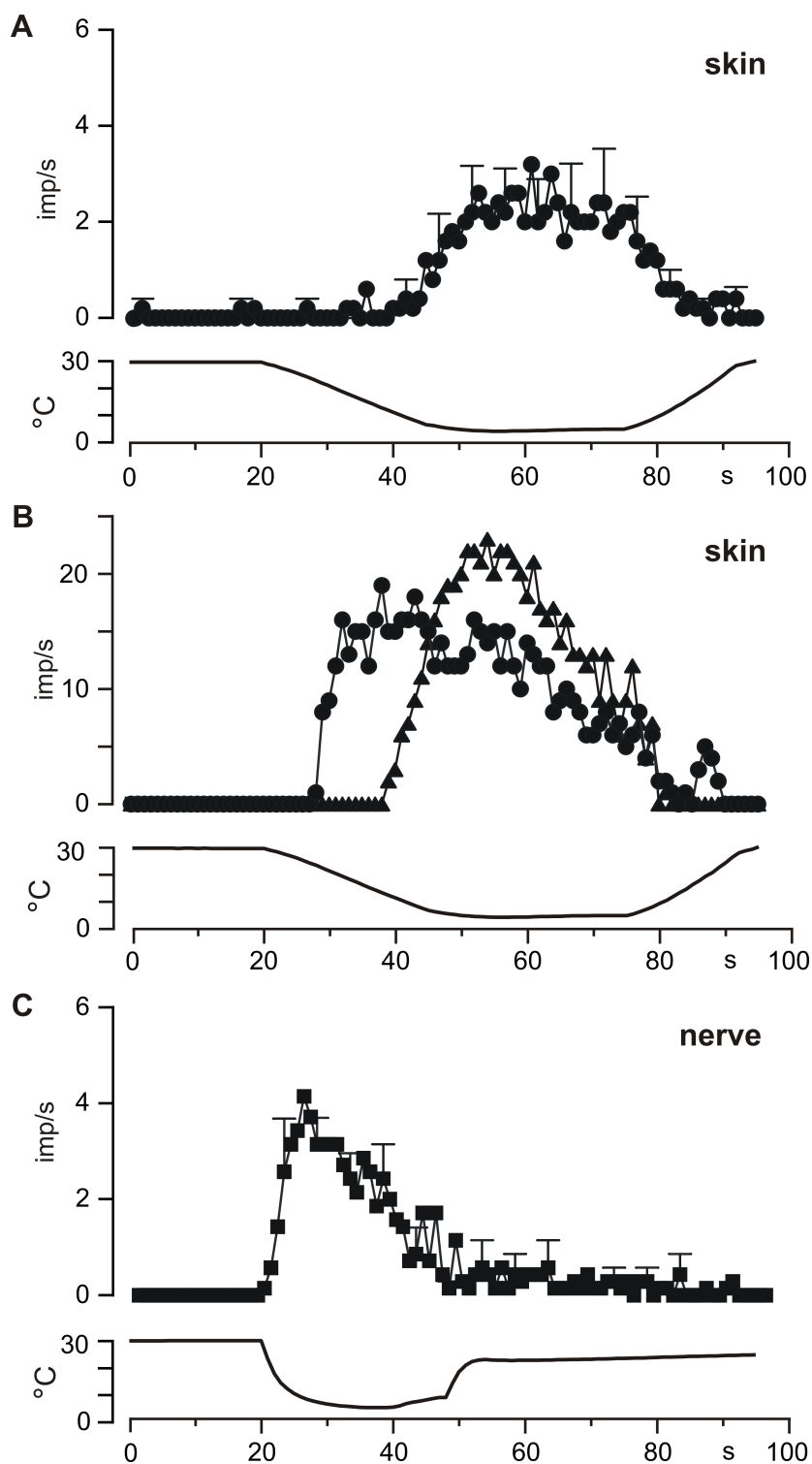


Figure 3.7. Mean responses of 7 C-fibers with type 2 cold-sensitivity to cold stimuli applied to the skin or the nerve. **A.** Response (mean + SEM) of 5 fibres (with a low rate of cold activity) to cold stimulation of the skin. The cold ramp stimuli started from a baseline temperature of 30°C and went down to a static temperature of 0°C. **B.** Responses of 2 C-fibres (with a high rate of cold-induced activity) to cold stimulation of the skin. The ramp stimuli started from a baseline temperature of 30°C and decreased to about 0°C. **C.** Response (mean + SEM) of 7 fibres (with a low rate of cold activity) to cold stimulation of the nerve. The cold stimuli were applied by a thermode, starting from an adaptation temperature of about 30°C and decreasing to 5°C.

C fibres were also tested for their responses to cold and mechanical stimulation of the skin: nine of these fibres were only heat-sensitive and the rest of them were additionally mechanosensitive and/or cold-sensitive (Table 3.2). We probably missed some heat-sensitive C-fibres which were also mechanosensitive with high mechanical thresholds, since we did not apply strong mechanical stimuli such as pinching to the skin. The activation threshold by heat stimuli applied to the skin was $45.5 \pm 0.67^\circ\text{C}$ (N=61).

Three heat-sensitive C-fibres, which were not type 1 cold-sensitive, showed ongoing activity at a temperature of the skin of 30°C : 0.15, 0.18 and 0.2 imp/s. These fibres probably were sensitized by the repeated application of heat stimuli to the skin. Five of the heat-sensitive C-fibres were activated additionally by non-noxious cold stimuli applied to the skin, these fibres being type 1 cold-sensitive C-fibres. Four of them exhibited a high rate of ongoing activity at a skin temperature of 30°C ; their activation by heat stimulation of the skin was always preceded by inhibition of the ongoing activity.

Twenty-one of 61 heat-sensitive C-fibres (34%), activated from the skin were also activated from the sural nerve by heat stimulation (Table 3.3, Fig. 3.8). Most heat-sensitive C-fibres could be activated from the nerve only from one to three of the five tested sites (see section Methods, Fig.2.2A). Therefore, the axonal heat-sensitivity was not continuously distributed along the nerve.

The comparison between the mean responses of 18 heat-sensitive C-fibres to heat stimuli applied to the skin ($\sim 53^\circ\text{C}$) and to the sural nerve ($\sim 52^\circ\text{C}$) is shown in Figure 3.9.

The mean peak response over 5 s to heat stimulation of the skin was not significantly larger than the mean peak response to heat stimulation of the nerve (skin, 5.0 ± 1.3 imp/s; nerve, 3.6 ± 0.92 imp/s; $p=0.6$, Wilcoxon paired test).

The maximal response rates to heat stimulation of the skin ranged from 1 to 30 imp/s and to heat stimulation of the nerve ranged from 1 to 24 imp/s (Fig. 3.10). There was a weak correlation between the maximal rates of response over 5 s to heat stimuli applied to the skin and the maximal rates of response over 5 s to heat stimuli applied to the nerve (Fig. 3.10).

3.2.1.4 Mechanosensitive C-fibres

Twenty-eight C-fibres were activated by mechanical stimuli applied to the skin. Eleven of them were only mechanosensitive and 17 of them additionally heat-sensitive and/or cold-sensitive (Table 3.2). Almost all mechanosensitive C-fibres had a high activation threshold (response induced by strong pressure, N=26); only two of them had a low activation threshold (response induced by light mechanical pressure). All mechanosensitive C-fibres

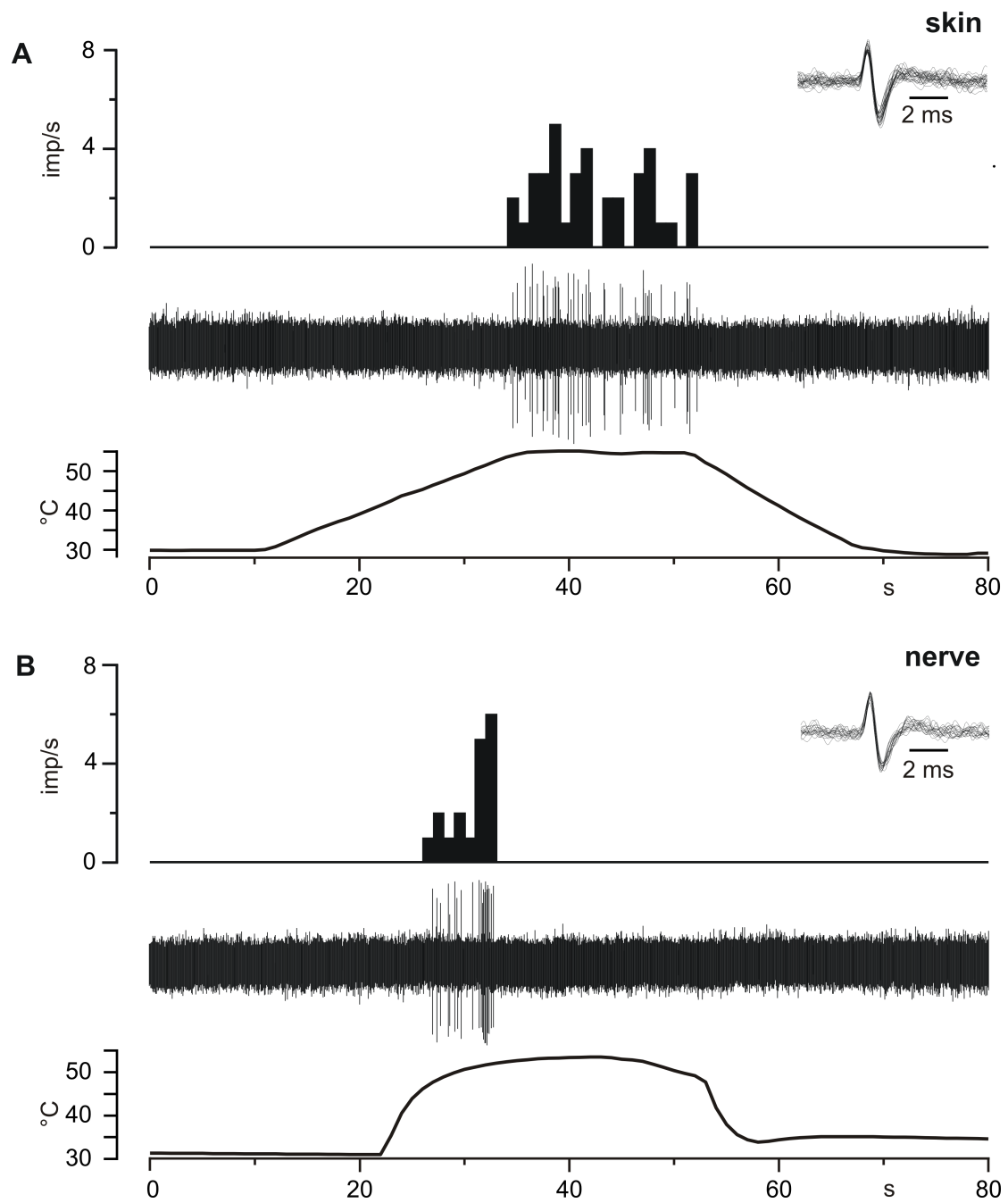


Figure 3.8. Responses of a C-fibre to heat stimuli applied to the skin (**A**: the ramp stimulus started from a baseline temperature of 30°C and increased to 55°C) or the nerve (**B**: the stimulus started from a baseline temperature of about 30°C and increased to about 50°C). Insets in **A** and **B**: action potential several times superimposed. The shapes of the action potentials elicited by heat stimulation of the skin and elicited by heat stimulation of the nerve are identical.

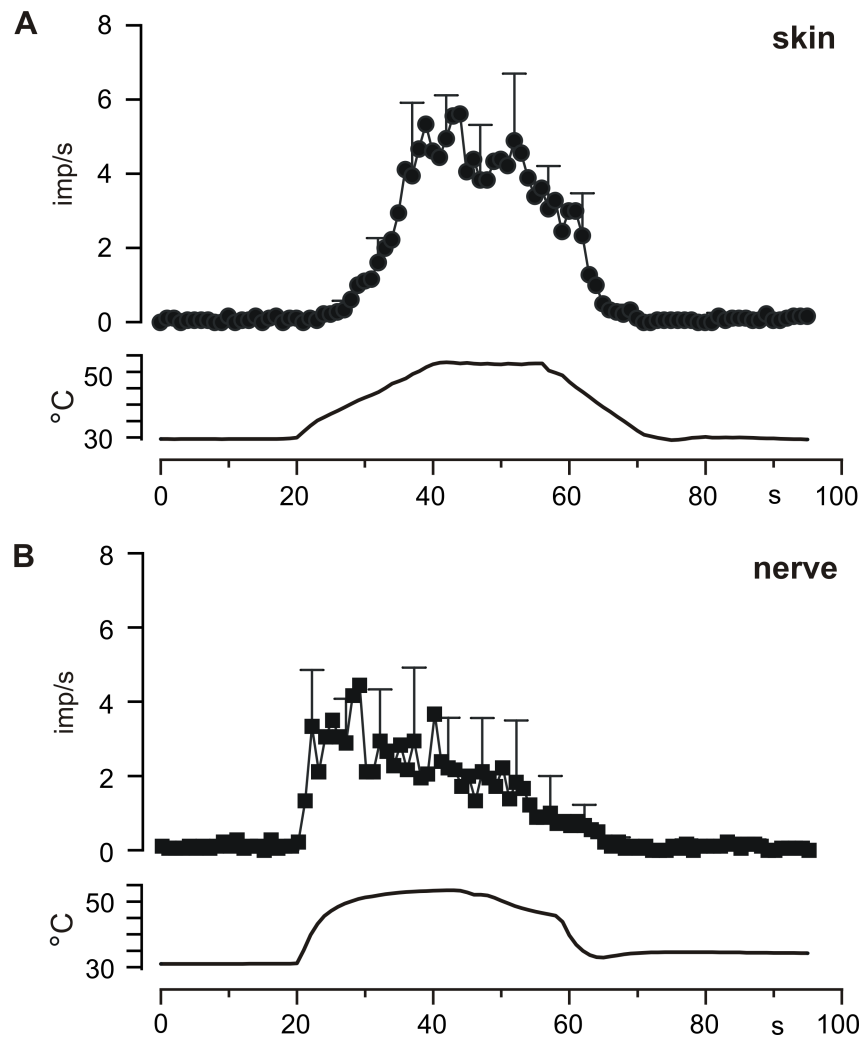


Figure 3.9. Mean responses of 18 C-fibers with heat-sensitivity to a heat stimulus applied to the skin or the nerve. **A.** Responses (mean+SEM) to heat stimulation of the skin. The heat ramp stimuli were delivered at a rate of $1^{\circ}\text{C}/\text{s}$, starting from an adaptation temperature of 30°C and going up to a plateau temperature of 53°C . **B.** Responses (mean + SEM) to heat stimulation of the nerve. The heat stimuli started from a baseline temperature of about 30°C and increased to about 52°C .

were silent at a skin temperature of 30°C (Table 3.2).

Only one of 28 mechanosensitive C-fibers could be activated by mechanical stimulation of both skin and nerve.

3.2.2 A-fibres

118 A-fibres were investigated in this study (Table 3.2). Most of them were mechanosensitive and only a few were thermosensitive.

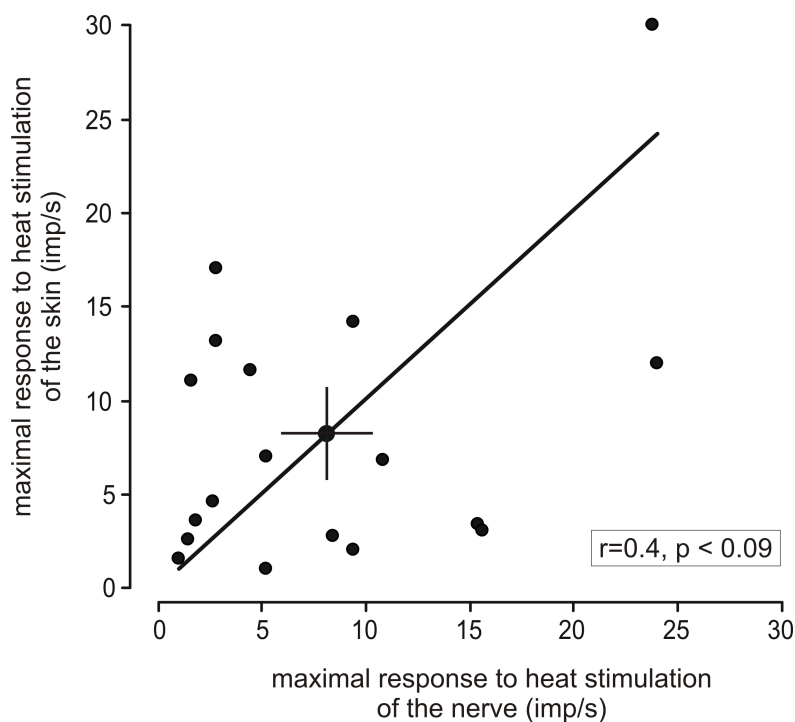


Figure 3.10. Maximal responses to heat stimulation of the skin (ordinate) and the nerve (abscissa) of 18 heat-sensitive C-fibres. The heat responses were counted over a time period of 5 seconds. Mean \pm SEM is indicated by the large closed circle and the bars. The correlation coefficient between the maximal responses to heat stimulation of the skin and of the nerve is $r=0.4$ ($p<0.09$).

3.2.2.1 Thermosensitive A-fibres

In all, five A-fibres were activated by thermal stimuli applied to the skin; three were activated by heat stimuli and two of them by cold stimuli (Table 3.2). One of the cold-sensitive A-fibre was type 1 cold-sensitive, having a low-activation threshold and ongoing activity at a skin temperature of 30°C (Table 3.2). The other cold-sensitive A-fibre was type 2 cold-sensitive with a high activation threshold and no ongoing activity at a skin temperature of 30°C (Table 3.2). The three heat-sensitive A-fibres were silent; their activation thresholds ranged from 46°C to 49°C.

All the thermosensitive A-fibres were activated only from the skin by the thermal stimuli.

3.2.2.2 Mechanosensitive A-fibres

In all, 113 A-fibres responded to mechanical stimuli applied to the receptive fields in the skin. Forty-nine of them were activated by strong mechanical stimuli, having a medium

to high mechanical threshold. The remaining A-fibres had low-activation thresholds. All mechanosensitive A-fibres were, with one exception (0.15 imp/s), silent (Table 3.2).

Only two of 113 mechanosensitive A-fibres activated from the skin could also be activated from the nerve (Table 3.3).

3.3 Responses of intact afferent nerve fibres to menthol

Menthol is an agonist of the transient receptor potential M8 (TRPM8) (McKemy et al., 2002). The hypothesis was tested that low-threshold cold-sensitive afferent nerve fibres (type 1 cold-sensitive fibres) are activated by menthol, whereas the high-threshold cold-sensitive C-fibres (type 2 cold-sensitive fibres), heat-sensitive and mechanosensitive afferent nerve fibres are insensitive to menthol. For this purpose, ten experiments where the sural nerve was intact were performed.

The intact afferent C-fibres and A-fibres, which were functionally characterized by their responses to physiological stimulation of the skin, were tested for their responses to menthol applied to their receptive fields in the skin. After application of menthol, these fibres were tested again for their responses to cold, heat and mechanical stimuli applied to their cutaneous receptive fields. 16 filaments with 30 intact afferent C-fibres and 11 afferent A-fibres activated by physiological stimulation of the skin were investigated. Table 3.4 shows the individual and the combinations of properties of the intact C-fibres and A-fibres tested for their responses to menthol.

3.3.1 C-fibres

In all, 30 functionally characterized C-fibres were studied in this work. The population of the C-fibres was heterogeneous, consisting of cold-sensitive (type 1 & type 2 cold-sensitive), heat-sensitive and mechanosensitive fibres.

3.3.1.1 Type 1 cold-sensitive C-fibres

Twelve intact C-fibres showed type 1 cold-sensitivity (Table 3.4). These fibres were activated by non-noxious cold stimuli applied to the skin. The cold ramp stimuli were applied by a Peltier element (see chapter Methods, Fig. 2.5). The responses to cooling were monitored during the decrease of the temperature (dynamic responses) and during the temperature plateau (static responses: Fig. 3.11A, 3.12A, 3.14A). The dynamic

responses were graded and had a maximal mean discharge rate of about 22.2 ± 4 imp/s at 10-25°C. During static cooling, the responses were also graded with a maximal mean discharge rate of 16 ± 1.9 imp/s at about 5°C. The mean activation threshold by cooling was $28.5^\circ\text{C} \pm 0.35^\circ\text{C}$. The high-sensitivity to cooling of these fibres was also reflected in their activation to a non-contact cold stimulus applied with a copper rod of about 5°C positioned 2-3 mm above their receptive fields (Fig. 3.11D).

In 11 C-fibres the activation by cooling was followed by a depression of the activity for up to one minute (Fig. 3.11A, 3.12A, 3.14A).

The cold sensitivity was also tested using acetone applied to the skin. Acetone can be used as a non-noxious cold stimulus. All 12 type 1 cold-sensitive C-fibres were activated by acetone applied to the skin (Fig. 3.11C). All type 1 cold-sensitive C-fibres showed ongoing activity at a baseline skin temperature of about 30°C (rate: 6.5 ± 0.7 imp/s, N=12). This ongoing activity was depressed by heating of the skin (Fig. 3.11B, 3.11E, 3.12B, 3.14B). In 10 C-fibres, the depression of the ongoing activity was followed by rebound activation (Fig. 3.11B, 3.12B, 3.14B). Only one type 1 cold-sensitive C-fibre was weakly activated by heating that followed the depression of the ongoing activity (Table 3.4). Figure 3.11E illustrates the suppression of the activity of a type 1 cold-sensitive C-fibre even by non-contact heat stimuli applied with a metal rod kept 2-3 mm above its cutaneous receptive field. All these C-fibres were mechano-insensitive.

All 12 type 1 cold-sensitive C-fibres were activated by menthol (10% or 20% in 96% ethanol) applied to their cutaneous receptive fields (Fig. 3.13). Figure 3.12 shows the responses to cold, heat stimuli or menthol of a typical type 1 cold-sensitive C-fibre. Before and during application of menthol or vehicle (ethanol 96%), the skin temperature was increased to 33-34°C by a radiant heat to depress the ongoing activity of the cold-sensitive C-fibre (Fig. 3.12E). In this way, it was possible to record the responses to menthol. The response to application of menthol consisted of an instant transient activation followed by a delayed increasing activation of the type 1 cold-sensitive C-fibre (Fig. 3.12E). Application of the vehicle to the skin has generated only the initial transient response (Fig. 3.12E). This initial response was generated by the transient decrease of the skin surface temperature at the receptive field when the pieces of cotton soaked in ethanol or menthol-solution were applied to the skin and by the evaporation of ethanol. It has to be mentioned that ethanol itself had also a cooling effect.

The insets in Figure 3.12A, C, E illustrate the interval histograms of an intact type 1 cold-sensitive C-fibre at temperatures of 5°C applied to the skin before or after application of menthol as well as during menthol application. Some studies, reporting about cold

Table 3.4. Numbers of intact A-and C-fibres activated or not activated by MENTHOL.

		A-FIBRES		C- FIBRES	
		Activation	No activation	Activation	No activation
Numbers of fibres activated from the skin		11		30	
		0	11	12	18
INDIVIDUAL PROPERTIES	Type 1 cold-sensitive	0	0	12	0
	Type 2 cold-sensitive	0	0	0	7
	Heat-sensitive	0	0	1	8
	Mechanosensitive	0	11	0	8
	Spontaneous activity	0	0	12	1
COMBINATIONS OF PROPERTIES	Type 1 cold-sensitive/SA*	0/0	0/0	11/11	0/0
	Type 1 cold- & heat-sensitive /SA*	0/0	0/0	1/1	0/0
	Type 2 cold-sensitive/SA*	0/0	0/0	0/0	3/1
	Type 2 cold- & heat-sensitive /SA*	0/0	0/0	0/0	1/0
	Type 2 cold- & mechanosensitive /SA*	0/0	0/0	0/0	3/0
	Heat-sensitive /SA*	0/0	0/0	0/0	6/0
	Heat- & mechanosensitive /SA*	0/0	0/0	0/0	1/0
	Mechanosensitive	0/0	11/0	0/0	4/0
	Spontaneous activity	0	0	0	0

*SA–Spontaneous activity. Numbers behind slash indicate the numbers of fibres with spontaneous activity; these fibres are included in the numbers before the slash.

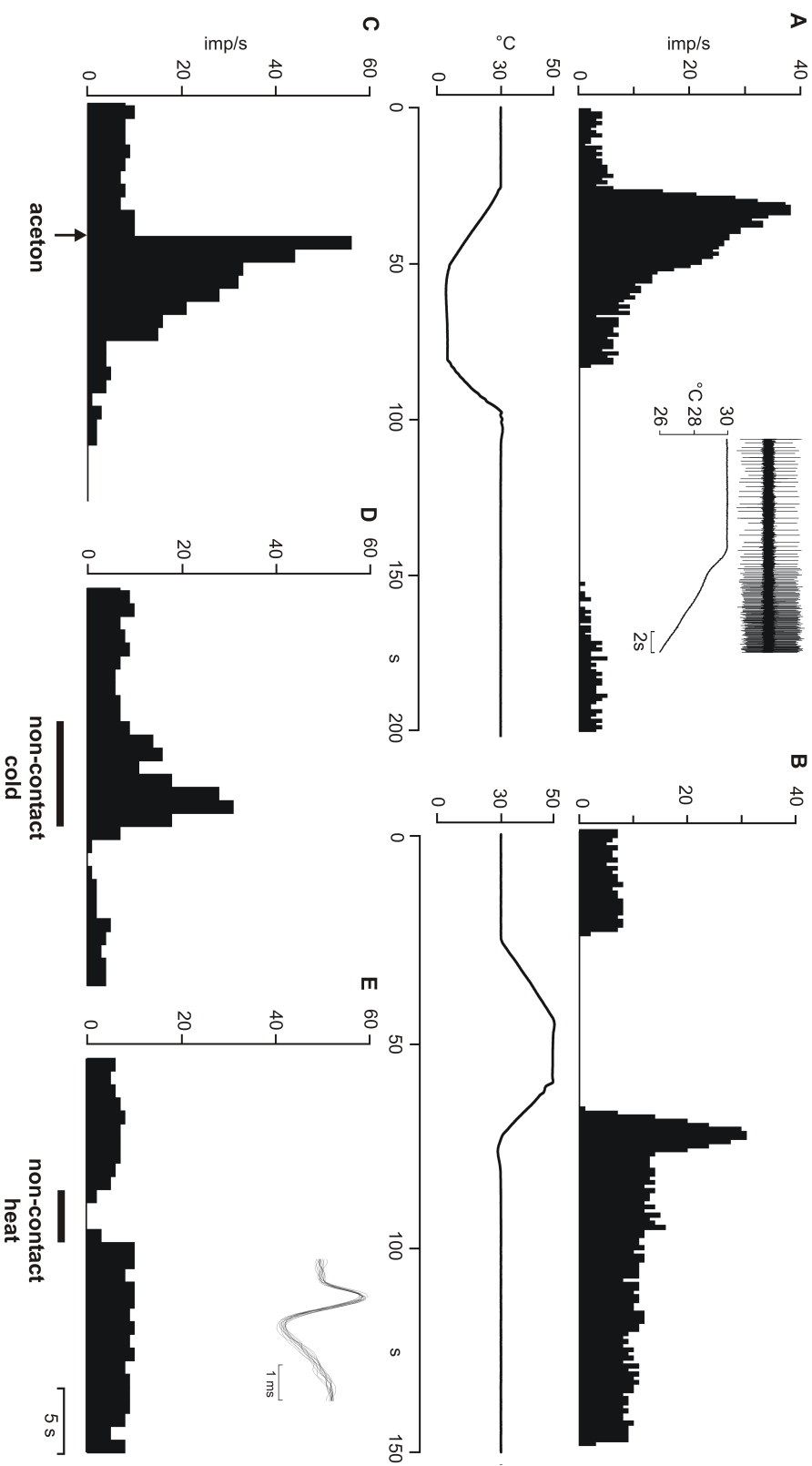


Figure 3.11. Responses of an intact C-fibre with type 1 cold-sensitivity to cooling or heating of the skin. **A.** The response of the C-fibre to cold stimulus applied to its cutaneous receptive field. The cold ramp stimuli were delivered at a rate of $1^{\circ}\text{C}/\text{s}$, starting from an adaptation temperature of 30°C and decreasing to a static temperature of 5°C . Inset: the response to cooling on an expanded time scale; note that the activation by cooling of the skin started in a few seconds. **B.** The response of the C-fibre to a heat stimulus applied to the receptive field. The heat stimulus started from a baseline temperature of 30°C and increased to 50°C . During the heat stimulation of the skin, the ongoing activity of the C-fibre was almost immediately suppressed; the heat suppression was followed by massive rebound activation. **C.** The activation of the C-fibre by a drop of acetone applied to the skin; note the huge response elicited by acetone and the suppression period of the ongoing activity which followed after that. **D, E.** Responses of the C-fibre to non-contact cold and heat stimuli. The tip of a copper rod of 5°C or 52°C was kept 2-3 mm above the cutaneous receptive field of the C-fibre. Inset in **E**: action potential several times superimposed.

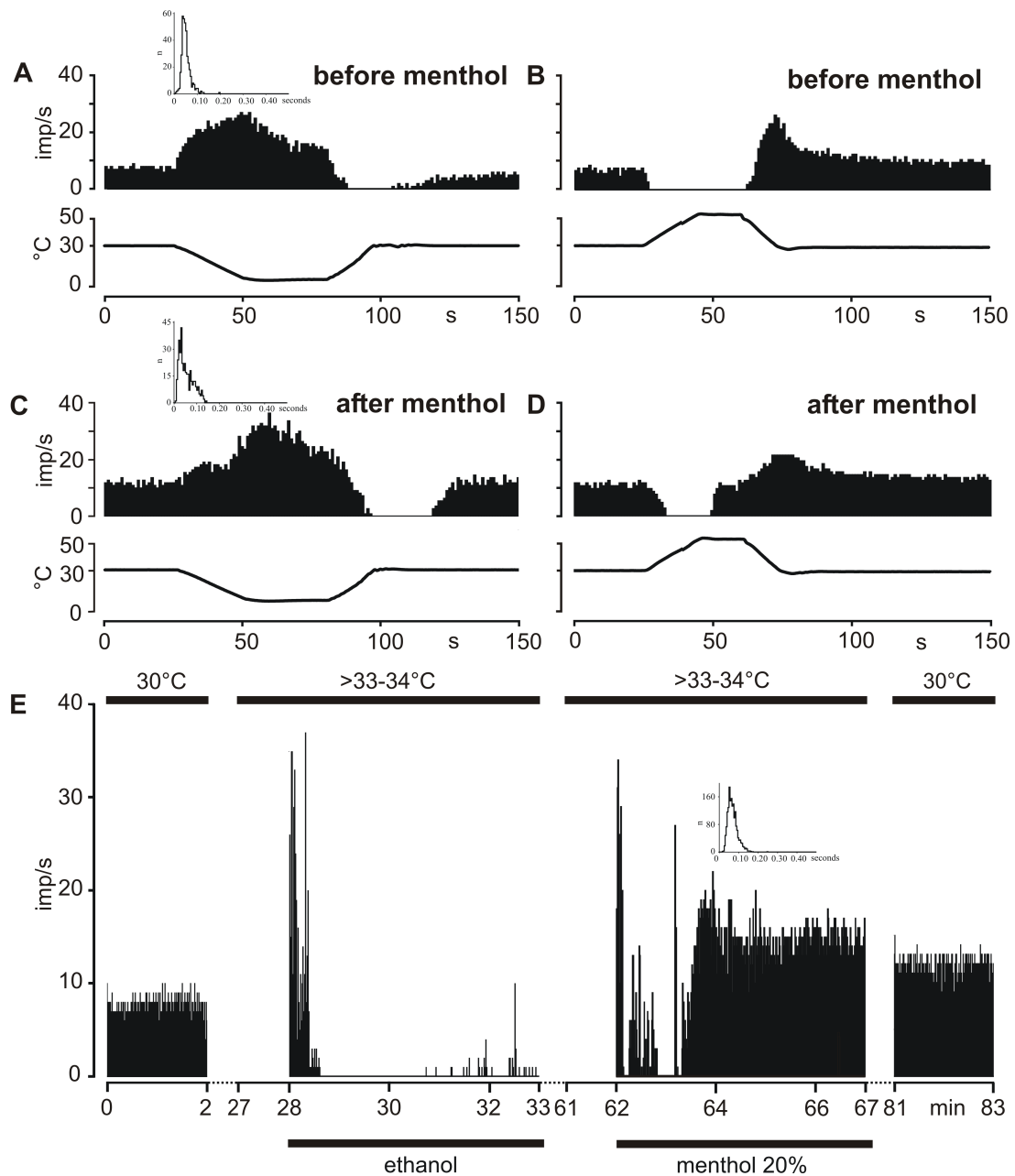


Figure 3.12. Responses of an intact type 1 cold-sensitive C-fibre to thermal stimuli and menthol. **A, B.** Responses of the C-fibre to a cold or heat stimulus applied to its cutaneous receptive field before application of menthol. The ramp stimuli ($1^{\circ}\text{C}/\text{s}$) started from a baseline temperature of 30°C and went to 5°C or 50°C . **C, D.** Responses of the C-fibre to a cold or heat stimulus applied to the receptive field after application of menthol. Note that the rate of the ongoing activity was higher after application of menthol than before application of menthol. **E.** Response of the C-fibre to vehicle (ethanol 96%) and menthol applied to its receptive field. During application of ethanol and menthol, the skin was heated at $33\text{--}34^{\circ}\text{C}$ with a radiant lamp, in this way, the ongoing activity was almost completely suppressed and the effect of menthol was clear. In minutes 0-2 and 81-83, the ongoing activity was measured at a skin temperature of 30°C . Application of vehicle and menthol was followed by an immediate response of the fibre. This response was evoked by the transient decrease of the skin surface temperature at the receptive field; the decrease of the temperature appeared when the pieces of cotton soaked in ethanol or menthol-ethanol solution were applied on the receptive field. Insets in **A, C, E:** interval histograms of the activity in the C-fibre during the cold ramp stimuli before and after application of menthol as well as during two minutes of maximal activation generated by menthol (bin width: 5 ms; interval: 0.5 s).

receptors in various species, show that the cold receptors develop a bursting discharge pattern (periodic bursts of 2 to 12 impulses interrupted by pauses) during cooling and during application of menthol (Hensel and Zotterman, 1951; Iggo, 1969; Hellon et al., 1975; Bade et al., 1979; Schäfer et al., 1986). With one exception, the type 1 cold-sensitive C-fibres did not develop a bursting discharge pattern during cooling or during application of menthol.

The mean response of 11 intact C-fibres to menthol (filled circles) and 8 intact C-fibres to vehicle (unfilled circles) is shown in figure 3.13. The ongoing activity in the 11 C-fibres was measured 10-20 min before application of menthol as well as 6-7 min and 20-40 min after application of menthol at a skin temperature of 30°C. The activity of these fibres was measured during the entire period (5 minutes) of menthol application. Two minutes before and during application of menthol or vehicle the ongoing activity was depressed by increasing the skin temperature to 33-36°C by radiant heat. It can be seen that the activity was significantly higher in minutes 2 to 5 of application of menthol compared to the activity 1-2 min before menthol application ($+p < 0.001$, paired t test) and the activity during application of the vehicle ($p < 0.001$, ANOVA) (Fig. 3.13). The latency of the response to menthol ranged from 12 s to 194 s (58 ± 16.5 s; $N=11$). The activity was still significantly increased one, two and ten min after removal of menthol compared to the activity before TP1 ($*p < 0.01$, $\#p < 0.05$, paired t test) (Fig. 3.13). After removal of menthol, the ongoing activity was measured at a skin temperature of 30°C. This explains the highest rate of ongoing activity measured in the first min after removal of menthol.

The type 1 cold-sensitive C-fibres were tested again about 10 to 25 min after application of menthol (test period 2(TP2)) for their responses to cold and heat stimuli applied to their receptive fields in the skin. The comparison between the responses of intact type 1 cold-sensitive C-fibres to cold stimulation (A) and heat stimulation (B) of the skin before and after application of menthol is illustrated in Figure 3.14.

Figure 3.14A shows the mean responses of 11 intact C-fibres by cold stimuli applied to their cutaneous receptive fields before (black circles) and after application of menthol (white triangles). The response profile did not change qualitatively. The cold activation during the static stimulus (5°C) was significantly higher after than before application of menthol ($p < 0.006$, ANOVA). The cold responses of these C-fibres were prolonged after application of menthol. Figure 3.14B illustrates the mean responses of 10 intact type 1 cold-sensitive C-fibres by heat stimulation of the skin before (black circles) and after application of menthol (white triangles). Only 10 of 11 intact type 1 cold-sensitive C-fibres were averaged since one fibre was activated by heating after the initial depression of

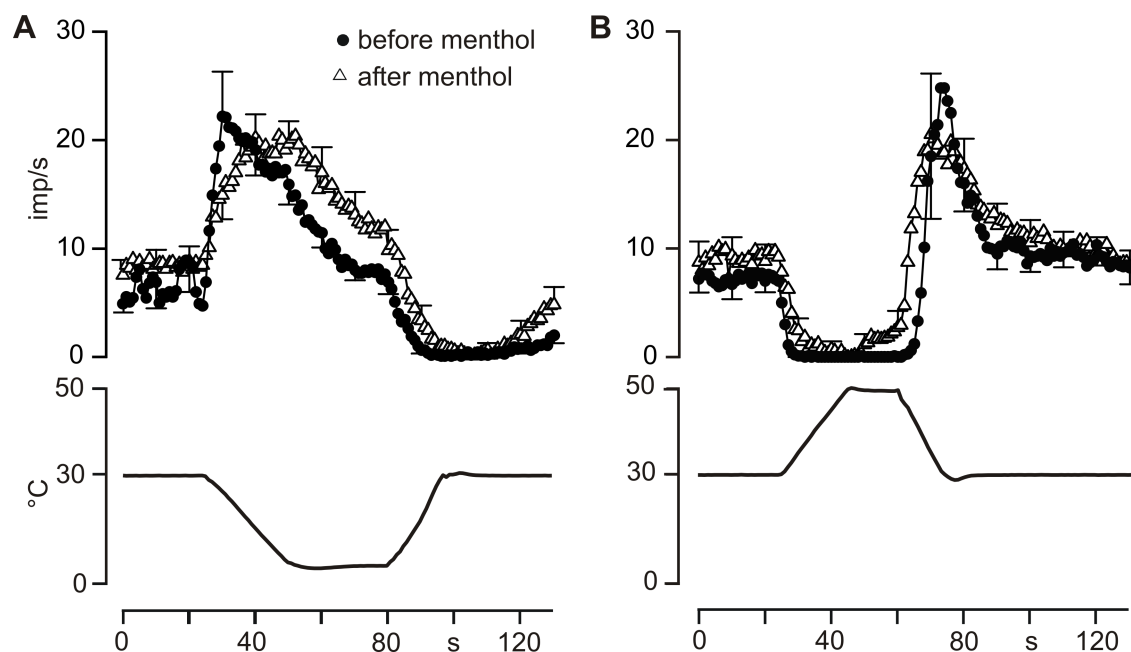


Figure 3.14. Responses of afferent C-fibres with type 1 cold-sensitivity to thermal stimuli applied to the cutaneous receptive fields before and after application of menthol. The ramp stimuli were delivered by a Peltier thermode at a rate of $1\text{ C}^\circ/\text{s}$, starting from an adaptation temperature of 30°C and going to 5°C or 50°C . **A.** Mean responses (+SEM) of 11 C-fibres to cold-ramp stimuli before application of menthol (black circles) and after application of menthol (white triangles). The cold activation before menthol application vs the cold activation after menthol application: $p < 0.006$ (ANOVA). **B.** Mean responses (+SEM) of 10 C-fibres to heat-ramp stimuli before application of menthol (black circles) and after application of menthol (white triangles). The responses of 10 fibres were averaged since one type 1 cold-sensitive fibre was activated by heating the skin. The rebound activation which follows after the heat stimulus started earlier after than before application of menthol. The rebound activation (after the heat stimulus) before menthol application vs the rebound activation (after the heat stimulus) after menthol application: $p < 0.007$ (ANOVA).

the ongoing activity. The rebound activation after the heat stimulus started earlier after than before application of menthol. The rebound activation after menthol application was significantly higher compared to the rebound activity before application of menthol ($p < 0.007$, ANOVA).

3.3.1.2 Type 2 cold-sensitive C-fibres

Seven intact afferent C-fibres showed type 2 cold-sensitivity (Table 3.4). They were activated by strong cold stimuli applied to their receptive fields in the skin. Four of them were additionally activated by mechanical and heat stimulation of the skin (Table 3.4). Their threshold for activation by cold stimuli was $12^\circ\text{C} \pm 2.2^\circ\text{C}$ ($N=7$). The cold responses

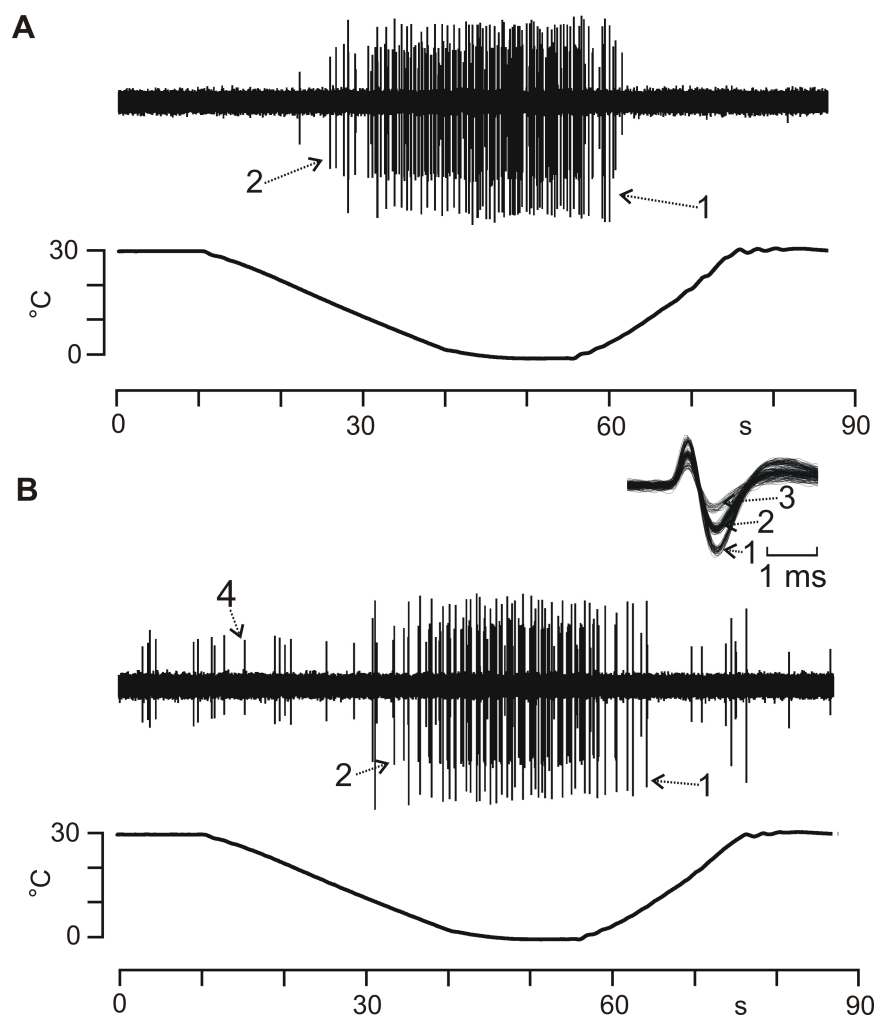


Figure 3.15. Responses of three type 2 cold-sensitive C-fibres (1, 2, 3) to cold stimulation of the skin before (**A**) and after application of menthol (**B**). The cold ramp stimuli were delivered at a rate of $1\text{ C}^\circ/\text{s}$ starting from a preset temperature of 30°C and decreasing to 0°C . The duration of the static cold stimulus was 15 seconds. One fibre (fibre 4 in the graph **B**) developed ongoing activity during the testing period 2 (TP2). Inset in **B**: action potentials of the three C-fibres several times superimposed.

had a maximal mean discharge rate of about 1.74 ± 0.63 imp/s. The type 2 cold-sensitive C-fibres showed, with one exception (0.44 imp/s), no ongoing activity at a skin temperature of about 30°C . The responses of three typical type 2 cold-sensitive C-fibres to cold stimulation of the skin are shown in Figure 3.15A.

All seven type 2 cold-sensitive C-fibres were menthol-insensitive (Table 3.4). The responses of three C-fibres with type 2 cold-sensitivity to cold stimuli applied to the skin before and after application of menthol are depicted in figure 3.15.

Figure 3.16 illustrates the population responses of type 2 cold-sensitive C-fibres to

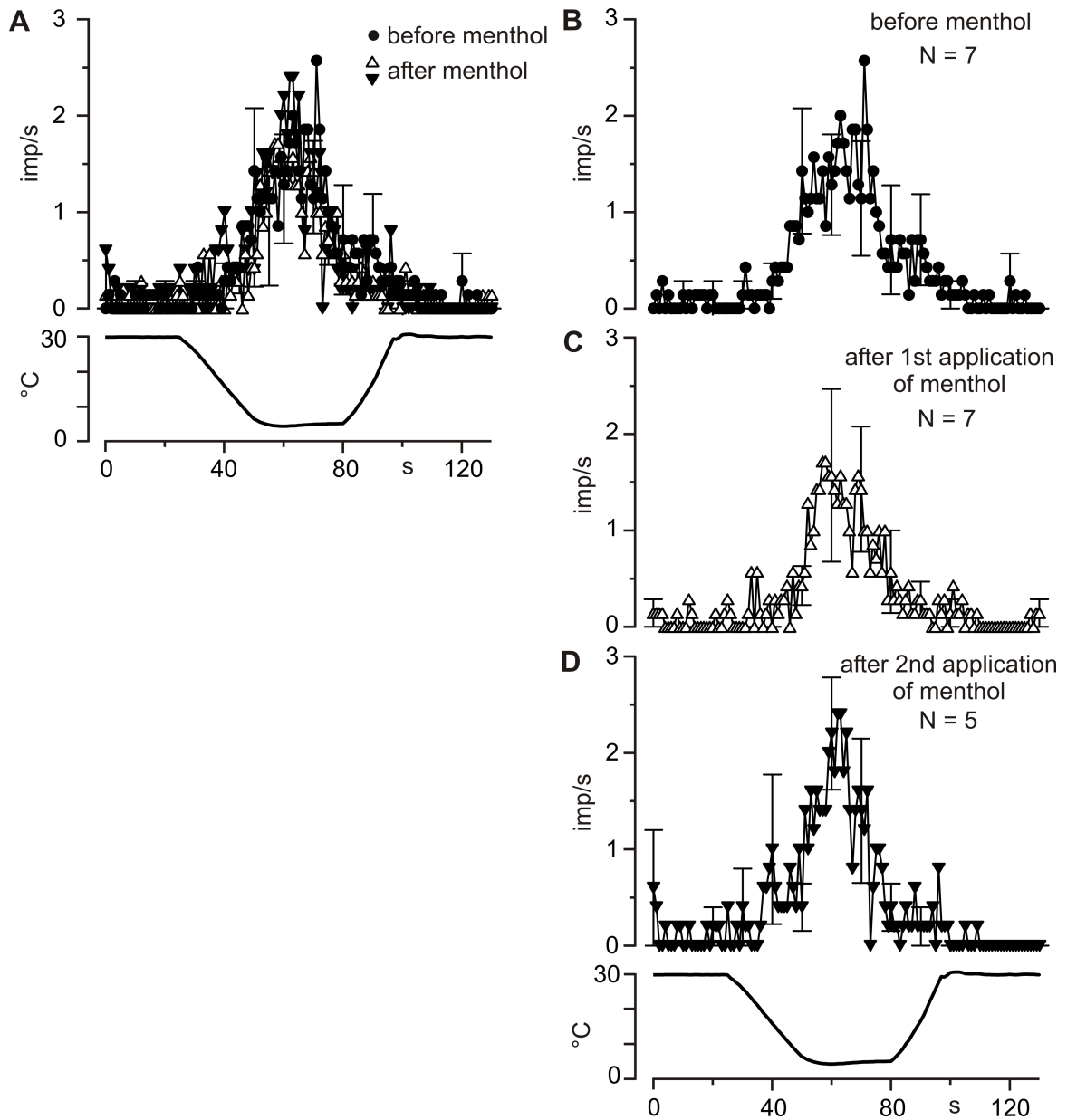


Figure 3.16. Population responses of C-fibers with type 2 cold-sensitivity to cold stimuli applied to their cutaneous receptive fields before and after application of menthol. **A.** Mean responses of 7 C-fibers to cold stimulation of the skin before (N=7, black circles) and after application of menthol (1st application of menthol: N=7, white triangles; 2nd application of menthol: N=5, inverted black triangles). The cold stimuli started from a preset temperature of 30°C and decreased to 5°C. **A** was disassembled in three separated graphs for a better comparison: **B.** Mean response before application of menthol. **C.** Mean response after first application of menthol (10% in 96% ethanol). **D.** Mean response after the second application of menthol (20% in 96% ethanol). Mean±SEM.

cold stimulation of the skin before (black circles) and after application of menthol (white and black triangles). The menthol solution was applied in ascending concentrations; in the second application, the solution of menthol had a higher concentration (20% in 96% ethanol) than in the first application (10% in 96% ethanol). The mean responses of seven type 2 cold-sensitive C-fibres to cold stimuli applied to the skin before application of menthol did not change after first and second application of menthol.

3.3.1.3 Heat-sensitive C-fibres

Nine intact C-fibres were activated by heat stimuli applied to the skin (Table 3.4). Three of them were also activated by cold and mechanical stimuli. The activation threshold by heating was high: $47.3^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$ (N=9). One of the nine heat-sensitive C-fibres was type 1 cold-sensitive and showed ongoing activity at a skin temperature of 30°C . The heat activation of this type 1 cold-sensitive C-fibre has followed after an initial depression of the ongoing activity. The activation of two C-fibres to heat stimuli applied to the skin is illustrated in Figure 3.17A.

Eight of nine heat-sensitive C-fibres were not activated by menthol applied to their receptive fields in the skin. Only the heat-sensitive fibre, which was a type 1 cold-sensitive C-fibre, was activated by menthol. For this reason, this C-fibre was included in the graphs of type 1 cold-sensitive C-fibres (see subchapter 3.3.1.1. Type 1 cold-sensitive C-fibres).

The comparison between the responses of heat-sensitive C-fibres to heat stimuli applied to the skin before and after application of menthol is shown in Figure 3.18.

The mean responses of seven afferent C-fibres to heat stimulation of the skin after application of menthol (10% in the first application; 20% or 40% in the second application) were not changed compared with the mean response before application of menthol (Fig. 3.18).

3.3.1.4 Mechanosensitive C-fibres

Eight C-fibres were mechanosensitive (Table 3.4). Four of these fibres were additionally activated by cold and heat stimuli. All mechanosensitive C-fibres were silent. The mechanical thresholds were high (activation by strong pressure applied to the skin) in 7 C-fibres and low (activation by light mechanical pressure) in one fibre.

All mechanosensitive C-fibres were menthol-insensitive. The responses to mechanical stimulation of the skin were not changed by menthol.

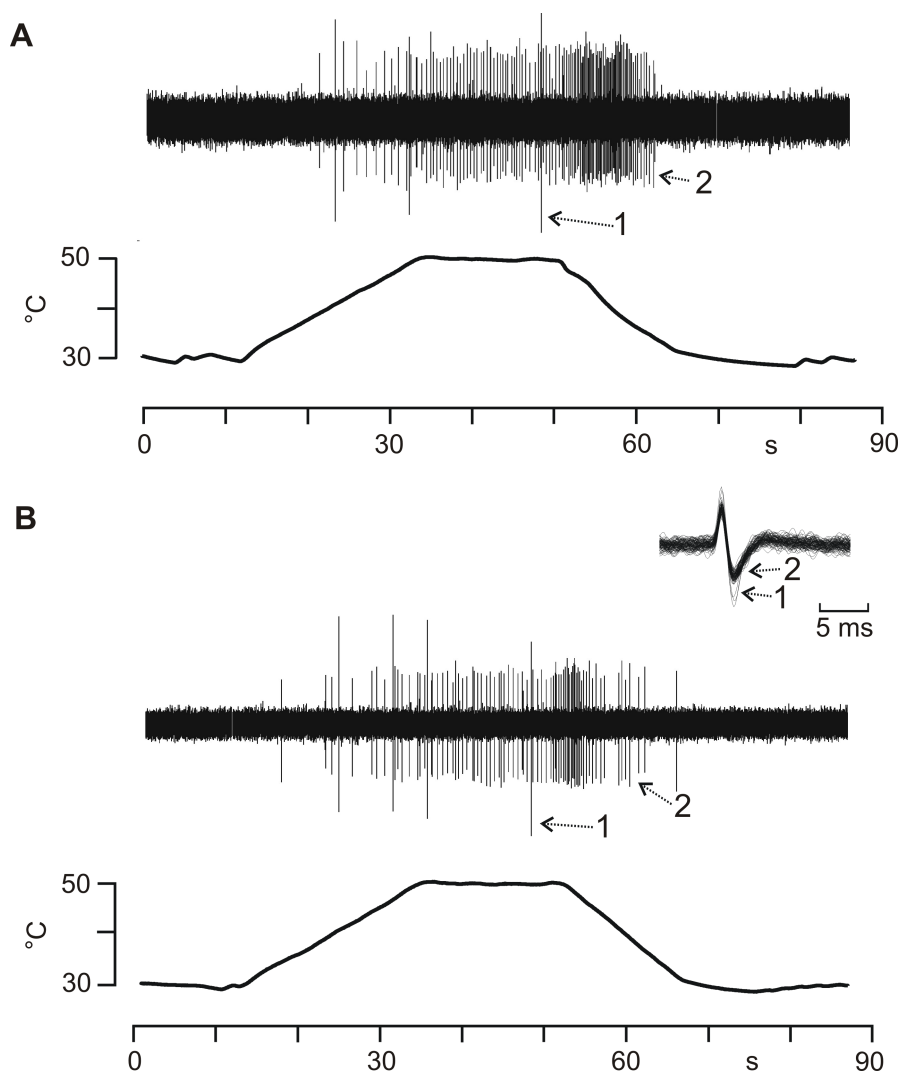


Figure 3.17. Responses of two C-fibres (1, 2) to heat stimuli applied to their cutaneous receptive field before (**A**) and after application of menthol (**B**). The heat stimuli were delivered at a rate of $1^{\circ}\text{C}/\text{s}$, starting from an adaptation temperature of 30°C and increasing to 50°C . The static phase of the heat stimulus lasted for 15 seconds. Inset in **B**: action potentials of the 2 C-fibres several times superimposed.

3.3.2 A-fibres

Eleven A-fibres were activated by mechanical stimuli applied to the skin (Table 3.4). They showed no ongoing activity at a skin temperature of about 30°C . Most of the mechanosensitive A-fibres had a low-threshold ($N=9$) and two of them had a high-threshold. All 11 mechanosensitive A-fibres were neither activated by menthol nor were their responses to mechanical stimuli changed.

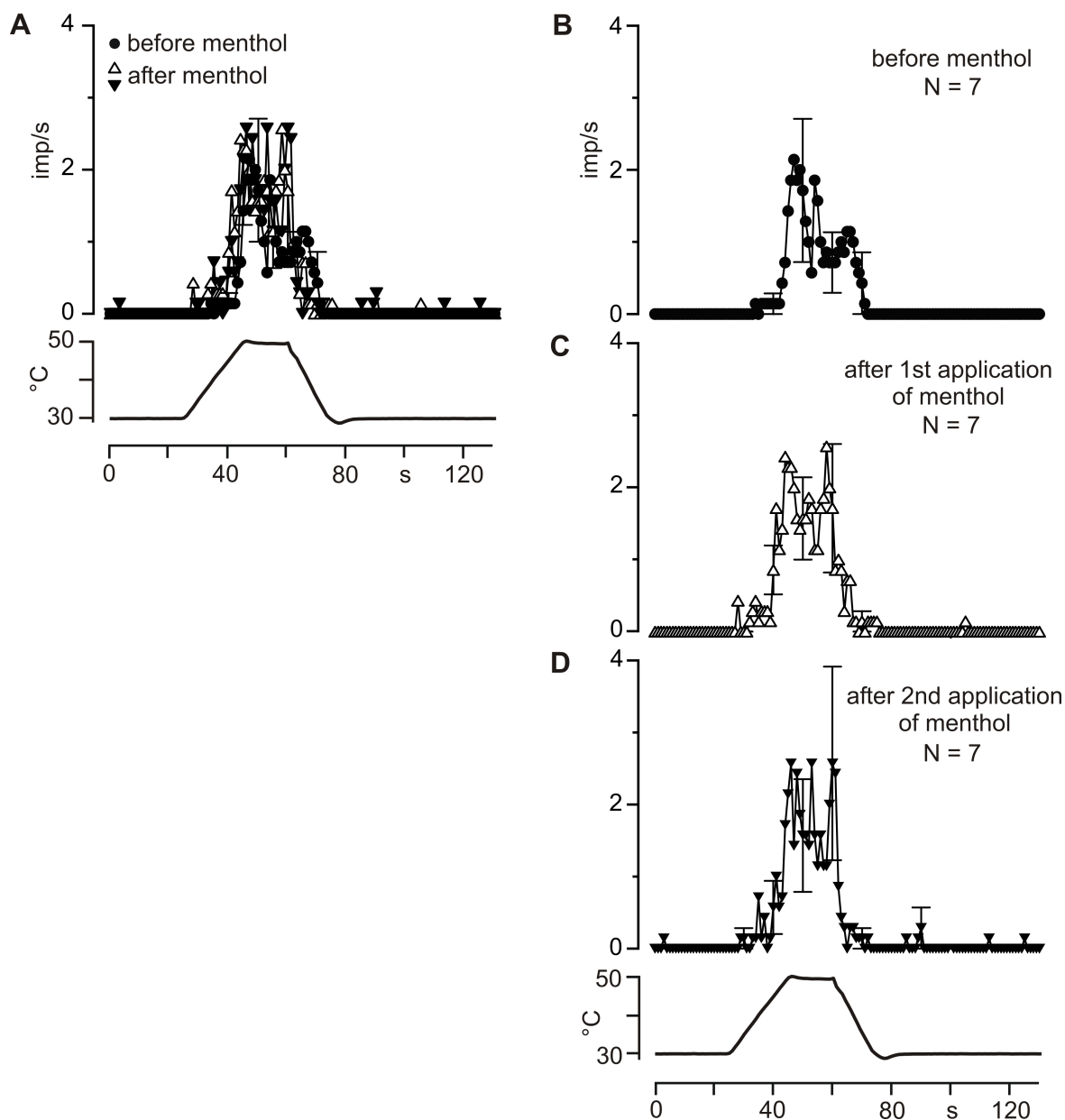


Figure 3.18. Responses of C-fibres to heat stimuli applied to the cutaneous receptive fields before and after application of menthol. The heat ramp stimuli started from a preset temperature of 30°C and increased to 50°C. **A.** Mean response of 7 C-fibres to heat stimulation of the skin before (black circles) and after application of menthol (1st application of menthol: white triangles; 2nd application of menthol: inverted black triangles). For a better comparison between the mean responses of the fibres before and after application of menthol, **A** was disassembled in three graphs (**B**, **C**, **D**): **B.** Mean response to heat stimuli before application of menthol. **C**, **D.** Mean response to heat stimulation after 1st application of menthol (10% in 5 fibres, 40% in 2 fibres) and 2nd application of menthol (20% in 5 fibres, 40% in 2 fibres). Mean \pm SEM.

3.4 Responses of injured afferent nerve fibres to menthol

As in the non-injured (intact) cutaneous C-fibres the hypothesis was tested in injured C- and A-fibres that the TRPM8 agonist menthol activate the innocuous type 1 cold-sensitive afferent nerve fibres but not high threshold cold-sensitive afferent nerve fibres and not cold-insensitive nerve fibres. In 23 rats the sural nerve was injured 6-12 days before the neurophysiological experiments. The injured afferent C- and A-fibres were functionally characterized by their responses to the mechanical and thermal stimuli applied to the nerve (at or distal to the injury site). Then, these fibres were tested for their responses to menthol applied to their receptive fields in the nerve. After application of menthol, the injured A-fibres and C-fibres were tested again for their responses to cold, heat and mechanical stimuli applied to the receptive fields in the nerve. In 28 filaments, 47 functionally identified injured C-fibres and 23 functionally identified A-fibres were investigated in this study. Table 3.5 shows the individual and the combinations of the functional properties of the injured afferent nerve fibres tested for their responses to menthol.

3.4.1 C-fibres

Forty-seven injured afferent C-fibres activated by physiological stimulation of the nerve were studied. As already discussed in chapter 3.1, the functional properties identified in intact C-fibres are qualitatively preserved up to 15 months after sural nerve injury in regenerating and regenerated injured nerve fibres (Jänig et al., 2009; Gorodetskaya et al., 2009). Therefore, the functional groups seen in intact C-fibres, such as type 1 and type 2 cold-sensitive fibres, heat- and mechano-sensitive fibres were present in injured C-fibres, too.

3.4.1.1 Type 1 cold-sensitive C-fibres

Twenty-two injured C-fibres have shown type 1 cold-sensitivity (Table 3.5). These fibres were activated by cooling of the nerve, showing strong dynamic responses (Fig. 3.19A). The mean threshold for activation was $22.7^{\circ}\text{C} \pm 0.56^{\circ}\text{C}$ (N=21). In 13 C-fibres the cold activation was followed by a depression of the activity. Most injured type 1 cold-sensitive C-fibres showed ongoing activity at a nerve temperature of about 30°C (1.9 ± 0.4 imp/s; N=19). This ongoing activity was completely depressed by heat stimuli of 35°C - 45°C

Table 3.5. Distribution of the ectopic properties in regenerating A-fibres and C-fibres activated or not activated by MENTHOL 6-12 days after lesion of the sural nerve.

		A-FIBRES		C- FIBRES	
		Activation	No activation	Activation	No activation
Number of fibres with ectopic activity		23		48	
		2	21	21	27
INDIVIDUAL PROPERTIES	Type 1 cold-sensitive	0	0	20	2
	Type 2 cold-sensitive	1	0	1	10
	Heat-sensitive	1	6	6	16
	Mechanosensitive	0	18	0	5
	Spontaneous activity	0	3	20	14
COMBINATIONS OF PROPERTIES	Type 1 cold-sensitive/SA*	0/0	0/0	15/15	2/2
	Type 1 cold-& heat-sensitive /SA*	0/0	0/0	5/4	0/0
	Type 2 cold-sensitive /SA*	0/0	0/0	0/0	8/3
	Type 2 cold-& heat-sensitive /SA*	1/0	0/0	1/1	2/0
	Heat-sensitive /SA*	1/0	3/2	0/0	9/5
	Heat-& mechanosensitive /SA*	0/0	3/1	0/0	5/3
	Mechanosensitive	0/0	15/0	0/0	0/0
	Spontaneous activity	0	0	0	1

*SA–Spontaneous activity. Numbers behind slash indicate the numbers of fibres with spontaneous activity; these fibres are also included in the numbers before the slash.

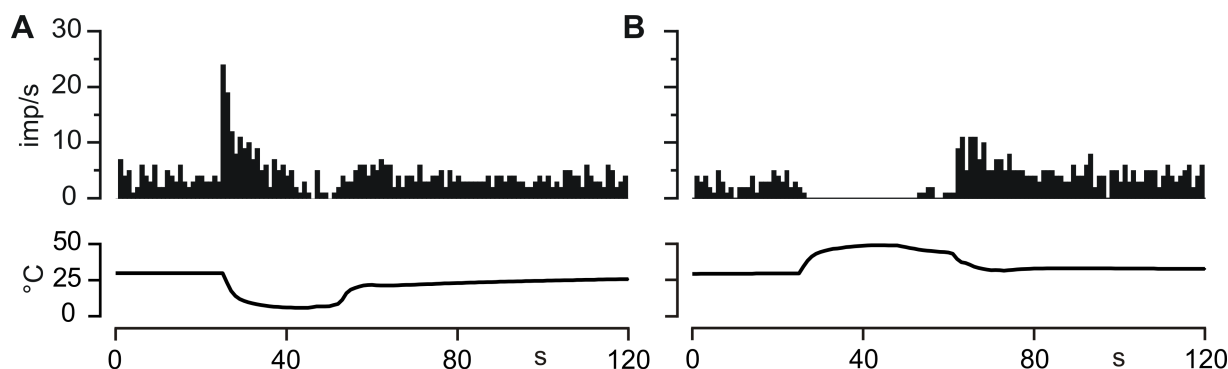


Figure 3.19. Responses of an injured afferent C-fibre with type 1 cold-sensitivity to cooling (**A**) or heating (**B**) of the sural nerve. The cold and heat stimuli were applied to the sural nerve by a thermode, starting from an adaptation temperature of about 29-30°C and going to temperatures of 5°C and 50°C, respectively. Note that the activation by cooling the nerve started in a few seconds (**A**). During the heat stimulus, the ongoing activity of the C-fibre was immediately depressed, this depression being followed by rebound activation (**B**).

applied to the nerve (Fig. 3.19B). After the heat-induced depression, 19 C-fibres exhibited rebound excitation (Fig. 3.19B). Five type 1 cold-sensitive C-fibres were also excited by heat stimulation of the nerve. All injured type 1 cold-sensitive C-fibres were mechano-insensitive.

Twenty of 22 injured C-fibres with type 1 cold-sensitivity were excited by menthol applied to their receptive fields in the nerve (Fig. 3.20E). 4 mM menthol concentration was applied to 19 C-fibres, while 8 mM menthol was used in one C-fibre. The other two C-fibres not activated by 4 mM menthol were not further tested for their responses to 8 mM. The response to menthol consisted of a small instant activation (in 9 fibres) and a delayed increase of activity (Fig. 3.20E). The immediate transient response was also seen, only in some cases when the vehicle (0.2% ethanol solution) was applied to the nerve. Figure 3.20 shows the responses of a typical injured type 1 cold-sensitive C-fibre to cold, heat stimuli and menthol applied to the nerve.

The insets from figure 3.20A, C, E show the interval histograms of an injured type 1 cold-sensitive C-fibre at cold stimuli of about 5°C applied before or after menthol application as well as during application of menthol. All injured type 1 cold-sensitive C-fibres showed no bursting pattern at a cold temperature of 20°C applied either before or after application of menthol as well as during application of menthol.

The activity in 17 injured C-fibres before, during and after application of menthol to the nerve (filled circles) and the activity in eight of these 17 C-fibres before and after application of vehicle to the nerve (unfilled circles) are depicted in Figure 3.21. The

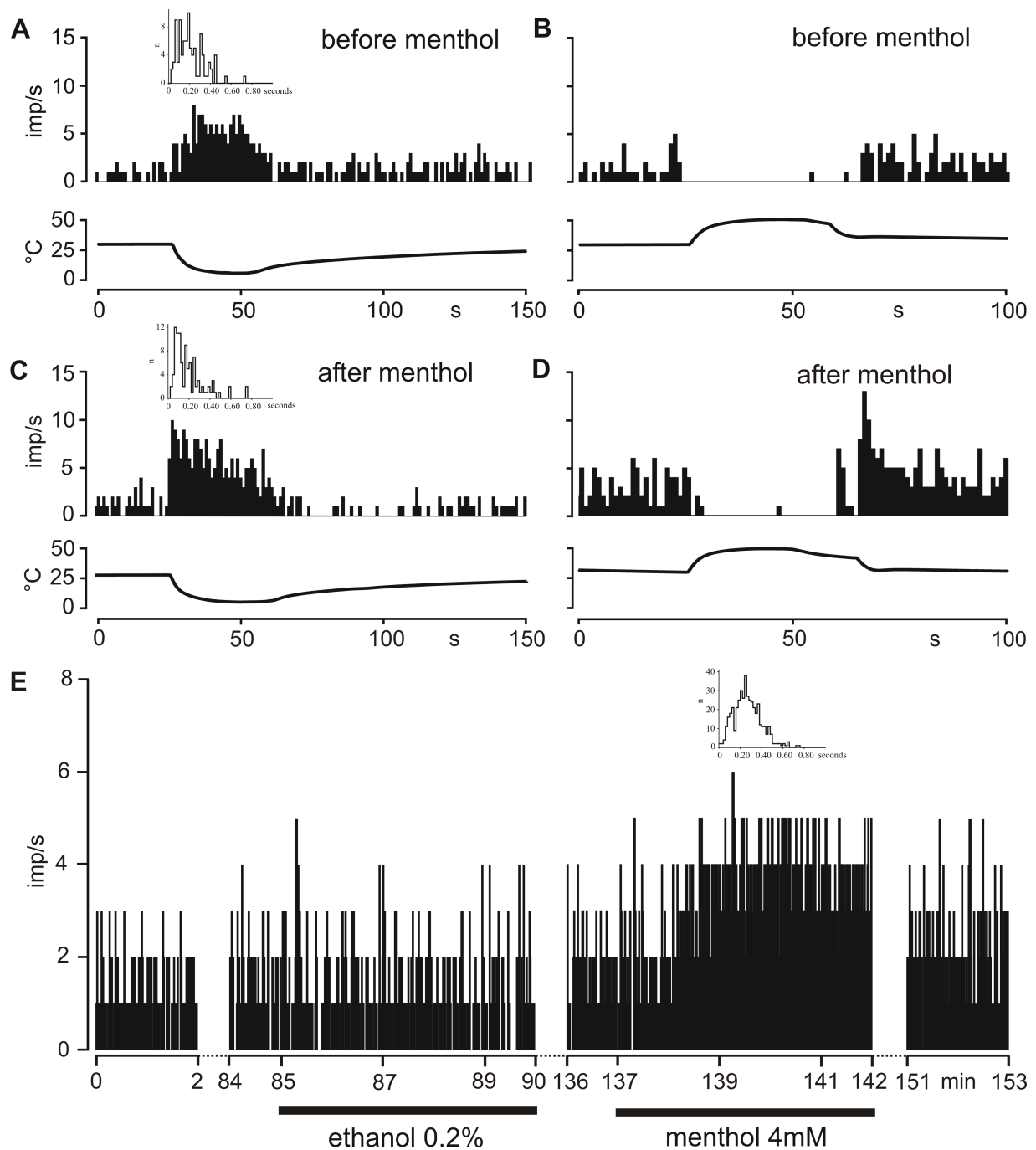


Figure 3.20. Responses of an injured type 1 cold-sensitive C-fibre to thermal stimuli and menthol. **A, B.** Responses of the C-fibre to cold and heat stimuli applied to the receptive fields in the nerve before application of menthol. The thermal stimuli started from a baseline temperature of about 30°C and went to 5°C or 50°C, respectively. **C, D.** Responses of the C-fibre to cold and heat stimulation of the nerve after application of menthol. Note that the response to cooling of the nerve was larger after application of menthol than before application of menthol. **E.** Responses of the C-fibre to vehicle (ethanol 0.2%) and to menthol applied to its receptive field in the nerve. The activation to menthol started in about 1 min and was maintained for about 15 min after removal of menthol (min. 151-153). Insets in **A, C, E:** interval histograms of the activity in the C-fibre during the cold stimuli applied before and after application of menthol as well as during two minutes of maximal response to menthol (bin width 20 ms; interval 1 s).

ongoing activity was measured 10–30 min and 1–2 min before application of menthol as well as 1–4 min (application of tyrode) and 20–40 min after application of menthol. The activity during application of menthol was recorded for 5 min. In eight type 1 cold-sensitive C-fibres (unfilled circles) the activity was recorded 1–2 min before and 5 min during application of vehicle. In order to observe the effect of menthol, immediately before and during application of menthol and ethanol as well as during tyrode application, the ongoing activity was subtracted in the C-fibres. The activity during application of menthol was significantly higher (starting within the first min of menthol application) than the activity immediately before application of menthol (*, $p < 0.01$, +, $p < 0.001$, #, $p < 0.0001$, paired t test). The activity was significantly higher during application of menthol when compared to the activity during application of vehicle ($p < 0.03$; ANOVA). The mean latency of the activation by menthol was 94.7 ± 14.4 s (22s–4min, $N = 20$). The activity one minute after removal of menthol was still increased and significantly higher than the activity before application of menthol (*, $p < 0.01$, paired t test).

About 11 to 35 min after application of menthol, the injured type 1 cold-sensitive C-fibres were tested again for their responses to thermal and mechanical stimuli applied to the receptive fields in the nerve (test period 2, TP2).

The population responses of the injured type 1 cold-sensitive C-fibres to cold and heat stimuli applied to the nerve before (TP 1; black circles) and after (TP 2; white triangles) application of menthol are shown in Figures 3.22, 3.23. The activation to nerve cooling after application of menthol was not significantly enhanced comparatively with the activation to cooling before application of menthol (Fig. 3.22). Furthermore, the response profiles did not change qualitatively (Fig. 3.23). On the other side, it was found that the rebound activation after heating is slightly increased after application of menthol, as shown in Figure 3.23B.

The relation between the maximal responses to menthol and the rate of ongoing activity at 30°C of the intact type 1 cold-sensitive cutaneous afferent fibres (10% menthol) and of the injured type 1 cold-sensitive cutaneous afferent fibres (4 mM menthol) is depicted in Figure 3.24. The correlation between these parameters was significant ($r = 0.68$, $p < 0.0001$) for the injured afferent fibres and not significant ($r = 0.13$) for the intact afferents. Furthermore, both parameters were significantly higher in intact than in injured C-afferents (maximal response to menthol: 11.7 ± 1.5 imp/s vs 3.1 ± 0.5 imp/s, $p < 0.0001$; ongoing activity: 6.5 ± 0.75 imp/s vs 1.6 ± 0.48 imp/s, $p < 0.0001$; Wilcoxon signed rank test). For these statistical tests, the results from the previous chapter have been used for the intact afferent fibres.

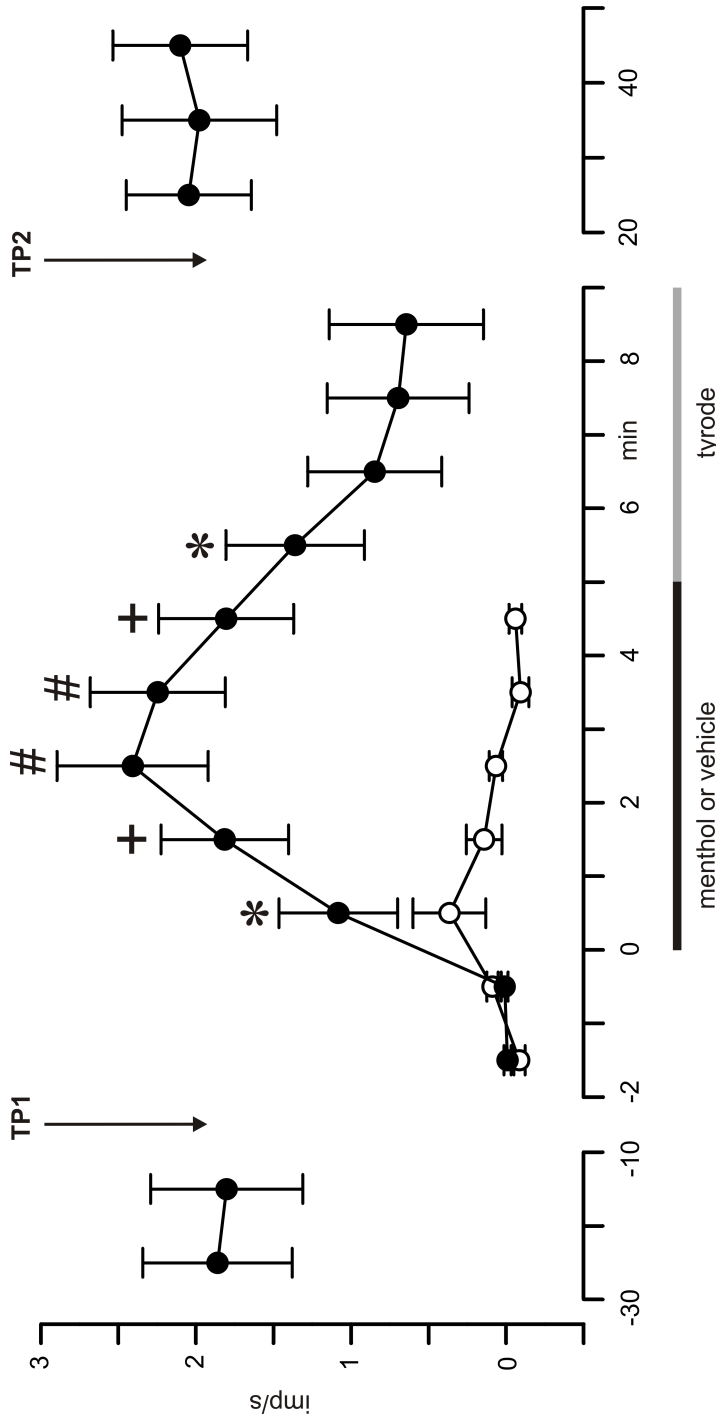


Figure 3.21. Rate of ongoing activity of injured type 1 cold-sensitive C-fibres before, during and after application of menthol and ethanol to their receptive fields in the nerve. Activity in 17 C-fibres recorded before, during and after application of menthol (4 mM, 8 mM) to the nerve (filled circles) or before and during application of ethanol (0.2%, 0.4%, 10%) in 8 of these C-fibres (unfilled circles). Before and during application of menthol and vehicle as well as during application of tyrode, the ongoing activity of the C-fibres was deducted in order to observe the response to menthol. In minutes -30-10 and 20-40, the ongoing activity of the fibres was not deducted. In test period 1 (TP1) and TP2, the C-fibres were tested for their responses to physiological stimuli applied to the nerve before and after application of menthol, respectively. The activity during application of menthol was significantly higher than the activity immediately before menthol: * $p < 0.01$, # $p < 0.0001$ (paired t test). Response to menthol vs. response to vehicle: $p < 0.03$ (ANOVA). Activity during application of tyrode vs activity immediately before menthol: * $p < 0.01$ (paired t test).

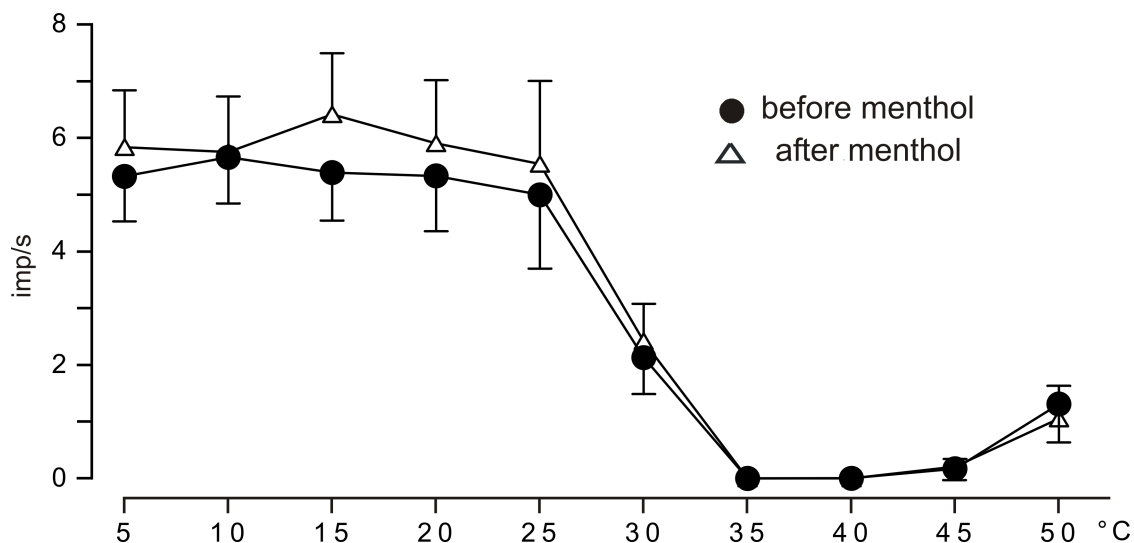


Figure 3.22. Stimulus-response functions of 14 injured afferent C-fibres with type 1 cold-sensitivity before (black circles) and after (white triangles) application of menthol. The activity of a C-fibre to cooling and heating was counted for a duration of 20 seconds following the start of the thermal stimuli. The cold and heat stimuli were applied by a thermode, starting from an adaptation temperature of about 30°C and leading to 5°C or 50°C, respectively. The responses to cold and heat stimulation of the nerve after application of menthol were not significantly different from those before application of menthol. Mean+SEM.

Figure 3.25 shows how and under which conditions the values of the previous statistics were counted. For the intact type 1 cold-sensitive afferents, the ongoing activity was measured for one minute before application of radiant heat and of menthol at a baseline skin temperature of 30°C (Fig. 3.25A). Five minutes before and during application of menthol, the skin was heated at a temperature of 33–36°C in order to depress the ongoing activity of the intact afferent fibres. Under these conditions, the maximal response to menthol was measured over a time period of one minute (Fig. 3.25A).

For the injured type 1 cold-sensitive C-fibres, the ongoing activity 1-2 min before application of menthol and the maximal response to menthol were both counted over a time period of one minute at a baseline nerve temperature of about 30°C (Fig. 3.25B). The response to menthol displayed in Figure 3.24 represents only the response to menthol, the ongoing activity being subtracted.

3.4.1.2 Type 2 cold-sensitive C-fibres

Eleven injured afferent C-fibres were activated by strong cold stimuli applied to their receptive fields in the nerve (Table 3.5). Three of them were additionally activated

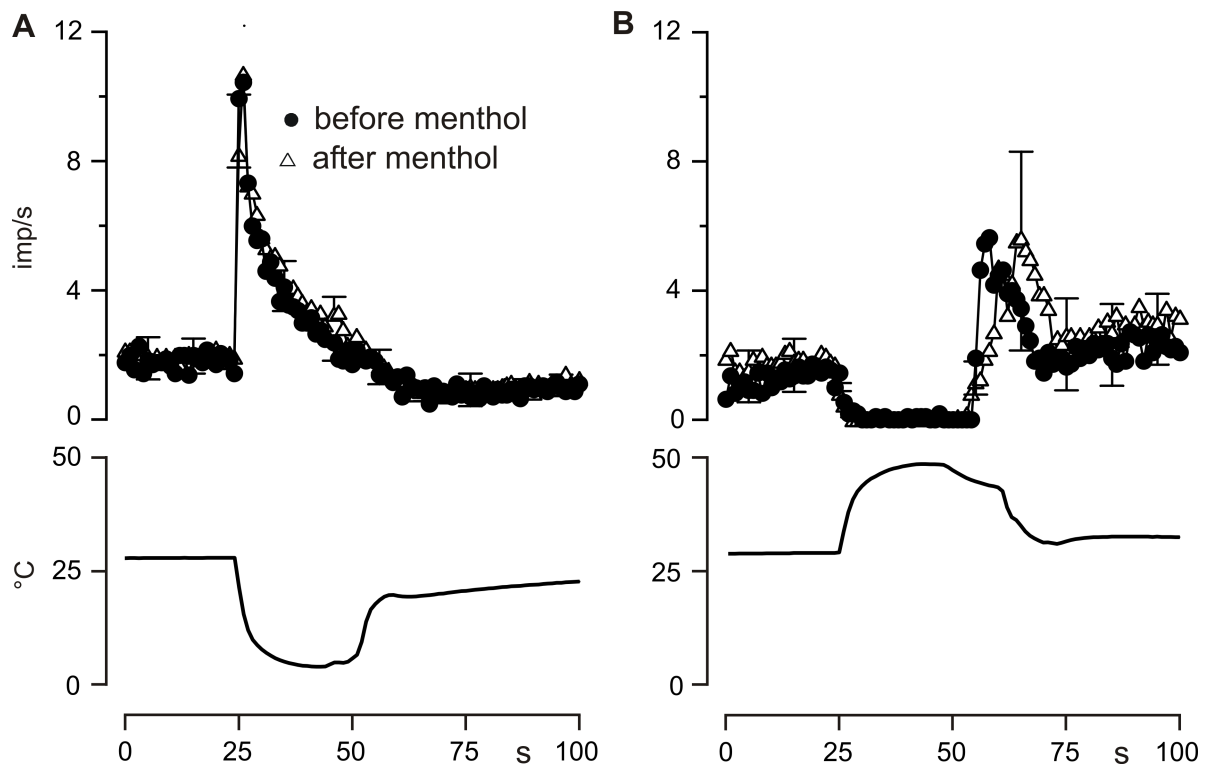


Figure 3.23. Responses of injured afferent C-fibers with type 1 cold-sensitivity to cold (**A**, $N=17$) and heat stimuli (**B**, $N=11$) applied to their receptive fields in the nerve before and after application of menthol. The cold and heat stimuli were delivered by a thermode, starting from a baseline temperature of about 30°C and going to temperatures of 5°C and 50°C . In **B** the number of fibres is 11, since six fibres were also activated by heating after an initial depression of the ongoing activity.

by heat stimuli applied to the nerve. Their cold activation threshold was significantly higher than the activation threshold of the type 1 cold-sensitive C-fibres ($13.6^{\circ}\text{C}\pm 1.2^{\circ}\text{C}$ vs $22.7^{\circ}\text{C}\pm 0.56^{\circ}\text{C}$; $p<0.0001$, Mann-Whitney U-test). The cold responses of these fibres had a maximal mean discharge rate of about 1.51 ± 0.38 imp/s. Four of 11 type 2 cold-sensitive C-fibres showed low rates of ongoing activity at a nerve temperature of about 30°C : 0.08 imp/s, 0.1 imp/s, 0.1 imp/s, 0.17 imp/s. Activation of two injured afferent C-fibres with type 2 cold-sensitivity to cooling of the nerve is depicted in Figure 3.26A.

All injured type 2 cold-sensitive C-fibres, with one exception were menthol-insensitive (Table 3.5). Menthol solution in concentration of 8 mM was applied to the receptive fields in the nerve. The typical responses of two C-fibres with type 2 cold-sensitivity to cold stimulation of the nerve before and after application of menthol are illustrated in Figure 3.26. The cold responses of these two fibres were similar before and after application of menthol.

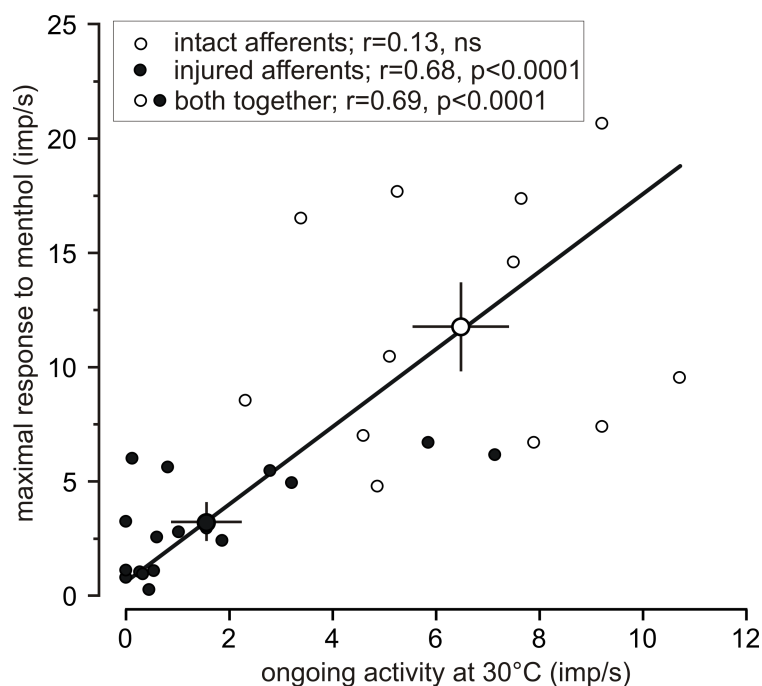


Figure 3.24. Rate of ongoing activity (abscissa scale) and maximal responses to menthol (ordinate scale; the ongoing activity was deducted [see Fig. 3.25]) of injured (filled circles; 4 mM menthol) and intact (unfilled circles; 10%, 20% menthol) type 1 cold-sensitive cutaneous afferent C-fibres. The ongoing activity was measured before application of menthol over a time period of one minute. The maximal response to menthol was counted for a time period of 1 minute. Means \pm SEMs are indicated by the large unfilled (intact afferents) and filled circles (injured afferents) and the error bars. The correlation coefficients between maximal responses to menthol and rate of ongoing activity are: $r=0.13$ ($p<0.69$) for intact afferents, $r=0.68$ ($p<0.0001$) for injured afferents and $r=0.69$ ($p<0.0001$) for both intact and injured fibres.

Figures 3.27 and 3.28 show the population responses of injured type 2 cold-sensitive C-fibres to cold stimuli applied to the receptive fields in the nerve before (black circles) and after (white triangles) application of menthol. It can be seen that the total responses were quantitatively the same before and after application of menthol (Fig. 3.27). Moreover, the response profiles did not change qualitatively (Fig. 3.28).

3.4.1.3 Heat-sensitive C-fibres

Twenty-two injured C-fibres were activated by heat stimuli applied to the receptive fields in the nerve (Table 3.5). Thirteen of them were additionally cold-sensitive and/or mechanosensitive. Five heat-sensitive C-fibres were activated also by non-noxious cold stimuli (type 1 cold-sensitive fibres) and three fibres by noxious cold stimuli (type 2 cold-sensitive fibres) (Table 3.5).

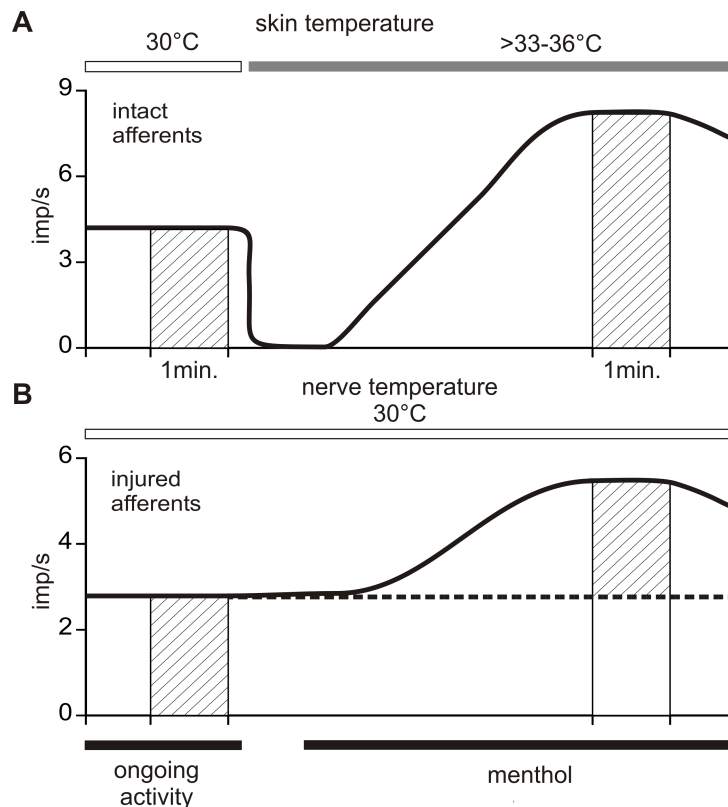


Figure 3.25. Quantitative measurements of ongoing activity and responses to menthol in intact and injured afferent nerve fibres. **A.** Intact afferents. The initial ongoing activity was counted over one minute at a skin temperature of 30°C. A few minutes before and during application of menthol, the skin was heated by a radiant lamp to a temperature of about 33 to 36°C to suppress the ongoing activity. The maximal response to menthol was counted over a time period of one minute. **B.** Injured afferents. Both the initial ongoing activity and the maximal response to menthol were measured over a time period of one minute at a nerve temperature of about 30°C. In order to have only the response to menthol, the ongoing activity (indicated by the interrupted line) was subtracted. This figure explains how the values from figure 3.21 were calculated.

Figure 3.29A illustrates the responses of three injured C-fibres to heat stimuli applied to the nerve.

The threshold of the response to heat stimuli applied to the nerve was $45.3^{\circ}\text{C} \pm 1.3^{\circ}\text{C}$ (N=19). Nine heat-sensitive C-fibres, which were not type 1 cold-sensitive, showed a low rate of ongoing activity at a nerve temperature of about 30°C ($0.23 \pm 0.03 \text{ imp/s}$). Four heat-sensitive fibres which were also type 1 cold-sensitive exhibited a high rate of ongoing activity. The activation of these type 1 cold-sensitive fibres by heat stimuli was always preceded by inhibition of the ongoing activity.

Sixteen of 22 injured heat-sensitive C-fibres were not excited by menthol (4 mM) applied to the nerve (Table 3.5). Six heat-sensitive C-fibres which were additionally cold-

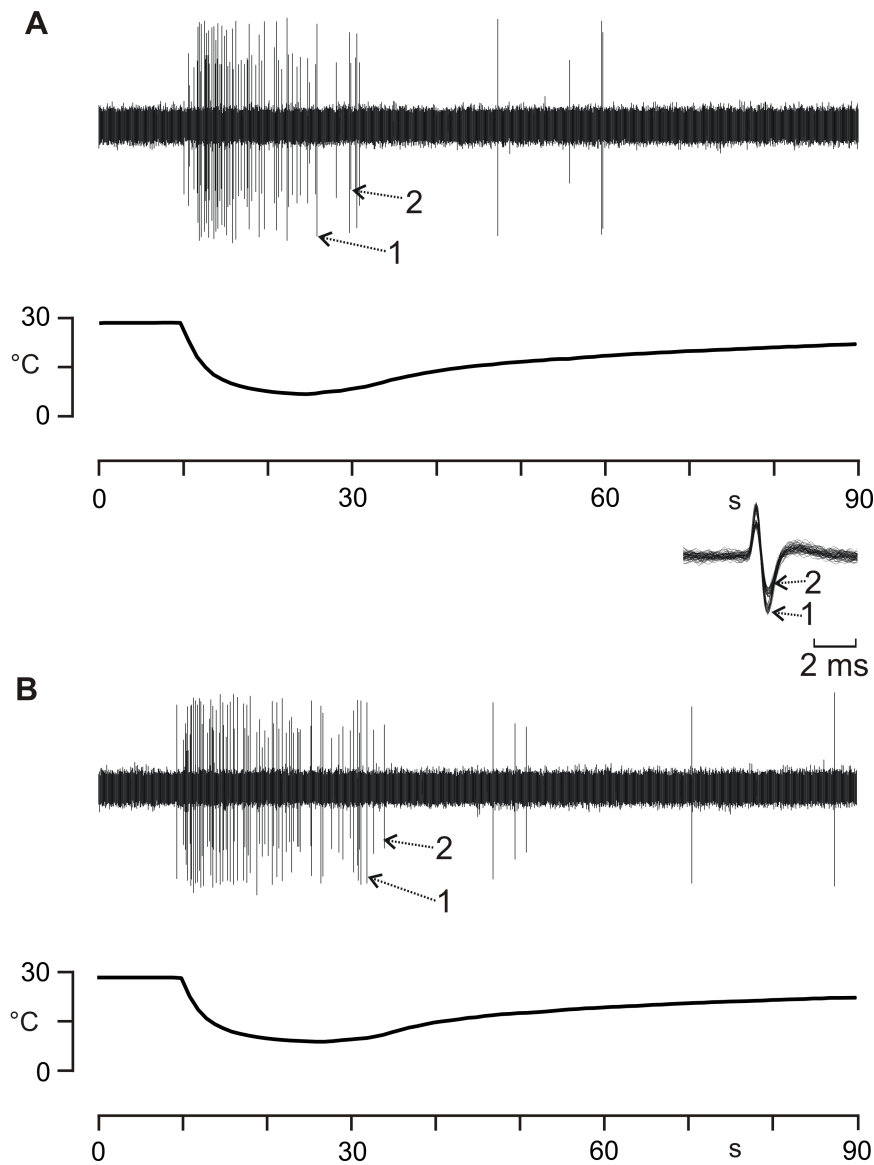


Figure 3.26. Responses of two injured afferent C-fibers with type 2 cold-sensitivity to cold stimuli applied to the receptive fields in the nerve before (**A**) and after application of menthol (**B**). The cold stimuli were delivered by a thermode, starting from an adaptation temperature of about 30°C and decreasing to 5°C. Inset: action potentials of these two C-fibers several times superimposed.

sensitive were excited by menthol. Five of them were also type 1 cold-sensitive C-fibres and one was also a type 2 cold-sensitive C-fibre. These cold-sensitive C-fibres were fully characterized in the previous subchapters and therefore, they were not included in the graphs of this subchapter. Responses of three heat-sensitive C-fibres to heating of the nerve before and after application of menthol are depicted in Figure 3.29.

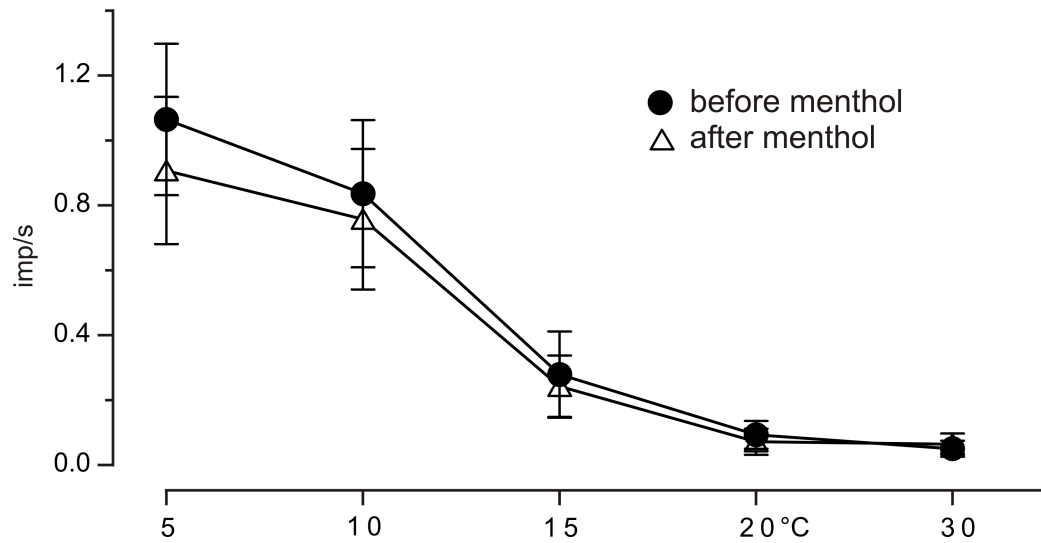


Figure 3.27. Stimulus-response curves of 7 injured afferent C-fibers with type 2 cold-sensitivity before (black circles) and after application of menthol (white triangles). Mean \pm SEM of the cold activity counted during 20 s stimulation. The cold stimuli started from an adaptation temperature of about 30°C and decreased to 5°C.

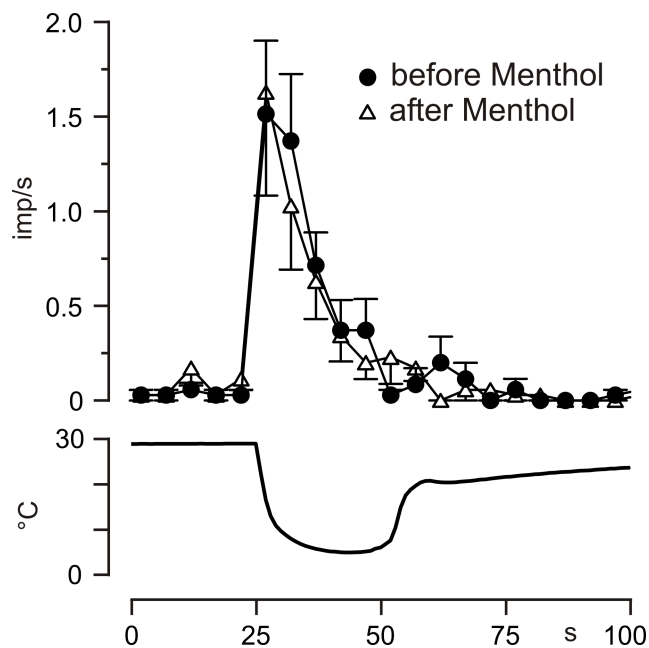


Figure 3.28. Responses of 7 injured type 2 cold-sensitive C-fibers to cold stimuli applied to the receptive fields in the nerve before (black circles) and after application of menthol (white triangles). The cold stimuli started from a baseline temperature of about 30°C and went to 5°C. Bin width, 5 s. Mean \pm SEM.

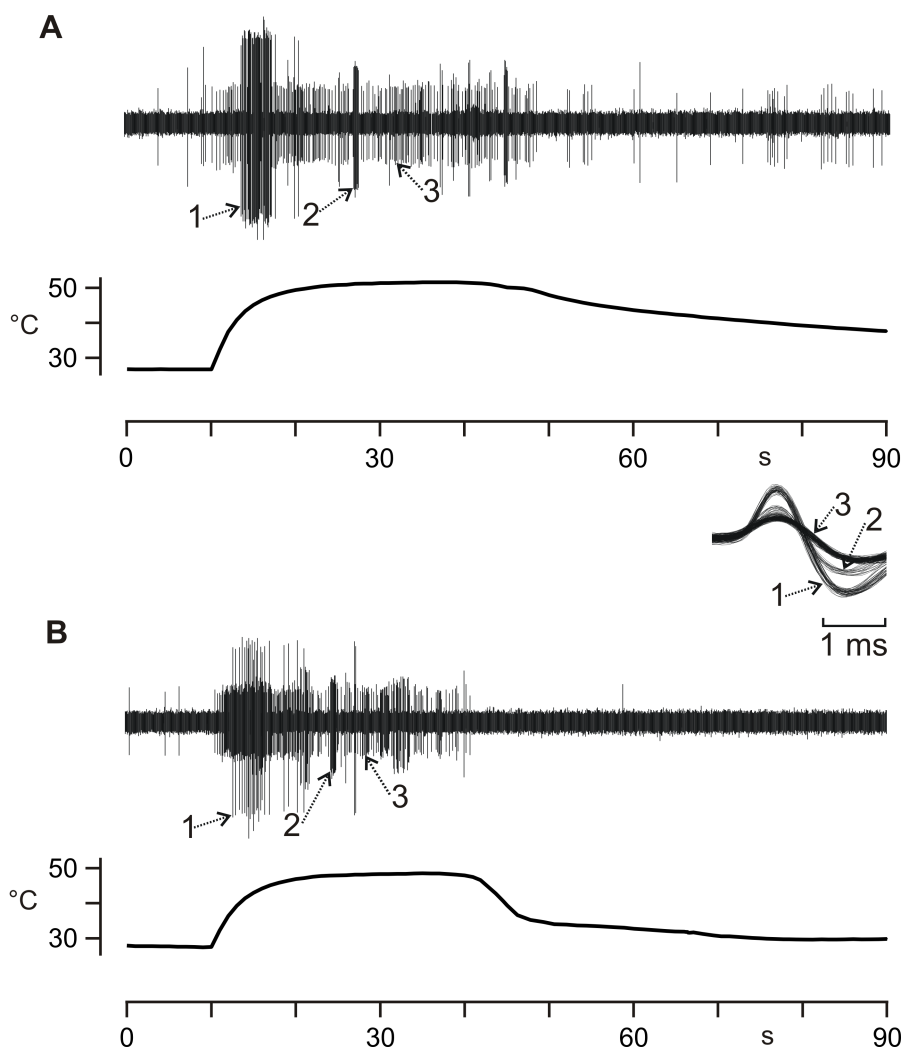


Figure 3.29. Responses of 3 injured C-fibers with heat-sensitivity to heat stimuli applied to the receptive fields in the nerve before (A) and after application of menthol (B). The heat stimuli started from a baseline temperature of about 30°C and increased to 52°C. Inset: action potentials of three C-fibers superimposed several times.

Figure 3.30 shows the population response of injured C-fibers to heat stimulation of the nerve before (black circles) and after (white triangles) application of menthol. It can be seen that the heat responses were not changed after application of menthol.

3.4.1.4 Mechanosensitive C-fibres

Five injured afferent C-fibers were activated by mechanical stimuli applied to the nerve (Table 3.5). These C-fibers were also heat-sensitive. Three of them exhibited ongoing activity: 0.15 imp/s, 0.23 imp/s, 0.43 imp/s. All injured mechanosensitive C-fibers were

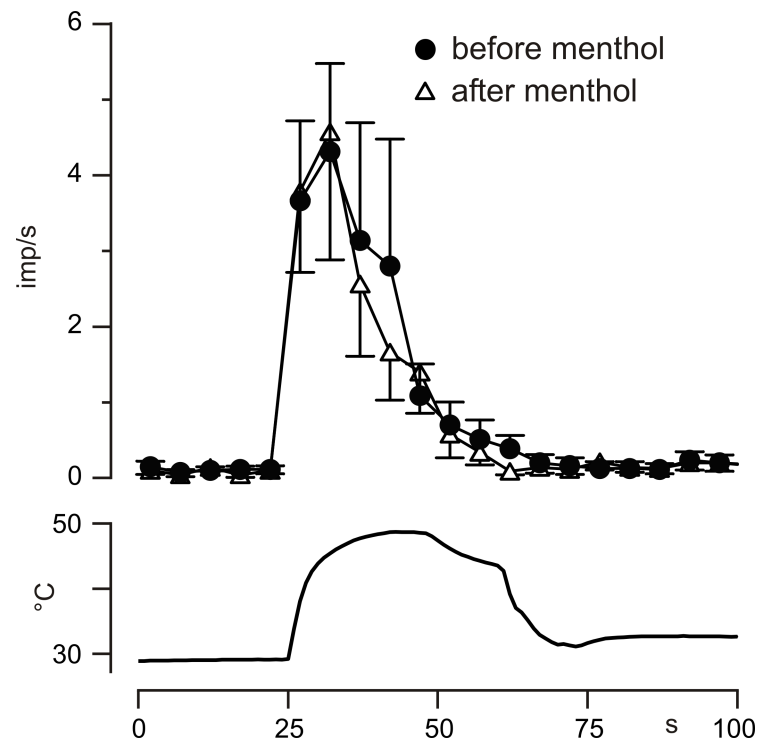


Figure 3.30. Mean response (+1 SEM) of 16 C-fibres to heat stimuli applied to the receptive fields in the nerve before (black circles) and after application of menthol (white triangles). The heat stimuli were delivered by a thermode, starting from an adaptation temperature of about 30°C and going over to 50°C. Time resolution 5 s.

not activated by menthol. Their responses to mechanical stimulation of the nerve after application of menthol were not influenced by menthol.

3.4.2 A-fibres

In all, 23 injured functionally identified A-fibres were tested for their responses to menthol (Table 3.5). They were tested for their responses to physiological stimuli before and after application of menthol following the same testing procedure as used for the C-fibres. Most injured A-fibres were mechanosensitive and very few were thermosensitive.

3.4.2.1 Thermosensitive A-fibres

Eight injured afferent A-fibres were activated to thermal (heat, cold) stimuli applied to the receptive fields in the nerve (Table 3.5). Seven of them were heat-sensitive or heat- and mechano-sensitive and one was type 2 cold-sensitive. Three thermosensitive A-fibres showed ongoing activity at a nerve temperature of about 30°C: 0.95 imp/s, 5.8 imp/s,

6.9 imp/s. Two of 8 injured thermosensitive A-fibres were activated by menthol applied to their receptive fields in the nerve. One was a type 2 cold-sensitive afferent A-fibre and the other a heat-sensitive fibre. The other six A-fibres were not excited by menthol and also their responses to thermal stimuli were not influenced by menthol.

3.4.2.2 Mechanosensitive A-fibres

Eighteen injured afferent A-fibres were activated by mechanical stimuli applied to the receptive fields in the nerve (Table 3.5). Three of them were also heat-sensitive. These fibres, with one exception, were silent. The injured mechanosensitive A-fibres were not activated by menthol. Their responses to mechanical stimulation of the nerve were not changed after application of menthol.

3.5 Patterns of discharge of intact and injured C-fibres and A-fibres to physiological stimulation and responses to menthol.

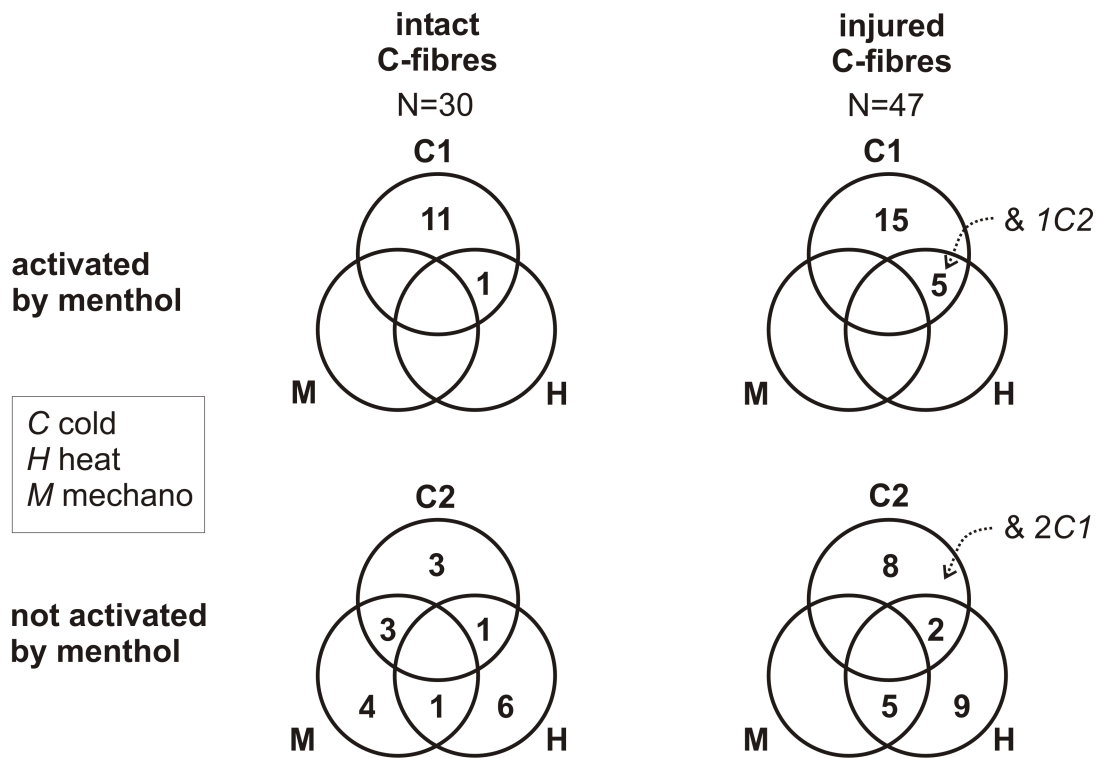
In this chapter, the responses to menthol of intact and injured C- and A-fibres in relation to their physiological properties are summarized and compared.

A comparison between the patterns of discharge to physiological (thermal and mechanical) stimulation of intact C-fibres and of injured C-fibres in relation to their responsiveness to menthol, as shown in the last two sections, is depicted in Figure 3.31.

All intact afferent C-fibres activated by menthol were type 1 cold-sensitive fibres. With one exception, all injured C-fibres excited by menthol were also type 1 cold-sensitive fibres. The exception was a type 2 cold-sensitive fibre. Thus, thirty-two of 34 intact or injured afferent C-fibres excited by menthol were type 1 cold-sensitive fibres.

The intact C-fibres not activated by menthol were type 2 cold-sensitive, heat-sensitive, mechanosensitive or showed combinations of these properties. With two exceptions, the injured C-fibres not excited by menthol were also type 2 cold-sensitive, heat-sensitive, mechanosensitive or showed combinations of these properties. The menthol-insensitive exceptions were two injured type 1 cold-sensitive fibres. Thus, forty-two out of 44 intact or injured afferent C-fibres not excited by menthol were type 2 cold-sensitive fibres and cold-insensitive fibres.

Figure 3.32 illustrates the patterns of discharge to thermal and mechanical stimulation in intact and injured A-fibres in relation to their responsiveness to menthol. All intact



	type 1 cold (C1)	type 2 cold (C2)	cold insensitive (H, M)
activated by menthol	32	1	0
not activated by menthol	2	17	25

Figure 3.31. Distribution of cold sensitivity (C1, C2), heat sensitivity (H) and mechanosensitivity (M) of intact and injured cutaneous afferent C-fibres activated/not activated by menthol. The overlaps represent the number of fibres which exhibit a combination of two of the mentioned properties. The intact and injured C-fibres excited by menthol were, with one exception, type 1 cold-sensitive C-fibres (C1). The C-fibre excited by menthol, but being not type 1 cold-sensitive, was an injured type 2 cold-sensitive C-fibre (C2). The menthol-insensitive intact and injured afferent C-fibres were, with two exceptions, type 2 cold-sensitive, heat-sensitive, mechanosensitive or showed combinations of these properties. The two exceptions of C-fibres not activated by menthol were injured type 1 cold-sensitive fibres. The table shows the total number of intact and injured afferent C-fibres activated or not activated by menthol.

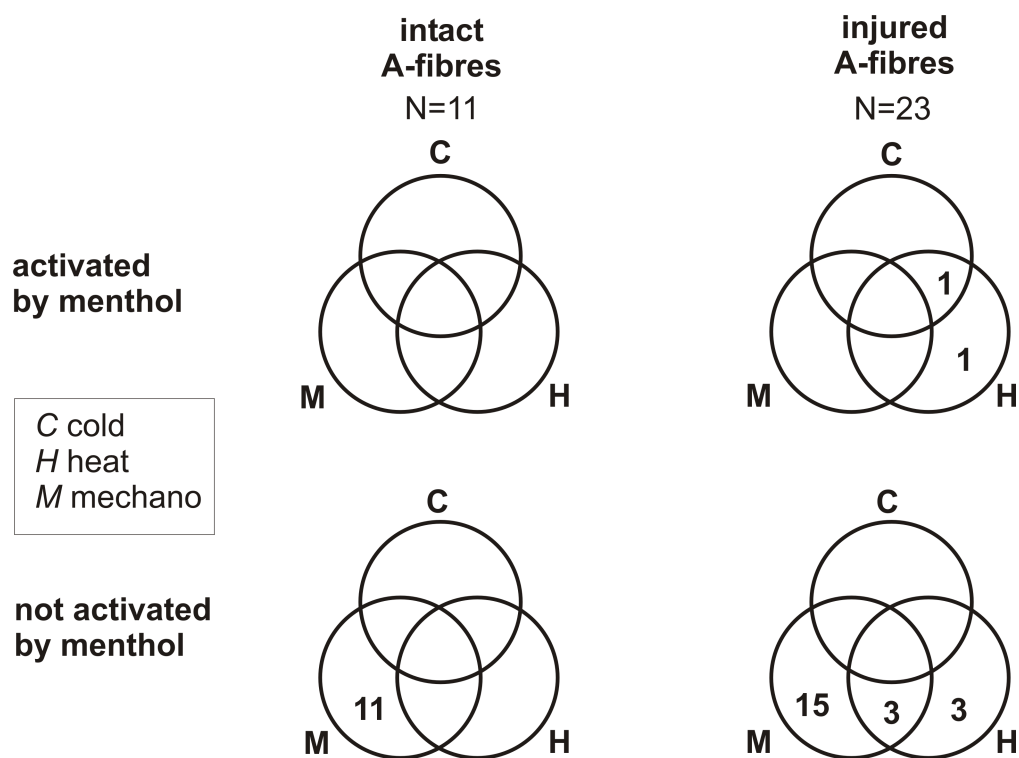


Figure 3.32. Distribution of cold sensitivity (C), heat sensitivity (H) and mechanosensitivity (M) in intact and injured cutaneous afferent A-fibres activated/not activated by menthol. All intact and injured afferent A-fibres were, with two exceptions, menthol-insensitive. The A-fibres activated by menthol were an injured type 2 cold-sensitive fibre and an injured heat-sensitive fibre.

A-fibres were menthol-insensitive. Twenty-one out of 23 injured afferent A-fibres were not activated by menthol. The remaining two injured A-fibres excited by menthol were a type 2 cold-sensitive A-fibre and a heat-sensitive fibre. Thus, with two exceptions, all intact and injured afferent A-fibres were menthol-insensitive.

3.6 Axonal responses of functionally identified C-fibres and A-fibres to menthol

In the previous chapter (3.2) referring to axonal thermo- and mechano-sensitivity of cutaneous afferent neurons it was shown that: 1) almost all type 1 cold-sensitive C-fibres that were activated by cold stimuli applied to the skin could also be activated by cold stimuli applied to their axons in the sural nerve; 2) about 1/3 of the type 2 cold-sensitive C-fibres and the nociceptive heat-sensitive C-fibres activated from the skin could also be

Table 3.6. Numbers of functional types of afferent neurons activated or not activated by menthol applied to their axons.

		ACTIVATION		NO ACTIVATION		
		C-fibres	A-fibres	C-fibres	A-fibres	Total
Identified from skin	Type 1 cold-sensitive	1	0	19	0	20
	Type 2 cold-sensitive	0	0	1	0	1
	Heat-sensitive	0	0	2	0	2
	Mechanosensitive	0	0	1	14	15
<i>Total</i>		<i>1</i>	<i>0</i>	<i>23</i>	<i>14</i>	<i>38</i>
Not identified from skin	Cold or cold-& heat-sensitive	3	0	12	0	15
	Heat-sensitive	0	0	8	0	8
<i>Total</i>		<i>3</i>	<i>0</i>	<i>20</i>	<i>0</i>	<i>23</i>

activated by cold or heat stimuli applied to the sural nerve. The question to be addressed in this chapter is if type 1 cold-sensitive C-fibres are activated by menthol applied to their axons in the nerve, while the type 2 cold-sensitive and cold-insensitive C-fibres are menthol-insensitive. This idea was tested in 19 experiments in which the sural nerve was not injured before.

The afferent C-fibres and A-fibres, which were functionally characterized by their responses to physiological stimulation of the skin and axon or only of the axon, were tested for their responses to menthol applied to their axons. In 30 filaments, 47 C-fibres and 14 A-fibres activated by physiological stimuli applied to the skin and axon or only to the axon were investigated in this study. Table 3.6 illustrates the distribution of properties in the C-fibres and A-fibres activated or not activated by menthol and functionally identified either from skin and axon or only from axon.

3.6.1 Sensitivity to menthol of C-fibres and A-fibres functionally identified from skin and axon

In total, twenty-four C-fibres and fourteen A-fibres characterized functionally from the skin and axon were tested for their excitability to menthol. These fibres were functionally identified from the skin on the basis of their responses to physiological stimuli as type 1 cold-sensitive, type 2 cold-sensitive, heat-sensitive and mechanosensitive fibres.

Twenty cold-sensitive C-fibres, identified as type 1 cold-sensitive from the skin and activated from the axon by cold stimuli, were tested for their responses to menthol applied to their axons in the sural nerve (Table 3.6). Two of them were additionally heat-sensitive fibres. They had an activation threshold to cold stimuli of $28 \pm 0.14^\circ\text{C}$. As we have seen in the previous chapter (3.4) the cold responses were dynamic and static. The dynamic responses were graded and had a maximal mean discharge rate of 17.9 ± 1.83 imp/s; the static responses were also graded and their maximal mean discharge rate was 15 ± 1.54 imp/s. The type 1 cold-sensitive C-fibres showed a high rate of ongoing activity (4.2 ± 0.47 imp/s) originating at the nerve terminals in the skin. In order to observe the effect of menthol applied to the axons in the nerve the ongoing activity in these C-fibres was depressed either by acute crushing of the sural nerve, after functional identification of the fibres from the skin (in 7 C-fibres), or by warming the cutaneous receptive field of the fibres up to $40\text{--}45^\circ\text{C}$ (in 13 C-fibres). Under this condition small pieces of filter paper soaked in menthol-solution (4 mM in 8 fibres or 8 mM in 12 C-fibres) were applied to the sural nerve for 5 min (4-16 mm proximal to the crush lesion in the acutely injured nerve). One out of 20 type 1 cold-sensitive axons investigated was activated by menthol (8 mM; Fig. 3.33) while the ongoing activity was depressed by a warm stimulus of 40°C applied to the cutaneous receptive field of the fibre. The response to menthol consisted of a delayed increase of activity.

Three C-fibres functionally identified as a type 2 cold-sensitive fibre or heat-sensitive fibres from the skin and showing axonal sensitivity to cold or heat stimuli were not activated by menthol applied to their receptive fields in the axon (Table 3.6).

Fifteen mechanosensitive afferent fibres (14 A-fibres and 1 C-fibre) were tested for their axonal menthol sensitivity (8 mM) and none of them were excited by menthol (Table 3.6).

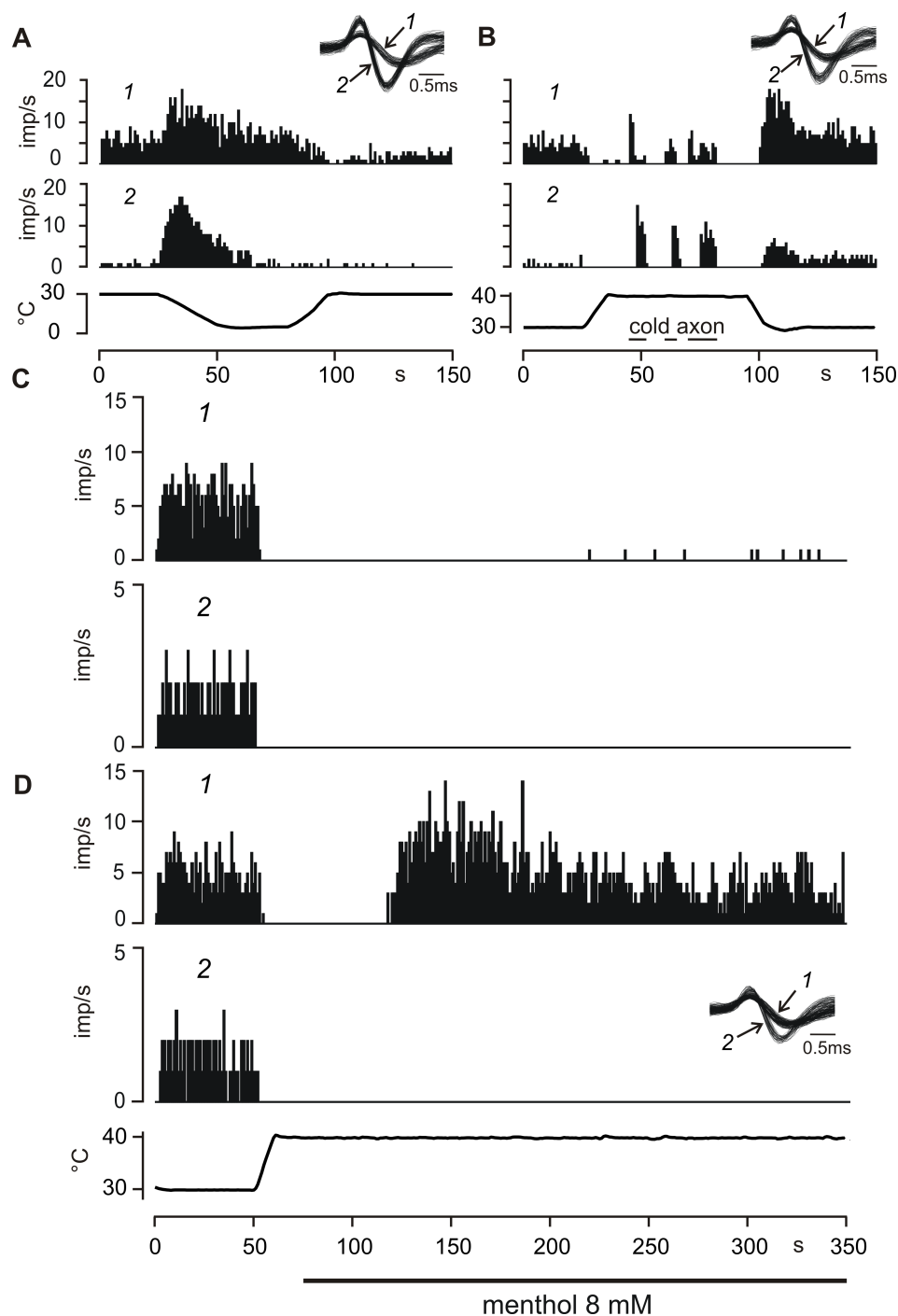


Figure 3.33. Responses of two intact afferent C-fibers with type 1 cold-sensitivity to cold stimuli and menthol. **A.** Responses to cold stimuli applied to their receptive fields in the skin. The cold ramp stimuli started from an adaptation temperature of 30°C and went to a temperature of 5°C. **B.** Activation of the C-fibers by a cold rod applied to the sural nerve. During application of the cold stimulus, the ongoing activity of these fibres was depressed by a warm stimulus of 40°C applied to the skin. **C.** Depression of the ongoing activity by a warm stimulus of 40°C applied to the skin. The ongoing activity was almost entirely depressed. **D.** Activation of fibre 1 by menthol (8 mM) applied to the sural nerve during depression of ongoing activity by warming the skin. Insets in **A**, **B** and **D**: action potentials of fibres 1 and 2 superimposed several times.

3.6.2 Sensitivity to menthol of acutely injured afferents C-fibres functionally identified from axon

Twenty-three C-fibres identified as heat-sensitive, cold-sensitive, cold- and heat-sensitive from the sural nerve (axon) after acute nerve crush were tested also for their responsiveness to menthol applied to the nerve. The distribution of their properties is shown in Table 3.6. In these fibres menthol (4 mM in 3 fibres and 8 mM in 20 C-fibres) was applied for 5 min to their axons about 4-16 mm proximal to the crush lesion.

Eight C-fibres were activated only by heat stimuli applied to the axon proximal to the lesion site. These C-fibres were not activated by menthol applied to the nerve (Table 3.6).

Fifteen C-fibres were activated by cold or cold and heat stimuli applied to their axons. Three of them were excited by menthol applied to their axons. The activation to menthol was weak in two fibres and strong in one fibre. One of the menthol-sensitive fibres was additionally activated by heat stimulation of the nerve. As these 15 C-fibres were not identified as type 1 or type 2 cold-sensitive fibres from the skin, the discrimination between these two types of cold-sensitivities from the axon was not possible.

Chapter 4

Discussion and conclusions

This experimental work had two main goals: **1)** to work out the axonal thermo- and mechano-sensitivity of intact afferent cutaneous neurons. Special emphasis was put on the axonal cold sensitivity which until now was not systematically studied. **2)** It was tested in intact and injured afferent nerve fibres if non-noxious (type 1) cold-sensitive C-fibres are sensitive to menthol, whereas noxious (type 2) cold-sensitive C-fibres and cold-insensitive C-fibres are menthol-insensitive. In this context it was tested whether menthol can excite intact type 1 cold-sensitive neurons when applied to their axons.

Unmyelinated axons of peripheral nerves have been shown to develop diverse properties at the proximal stump after nerve injury (Blenk et al., 1996; Gorodetskaya et al., 2003; Grossmann et al., 2009b). These sensory properties including the abnormal spontaneous activity, thermo- and mechano-sensitivity are developing on the axon at the neuroma site (Michaelis et al., 1995; Blenk et al., 1996). This abnormal (pathological) activity can trigger, in patients with nerve injury, burning pain and mechanical and/or thermal (cold or heat) hyperalgesia and/or allodynia. Cold allodynia is a common feature of neuropathic pain in the clinical setting. However, the underlying mechanisms of this enhanced sensitivity to innocuous cold stimuli (allodynia) are until now poorly understood.

The ectopic activity developing at the lesion site of an afferent neuron could be explained by the fact that following a nerve lesion, the anterograde axonal transport of sensory transduction molecules such as heat-, cold- and mechanically activated channels is interrupted and therefore, these molecules will accumulate at the injury site (Gorodetskaya et al., 2003; Grossmann et al., 2009b; Jänig et al., 2009). This accumulation of receptors and channels at the lesion site will provide the regenerating axonal sprouts with the same functional properties (thermo- and mechano-sensitivity) their sensory nerve endings possessed before injury (Devor and Govrin-Lippmann, 1983; Koschorke et al., 1991, 1994; Blenk et al., 1996; Michaelis et al., 1999). Some authors (Zimmermann and Sanders,

1982; Blenk et al., 1996; Hoffmann et al., 2008) reported the presence of the sensory properties (i.e. heat sensitivity) not only in the regenerating axons but also in the intact axons. Thus, the sensory properties probably are present in the axonal membrane as well.

Thermo- and mechano-sensitivity of the afferent neurons are subserved by a variety of transduction molecules, including those of the transient receptor potential (TRP) ion channel superfamily. The TRP superfamily consists of proteins with six transmembrane domains (6TM) that are assembled as homo- or heterotetramers to form cation-permeable ion channels (Voets et al., 2005). Based on sequence homology, the 28 mammalian TRPs are classified into six subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP and TRPML (Voets et al., 2005; Montell, 2005). A seventh subfamily, TRPN, has members in lower vertebrates and invertebrates only. A subset of the TRP superfamily, dubbed thermo-TRPs, is highly sensitive to temperature, and several thermo-TRPs essentially serve as molecular thermometers in different cell populations of the peripheral sensory system (Voets et al., 2005).

TRP channels involved in cold sensing

TRPM8 is a non-selective cation channel responding to non-noxious cold stimuli and also to substances causing a sensation of coolness such as menthol, icilin and eucalyptol (McKemy et al., 2002; Peier et al., 2002; Story et al., 2003; Brauchi et al., 2004; Bandell et al., 2004; Madrid et al., 2006; Bautista et al., 2007; Dhaka et al., 2007; Colburn et al., 2007). This channel may underlie the type 1 (low-threshold) cold-sensitivity of intact A δ -fibres and C-fibres and of regenerating C-fibres. As shown in chapter Results 3.3, 3.4, 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.

The role of TRPM8 in cold transduction was recently worked out by three independent groups studying the behavior of TRPM8-deficient mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Using a two temperature preference assay, the mice were challenged to choose between a preferred warm temperature (30-34°C) and a cool temperature usually avoided by them. The studies show that mice exhibit strong deficits in the ability to discriminate warm from cool temperatures. For example, whereas wild-type mice show a clear preference for the warm temperature range (30-35°C) compared to lower temperatures, TRPM8-deficient mice show no clear thermal preference in the range between 15 and 35°C. Moreover, two studies showed that TRPM8 knockout mice regained aversion to cold temperatures at or below 10°C (Bautista et al., 2007; Dhaka et al., 2007), whereas one study showed that the deficit in cold temperature detection persists down to

0°C (Colburn et al., 2007). Hence, all three studies indicate that TRPM8 plays a central and essential role in cold temperature transduction and perception.

The role of TRPM8 in mediating cold, mechanical and heat hypersensitivity under pathophysiological (neuropathic pain) conditions remains evasive. TRPM8 expression is increased in the ipsilateral DRG neurons after the development of neuropathic pain (Frederick et al., 2007; Proudfoot et al., 2006; Xing et al., 2007). Furthermore, different studies indicate that both agonism and antagonism of TRPM8 may be involved in mediating the analgesia in neuropathic pain. 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) (Proudfoot et al., 2006). This demonstrates that central expression of TRPM8 in primary afferents innervating the dorsal horn can contribute to the analgesic effect. TRPM8 agonists were also effective in reversing both thermal and mechanical hypersensitivity in the Complete Freund's Adjuvant (CFA) model of inflammatory pain and in the cinnamaldehyde-induced hypersensitivity (Proudfoot et al., 2006). A more recent study using a subtle modification of the formalin test elegantly showed that while wild-type mice exhibit reduced formalin induced nocifensor behavior when placed on plates set at 17°C, mice lacking TRPM8 develop similar nociceptive responses at 17°C and room temperature (Dhaka et al., 2007). While these studies clearly implicate TRPM8 agonism in reversing the hypersensitivity observed across a wide spectrum of pain models, recent data suggest that TRPM8 blockade may lead to an analgesic effect as well. Colburn et al. (2007) showed that TRPM8 deficient homozygous mice develop virtually no cold allodynia in both CCI and CFA models, while tactile allodynia is not affected in both genotypes. In summary, there is strong evidence suggesting that TRPM8 may play an important role in nociception. However, it remains unclear whether agonism or antagonism of this target should be pursued to treat clinical pain indications.

TRPA1 is a distantly related TRP channel expressed in DRG neurons and in the inner ear (Corey et al., 2004; Story et al., 2003). Electrophysiology data demonstrate that TRPA1 is activated by temperatures of about 17°C (i.e. at temperatures about 5°C lower than for TRPM8) making it a prime candidate for transducing noxious cold (Story et al., 2003; Story and Gereau, 2006). It was shown, that TRPA1 is also sensitive to mustard oil or cinnamaldehyde (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Karashima et al., 2009). However, 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 owing to complete Freund's

adjuvant (CFA) or after nerve injury (Obata et al., 2005). Our type 2 (high-threshold) cold-sensitivity in intact and regenerating C-fibres is suggested to be mediated by this channel. However, it is debatable whether this channel is specifically responsible for the transduction of noxious cold stimuli (Reid, 2005; 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 not express either the TRPM8 or the TRPA1 protein, and it is suggested that several other channels, in particular potassium channels, may be involved in the responses of afferent neurons to cold stimuli (Munns et al., 2007; Babes, 2009).

TRP channels involved in heating sensation

Four members of the TRPV family of nonselective cation channels can be activated by warm to hot temperatures. TRPV1 (43°C) and TRPV2 (52°C) have properties consistent with noxious heat sensors. Both TRPV3 (33°C) and TRPV4 (25°C-34°C) respond to warm temperatures.

Also known originally as VR1, **TRPV1** is the founding member of the subfamily of TRP channels expressed in sensory neurons and is most widely studied. TRPV1 is a Ca²⁺-permeable channel, and is activated by temperatures in the noxious range >43°C and by capsaicin, the pungent ingredient of chili peppers (Caterina et al., 1997; Jordt and Julius, 2002). Consistent with a role in nociception, 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 and Caterina, 2004).

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 (Montell et al., 2002; Beech et al., 2004; Muraki et al., 2003). This channel is activated by high temperatures (> 52°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 in [Ca²⁺]_i in vascular smooth muscle cells (Beech et al., 2004; Muraki et al., 2003).

TRPV3 is expressed in DRG neurons, TG neurons, brain, keratinocytes, tongue, and testis (Smith et al., 2002; Peier et al., 2002; Chung et al., 2003; Moqrich et al., 2005). It is activated by innocuous (≥ 30 -33°C) warm temperatures and by the natural compound camphor, which is known to sensitize skin to warm stimuli, and which does not activate any of the other TRPVs (Smith et al., 2002; Peier et al., 2002; Chung et al., 2003; Moqrich et al., 2005).

TRPV4 is widely expressed in the brain, DRG neurons, and multiple excitable and non-excitable peripheral cell types (Nilius et al., 2004). TRPV4 can be activated by surprisingly diverse stimuli, including moderate heat ($>24^{\circ}\text{C}$, i.e. TRPV4 is constitutively active at normal body temperature), cell swelling, shear stress, the endocannabinoid anandamide (a polyunsaturated fatty acid) and the arachidonic acid metabolite 5,6-epoxyeicosatrienoic acid (5,6 EET) (Nilius et al., 2003, 2004; Watanabe et al., 2002, 2003).

Heat sensitivity of our afferent (intact and injured) nerve fibres is primarily related to the activation of the TRPV1 channel (Caterina et al., 1997, 1999; Caterina, 2007), but probably also to the activation of other channels such as TRPV3 and TRPV4 (Güler et al., 2002; Smith et al., 2002; Xu et al., 2002; Woodbury et al., 2004; Caterina, 2007). The TRPV2 channel can also be involved in transduction of heat sensitivity (Lawson et al., 2008; Jankowski et al., 2009). In our laboratory, we showed that the injured and regenerating C-fibres with heat-sensitivity were excited by the TRPV1 agonist, capsaicin.

Channels involved in mechanosensation

Indirect evidence suggests that the heat-sensitive channels **TRPV2** and **TRPV4** are also mechanosensitive (Alessandri-Haber et al., 2006; Mutai and Heller, 2003; O'Neil and Heller, 2005; Suzuki et al., 2003). A sensitization to heat and mechanical stimuli may therefore be correlated. Besides these two TRP channels, in transduction of mechanical stimuli can be also involved some specific mechanically gated cation channels belonging to epithelial Na^+ channel (ENaC) family and to acid sensing ion channel (ASIC) family (Lumpkin and Caterina, 2007; Price et al., 2000; Waldmann and Lazdunski, 1998). These channels do not seem to be involved in mechanonociception.

The mechanism(s) underlying the mechanosensitivity in our afferent neurons is (are) until now not well understood. It is not far-fetched to postulate more than one mechanism underlying mechanosensitivity (Lewin and Moshourab, 2004; Hu et al., 2006; Julius and McCleskey, 2006; Lumpkin and Caterina, 2007).

4.1 Limitations of methods

In vivo experimentation is important for basic research because it gives us more information about the cellular processes directly from a living subject. However, the experimental work on living experimental animals enables to obtain correct results only when the conditions as defined by the arterial blood pressure (>70 mmHg), body temperature ($\approx 37^{\circ}\text{C}$),

oxygen saturation and acid-base balance are kept in the physiological range. Thus, these vital parameters should be checked and kept under control during the whole experiment, otherwise we will not be able to account (at least correctly or fully) for what we are observing.

4.2 Axonal thermo- and mechano-sensitivity of cutaneous afferent neurons

In 32 experiments without nerve lesion, 56/109 (51.4%) of the C-fibres and 2/118 (1.69%) of the A-fibres identified as thermo- and/or mechano-sensitive from the skin by thermal and mechanical stimuli could also be activated by these stimuli from the nerve. The main results of this study can be detailed as follows: **(1)** Almost all non-nociceptive (type 1) cold-sensitive C-fibres could be activated by cold stimuli from both skin and nerve. **(2)** About one third of the nociceptive (type 2) cold-sensitive C-fibres or heat-sensitive C-fibres can also be activated by cold or heat stimuli applied both to skin and nerve. **(3)** Mechanosensitivity in A- and C-fibres is restricted to the receptive afferent endings in the skin in practically all fibres. **(4)** With the exception of a few type 1 cold-sensitive C-fibres, which were activated by heat stimuli applied to the nerve but not by heat stimuli applied to the skin, no afferent fibre activated from the skin by a specific physiological stimulus was activated from the nerve by another physiological stimulus.

4.2.1 Type 1 cold-sensitive C-fibres

In this study, we showed that about 97% of the non-noxious (type 1) cold-sensitive C-fibres activated by cold stimuli applied to the skin can also be activated by cold stimuli applied to their axons. The mean peak response (over 5 s) to cold stimulation of the skin (after subtracting the ongoing activity) was not significantly larger than the mean peak response to stimulation of the nerve (12.3 ± 2.1 imp/s vs 9.9 ± 1.8 imp/s, $p=0.5$, Wilcoxon paired test). This axonal innocuous cold-sensitivity is a novel finding. Our study clearly argues that the transduction molecules for cold stimuli are not only present in the receptive terminals of the axons, but also in the membranes of axon.

Type 1 cold-sensitive afferent nerve fibres are activated by non-noxious cold stimuli and a few of them are also by strong heat stimuli. The molecular sensor involved in transducing non-noxious stimuli into an electrical signal is suggested to be the TRPM8 channel (McKemy et al., 2002; Story et al., 2003; Bandell et al., 2004; Madrid et al., 2006;

Bautista et al., 2007; Dhaka et al., 2007; Colburn et al., 2007).

4.2.2 Type 2 cold-sensitive C-fibres

Eleven out of 28 (39%) of the C-fibres exhibiting noxious cold-sensitivity from the skin (type 2 cold sensitivity) can also be activated by cold stimuli applied to the nerve. These results are in accordance with those published by Hoffmann et al. (2008) who showed that 25% of the mechano-& cold-sensitive and mechano-, heat-& cold-sensitive C-fibres (type2) identified in the isolated mouse skin responded clearly to axonal cold stimulation. The frequency of ectopic cold-sensitivity was smaller than in our study (39%), one reason for this quantitative difference could be that this group did not search systematically for cold-sensitive fibres. Moreover, Jankowski et al. (2009) and Lawson et al. (2008), describing intact or regenerated C-fibres in the saphenous nerve or a dorsal cutaneous nerve to the hairy skin of the mouse, found cold-sensitive C-fibres to be virtually absent. Thus, there may be species differences in this respect between rat and mouse.

Type 2 cold-sensitive C-fibres are excited by noxious cold stimuli (nociceptive fibres). Most of these fibres are also excited by heat stimuli and/or mechanical stimuli. The transduction channel underlying the type 2 cold sensitivity (noxious cold sensitivity) may be related to the activation of the TRPA1 channel, a transduction channel that is also sensitive to mustard oil or cinnamaldehyde (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Karashima et al., 2009).

4.2.3 Heat-sensitive C-fibres

Twenty-one of 61 (34%) noxious heat-sensitive C-fibres activated by noxious heat stimuli from the skin can be activated also by heat stimuli from the axon. The mean peak response (over 5 s) to heat stimulation of the skin was not significantly larger than the mean peak response to heat stimulation of the nerve (skin, 5.0 ± 1.3 imp/s; nerve, 3.6 ± 0.92 imp/s; $p=0.6$, Wilcoxon paired test).

Heat-sensitive C-fibres are nociceptive, most of them being probably polymodal. Here, we may have missed the high-threshold mechanosensitivity of some of these heat-sensitive fibres, as we did not apply pinch stimuli to the skin. Heat sensitivity is primarily related to the activation of the TRPV1 channel (Caterina et al., 1997, 1999; Caterina, 2007). Using electron microscopy, it has been shown that the capsaicin receptor TRPV1 is expressed in the axonal membranes of unmyelinated fibres of peripheral nerves, and that vesicular exocytosis of calcitonin gene-related peptide from these nerve fibres is stimulated

by capsaicin (Bernardini et al., 2004).

Indirect evidence of axonal heat sensitivity has previously been suggested from electrophysiological experiments. Recordings obtained from cat dorsal horn neurons showed that noxious heat applied to the peripheral nerve of the segmentally corresponding dermatome leads to an increase in ongoing discharge rate (Zimmermann and Sanders, 1982). More directly, Blenk et al. (1996) have reported that in vivo single fibre recordings from the rat sural nerve, five of 172 ($\approx 3\%$) afferent fibres could be activated by warm stimuli applied to the nerve stem through a contact thermode. The axonal heat sensitivity was also noted by Sauer et al. (1999, 2001) and Fischer et al. (2003), reporting that in vitro, the noxious heat or proton ions applied to an isolated desheathed sciatic nerve of rats leads to release of calcitonin gene-related peptide expressed in C- and A δ -fibres.

Our results are in accordance with those obtained by Hoffmann et al. (2008), who showed on a skin-nerve preparation of mice that about two-thirds (25/38; 66%) of heat-sensitive C-fibres can also be activated by heat stimuli applied to their axons in the nerve. The frequency of ectopically heat-activated C-fibres (in Hoffmann et al., 2008) was significantly higher than in our study (34%). The reason for this quantitative difference is unclear, in particular as we tested the ectopic axonal heat sensitivity regularly at five sites of the nerve and with stimuli normally exceeding 50°C. Moreover, the response magnitude of the afferent fibres to axonal heat stimulation in Hoffmann et al. (2008) was in the same range as in the present study.

4.2.4 Mechanosensitive C-fibres

In this study we clearly showed that the transduction mechanisms for weak or strong mechanical stimuli in cutaneous afferent A-fibres and C-fibres are restricted to the nerve terminals in the skin or to the sprouts in an injured nerve when the fibres regenerate (Gorodetskaya et al., 2003, 2009; Jänig et al., 2009). Also, in the chronic neuroma preparation, no afferent fibres could be excited by mechanical stimuli applied to the neuroma nerve ≥ 1 mm proximal to the neuroma (Blumberg and Jänig, 1984), although unmyelinated peptidergic afferents keep sprouting proximally to the neuroma in a neuroma nerve for > 100 days after nerve injury (Barrett and McLachlan, 1999). Hoffmann et al. (2008) have shown on a skin-nerve preparation of mice that only a small percentage (14%) of the tested fibres are activated to mechanical stimulation of the nerve-stem.

Strong mechanical stimulation of axons may depolarize their membrane and generate action potentials. So, we did not apply very strong mechanical stimuli to the nerve in order to avoid damaging the axons. In a separate study, using calibrated von Frey glass

fibre filaments, we have shown that mechanical stimuli of 100 mN applied to the nerve do not excite intact or sprouting myelinated or unmyelinated axons except at their terminals or lesion site (Gorodetskaya et al., 2003; A. Teliban, F. Bartsch, M. Struck and W. Jänig, unpublished observation). This situation changes during experimental perineural inflammation generated by complete Freund's adjuvant, simulating a neuritis. 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, 2008b). One reason for the axonal mechanosensitivity in these conditions could be that the inflammation can disrupt axoplasmic transport (Armstrong et al., 2004). 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, 2008a,b). 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.

Can the axonal sensitivity of intact afferent fibres generate pain sensations?

Whether the heat or cold stimulation of an intact (uninjured) cutaneous nerve in humans can generate distinct painful heat or cold sensations is unknown, since this has not been systematically tested, to our knowledge. Preliminary experiments in our laboratory (on rats), using graded cold or heat stimuli applied to an acutely injured nerve show that the axonal activation thresholds for cold or heat stimuli are significantly higher than the activation thresholds from skin (Teliban, Bartsch, Struck, Jänig, in preparation). Trotter and Davies (1909) have shown that transcutaneous stimulation of experimentally injured skin nerves in humans by cold or heat stimuli elicited non-painful or painful thermal sensations even months after a nerve injury (transection and resuturing). However, these distinct thermal sensations may have been triggered by excitation of afferent fibres that have not been regenerated to skin, but could still be activated from the nerve by these physiological thermal stimuli (see Gorodetskaya et al., 2009).

In a recent study, Hoffmann et al. (2009) have shown in a healthy human subject

that axonal activation of the exposed superficial radial nerve by radiant heat produces burning pain sensations projected into the innervation area of the nerve. These burning pain sensations were similar to a neuropathic pain attack. The projected pain intensity generated by supra-threshold heat stimuli was in the range of 4 to 7 (on a 1-10 numerical pain scale). However, repeated strong mechanical stimuli (glass rod pressure and pinching of the epineurium) or noxious cold stimuli (3°C) applied to the superficial radial nerve did not generate any local or projected pain or cold sensations. The cold sensation in humans is mainly mediated by the activation of A δ -fibres but not by cold-sensitive C-fibres. Thus, the absence of a cold sensation in the experiment of Hoffmann et al. (2009) may be explained by the absence of axonal cold sensitivity of A δ -fibres. The absence of pain during noxious cooling of the superficial radial nerve is not easily explained. Many cutaneous polymodal (heat-sensitive and mechanosensitive) C-fibres are also cold-sensitive (Campero et al., 1996). Furthermore, the human skin is innervated by C-fibres that are cold-sensitive and heat-sensitive and can be activated by menthol (Campero et al., 2001, 2009); these fibres are suggested to be involved in hot burning pain sensations in response to noxious cold or heat stimuli (Campero et al., 2009). Thus, either the axons of these cold-sensitive fibres are not cold-sensitive, or the absence of pain during noxious cooling of the skin nerve is attributable to central masking (see Campero et al., 2009 for discussion).

4.3 Responses of intact and injured afferent nerve fibres to menthol

The present study revealed for the first time, for the afferent innervation of the rat hindlimb skin, that innocuous (type 1) cold-sensitive intact or injured afferent nerve fibres are activated by menthol whereas practically all other functional types of afferent fibres, including noxious (type 2) cold-sensitive C-fibres, are insensitive to menthol. This applied to all intact afferent C-fibres investigated. In the population of injured C-fibres only three out of 47 fibres identified as either type 1 or type 2 cold-sensitive by physiological criteria were excited by menthol and contradicted the previous statement. Two out of 34 intact or injured A-fibres tested were excited by menthol; one was a type 2 cold-sensitive and one a heat-sensitive fibre by our physiological criteria. These two afferent fibres were injured afferents.

We have shown that almost all type 1 cold-sensitive C-fibres exhibit axonal cold-sensitivity and only 35-40% of the type 2 cold-sensitive C-fibres and the nociceptive heat-sensitive C-fibres exhibit axonal cold- or heat-sensitivity, respectively. Here we have

reproduced these results on sural nerve type 1 cold-sensitive afferent neurons. These fibres were tested for their sensitivity to menthol applied to their axons. To our surprise menthol applied to the sural nerve in concentrations of 4 mM or 8 mM excited only 1 out of 20 of these cold-sensitive axons. Menthol may not penetrate the nerve explaining these negative results although menthol is highly lipophilic. However, this explanation does not accord with the observation that menthol applied to the injured sural nerve activated injured type 1 cold-sensitive C-fibres 6-12 days after crush lesion.

4.3.1 Intact and injured type 1 cold-sensitive C-fibres

All investigated intact type 1 cold-sensitive C-fibres (12/12) and almost all injured type 1 cold-sensitive C-fibres (20/22) were excited by menthol. The maximal response to menthol was significantly higher in intact than in injured C-afferents (11.7 ± 1.53 imp/s vs 3.1 ± 0.50 imp/s, $p < 0.0001$; Wilcoxon signed rank test). Moreover, the correlation between the maximal responses to menthol and the ongoing activity at 30°C was significant for the injured afferent C-fibres ($r = 0.68$, $p < 0.0001$) but not significant for the intact C-fibres ($r = 0.13$).

The responses of the intact and injured type 1 cold-sensitive fibres to cold stimuli applied to the skin or the nerve were tested again after application of menthol. These responses were significantly larger after application of menthol in the intact innocuous afferent fibres ($p < 0.006$; ANOVA). In the injured afferent fibres, the activation to nerve cooling after application of menthol was not significantly enhanced after application of menthol.

Type 1 cold-sensitive C-fibres (in intact and injured populations) are activated by innocuous cold stimuli and some of them were additionally heat-sensitive. Almost all of them exhibited ongoing activity at a temperature of about 30°C and they were mechano-insensitive fibres. The frequency of these type 1 cold-sensitive afferent C-fibres in the population of intact or injured sural nerve C-fibres is about 20% (see Gorodetskaya et al., 2009; Grossmann et al., 2009b; Jänig et al., 2009). This percentage is comparable with the percentages obtained in the *in vitro* studies if one takes into account that many dorsal root ganglion cells innervate deep somatic tissues and some visceral tissues. These *in vitro* studies performed in rat and mouse, show that 5 to 16% of the cells in the dorsal root ganglia (Babes et al., 2004; Dhaka et al., 2008; Peier et al., 2002; Reid et al., 2002; Xing et al., 2006, 2007) or in the trigeminal ganglion (Abe et al., 2005; Dhaka et al., 2008; Peier et al., 2002; Thut et al., 2003; Viana et al., 2002) are cold-sensitive, menthol-sensitive and/or express TRPM8.

Table 4.1. Mechano- and heat sensitivity of cold-sensitive C-fibres^a.

	Type 1 cold-sensitive		Type 2 cold-sensitive	
	<i>intact</i>	<i>injured</i>	<i>intact</i>	<i>injured</i>
<i>Numbers</i>	N=61	N=89	N=54	N=81
<i>Mechanosensitivity</i>	0 (0%)	1 (1.1%)	25 (46.3%)¹	45 (55.6%)¹
<i>Heat sensitivity</i>	7 (11.5%)	23 (26%)²	29 (53.7%)³	57 (70.4%)^{3,4}

Data from present study, Gorodetskaya et al. (2009); Jänig et al. (2009);
Teliban et al. (2011).

¹ Comparison type 1 vs type 2 cold-sensitive neurons: $p < 0.0001$

² Comparison injured vs intact type 1 cold-sensitive neurons: $p < 0.05$

³ Comparison type 1 vs type 2 injured or intact cold-sensitive neurons: $p < 0.0001$

⁴ Comparison injured vs intact type 2 cold-sensitive neurons: $p = 0.067$

^a*chi*² test

The molecular sensor involved in transduction of the innocuous cold stimuli is suggested to be the TRPM8 channel (McKemy et al., 2002; Peier et al., 2002). This channel is also activated by menthol (Peier et al., 2002; Story et al., 2003). In vivo studies on low-threshold cold-sensitive primary afferent neurons innervating the cat cornea (Gallar et al., 1993; Parra et al., 2010), the cat tongue (Hensel and Zotterman, 1951), the guinea pig nasal mucosa (Sekizawa et al., 1996) or the dog laryngeal mucosa (Sant'Ambrogio et al., 1991) show that the TRPM8 agonist menthol activates these afferent neurons. Furthermore, studies in vitro on isolated dorsal root or trigeminal ganglion cells have shown that menthol excites neurons expressing the TRPM8 channel (Peier et al., 2002; Story et al., 2003; Viana et al., 2002; Babes et al., 2004; Belmonte et al., 2009; Dhaka et al., 2008; Xing et al., 2006). These in vivo or in vitro studies fully agree with our results showing that almost all intact or injured type 1 cold-sensitive afferent fibres can be activated by menthol.

Table 4.1 shows the percentages of type 1 (low-threshold and menthol-sensitive) cold-sensitive and type 2 (high-threshold and menthol-insensitive) cold-sensitive afferent neurons projecting in the sural nerve which are heat-sensitive or mechanosensitive from our present study and three recent studies (Gorodetskaya et al., 2009; Jänig et al., 2009;

Teliban et al., 2011).

These cold-sensitive afferents were divided in two populations: **1)** intact (non-injured) afferents; **2)** injured afferents. The comparison shows that mechanosensitivity and heat sensitivity have a significantly lower representation in the populations of intact and injured type 1 cold-sensitive afferent neurons than in the populations of intact and injured type 2 cold-sensitive afferent neurons ($p < 0.0001$, χ^2 -test). Furthermore, the results show that heat sensitivity has a higher representation in injured than in intact cold-sensitive afferent neurons although this did not reach statistical significance in the population of type 2 cold-sensitive afferents ($p < 0.05$ for type 1 cold afferents, $p = 0.67$ for type 2 cold-sensitive afferents).

The frequency of heat sensitivity of our intact menthol-sensitive C-fibres is comparable to the measurements reported by Peier et al. (2002); Story et al. (2003); Dhaka et al. (2008), showing that menthol-sensitive and/or low threshold cold-sensitive dorsal root or trigeminal ganglion cells are either not capsaicin-sensitive or do not express TRPV1 or that some 12 to 19% of the cells expressing TRPM8 also express TRPV1. Other in vitro studies on rat or mouse dorsal root ganglion or trigeminal afferent neurons have identified a significantly higher percentage of cold- and menthol-sensitive neurons that were activated by capsaicin (Reid et al., 2002; Viana et al., 2002; Babes et al., 2004; Okazawa et al., 2004; Xing et al., 2006, 2007). Similarly, other authors (McKemy et al., 2002; Okazawa et al., 2004; Takashima et al., 2007) reporting about the 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. Initially, these observations led to the hypothesis that neurons expressing cold-activated TRPM8 and heat-activated TRPV1 transduce noxious cold as well as heat and could contribute to phenomena such as paradoxical cold [(resulting from the discharge of cold fibres in response to high heat stimuli)(Dodt and Zotterman, 1952)]. Dhaka et al. (2006, 2008) argue that the high percentage of dorsal root and trigeminal ganglion cells activated in vitro by small cooling steps and/or menthol as well as by heat and/or capsaicin may be related to changes of the expression of the TRPV1 channel in culture due to an inflammatory or injury state of axotomized cultures. The presence of NGF (which is routinely used at high levels in these cultures) is known to have profound effects on capsaicin/heat-responsive DRG neurons in culture (Aguayo and White, 1992) because it seems that this factor may generate an inflammatory condition for the DRGs neurons (Story et al., 2003). So, NGF sensitizes TRPV1 activity, and has been shown to up-regulate TRPV1 mRNA and protein in cultured DRG neurons (Shu and Mendell, 1999;

Winston et al., 2001; Chuang et al., 2001; Ji et al., 2002). In fact, our injured cutaneous type 1 cold-sensitive C-fibres show a significantly higher incidence of heat sensitivity than the intact type 1 cold-sensitive C-fibres innervating skin (Table 4.1). Thus, the high frequency of neurons co-expressing TRPM8 and TRPV1 may turn out to be a culture artefact (see Dhaka et al., 2006). Furthermore, not all low-threshold cold-sensitive and menthol-sensitive dorsal root ganglion cells may innervate skin.

The absence of mechanosensitivity in all our intact cutaneous type 1 cold-sensitive C-fibres is in agreement with some other studies reporting about spinal cutaneous non-nociceptive afferents in different species (Fleischer et al., 1983; Shea and Perl, 1985; Spray, 1986; Kress et al., 1992; Leem et al., 1993). However, the mechanosensitivity of innocuous cold-sensitive afferent fibres has to date not been systematically studied. The only study in which the sensitivity of injured cutaneous afferents to menthol and to mechanical stimulation was tested has to our knowledge been conducted by Roza et al. (2006). In this *in vitro* study, the axotomized sensory fibres of 21 days-old neuromas of the saphenous nerve of mice were investigated. Nine of 17 cold-sensitive units were activated by menthol or showed changes in their threshold to cold stimuli. The authors of this study did not discriminate between high- and low-threshold cold-sensitive afferents; they also did not test the heat sensitivity of these cold-sensitive units. Furthermore, more than half (7/9) of the cold- and menthol-sensitive fibres were mechanosensitive, whereas almost all cold- and menthol-insensitive fibres were mechano-insensitive. These results obtained by Roza et al. (2006) are at variance with our data about injured cold-sensitive afferent fibres. This difference in results may be related to difference in preparation (*in vitro*, *in vivo*) and/or difference in species (mouse, rat).

Separation of cold- and menthol-sensitive C-fibres into two groups

Some studies (Belmonte et al., 2009; Madrid et al., 2009; Takashima et al., 2007) have reported about a separation of the cold- and menthol-sensitive C-fibres into non-nociceptive and nociceptive units, the criteria of this separation being their responses to heat stimuli or their response thresholds to cold stimuli. Here, the separation of our menthol-sensitive C-fibres in these two groups seems to be not justified. Our intact (non-injured) menthol-sensitive (type 1) C-fibres have very little heat sensitivity and they are activated by innocuous cold stimuli (threshold $\approx 28^{\circ}\text{C}$). In fact, the heat sensitivity was present only in 7 out of 61 intact type 1 cold-sensitive afferent fibres of the present study and of the other three previous studies (Gorodetskaya et al., 2009; Jänig et al., 2009;

Teliban et al., 2011) (table 4.1).

Comparison of our results with those obtained on human beings

Several studies have shown that the cutaneous cold sense in primates and humans is primarily mediated by A δ -fibres (Darian-Smith et al., 1973; Dubner et al., 1975; Hensel and Boman, 1960; Iggo, 1969; Mackenzie et al., 1975; Long, 1977; Hensel, 1981, 1982). The functional characteristics of our type 1 cold-sensitive afferents compare closely to those of cutaneous cold-sensitive A δ -fibres in primates. Campero et al. (2001, 2009) describe a population of C fibres innervating hairy skin of the human foot which are low-threshold cold-sensitive, menthol-sensitive and mechano-insensitive. These physiological features match closely those of our type 1 cold-sensitive afferents. However, most of these cold-sensitive fibres in humans can be activated by heat stimuli, this heat activation being poorly represented in our low-threshold cold-sensitive fibres. Campero et al. (2009) suggest that these cold-sensitive C-fibres play a little role in cold sensation but are instead involved in cold discomfort (together with the polymodal cold-sensitive C-fibres) and burning hot and heat pain (together with the polymodal cold-insensitive C-fibres). The type 2 cold-sensitive C-fibres in our study compare to the cutaneous C-polymodal nociceptors in humans that respond to noxious low temperatures of 10 to 0°C and also to heat and/or strong mechanical stimuli (Campero et al., 1996, 2009). They show rather low discharge rates to the cold stimuli and are menthol-insensitive.

Involvement of menthol in cold unpleasant sensations

The first molecular studies on cold thermoreception implicated the menthol receptor TRPM8 in innocuous cold sensation and the mustard oil and cinnamaldehyde-sensitive receptor TRPA1 in cold-induced pain (McKemy et al., 2002; Reid, 2005; Foulkes and Wood, 2007). Recent studies reported that TRPM8 can also be involved in unpleasant sensations. In human studies, Wasner et al. (2004) showed that 40% menthol applied to the hairy skin of healthy volunteers induces spontaneous burning pain and cold hyperalgesia, which were further enhanced during an A-fibre block that abolished cold sensation. They also reported a flare reaction to menthol, suggesting activation of C nociceptors responsible for axon reflex vasodilatation. However, a subsequent study by Namer et al. (2005), while confirming that menthol induced cold hyperalgesia, found that it evoked only local vasodilatation and not an extended axon reflex flare, in contrast to the TRPA1 agonist cinnamaldehyde. These studies suggest that menthol sensitizes and activates cold-sensitive peripheral afferent nociceptive neurons with C-fibres. The results of these

two studies obtained on human subjects are not supported by our study on intact and injured cutaneous afferent neurons, showing that nociceptive high-threshold (type 2) cold-sensitive afferents are menthol insensitive. However, two points must be kept in mind: **1)** these authors used a concentration of 40% menthol in ethanol, whereas in most experiments we used concentrations of 10% menthol; **2)** differences in species cannot be excluded.

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Publications

Journal papers

1. Grossmann, L., Gorodetskaya, N., Teliban, A., Baron, R. and Jänig, W.
"Cutaneous afferent C-fibers regenerating along the distal nerve stump after crush lesion show two types of cold sensitivity"
European Journal of Pain, 13:682-690, 2009.
2. Teliban, A., Bartsch, F., Struck, M., Baron, R. and Jänig, W.
"Axonal thermosensitivity and mechanosensitivity of cutaneous afferent neurons"
European Journal of Neuroscience, 33:110-118, 2011.
3. Kirillova, I., Teliban, A., Gorodetskaya, N., Grossmann, L., Bartsch, F., Rausch, V., Struck, M., Tode, J., Baron, R. and Jänig, W.
"Effect of local and intravenous lidocaine on ongoing activity in injured afferent nerve fibers"
Pain, 152:1562-1571, 2011.

Conference contributions

1. Gorodetskaya, N., Teliban, A., Grossmann, L. and Jänig, W.
"Lidocaine suppresses ectopic spontaneous activity in lesioned A-afferents, but not C-afferents"
Der Schmerz (Supplement), 20:81, 2006 (Deutscher Schmerzkongress, Berlin).
2. Gorodetskaya, N., Teliban, A., Grossmann, L. and Jänig, W.
"Systemic and local lidocaine suppresses ectopic spontaneous activity in lesioned A-afferents, but not in C-afferents"
European Journal of Pain (Supplement), 11:119, 2007 (Second International Congress on Neuropathic Pain, Berlin).
3. Gorodetskaya, N., Grossmann, L., Teliban, A., Baron, R. and Jänig, W.
"Intact and regenerating cutaneous afferent fibers show two types of cold-sensitivity"
Der Schmerz (Supplement), 21:93, 2007 (Deutscher Schmerzkongress, Berlin).
4. Struck, M., Teliban, A., Bartsch, F., Baron, R. and Jänig, W.
"Excitability of unmyelinated afferent nerve fibers by physiological stimuli along the

sural nerve”

Third International Congress on Neuropathic Pain, 27, 2010 (Athens).

5. Teliban, A., Bartsch, F., Struck, M., Baron, R. and Jänig, W.

”Thermo- and mechanosensitivity of cutaneous afferent axons”

Third International Congress on Neuropathic Pain, 27, 2010 (Athens).

6. Teliban, A., Bartsch, F., Struck, M., Baron, R. and Jänig, W.

”Axonal thermosensitivity and mechanosensitivity of cutaneous afferent neurons”

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Curriculum Vitae

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation, abgesehen von der Beratung durch meinen wissenschaftlichen Lehrer und der Verwendung der angegebenen Hilfsmittel, nach Inhalt und Form meine eigene Arbeit ist. Die Arbeit hat nicht an einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Teile der Arbeit sind unter Teliban et al. (2011) veröffentlicht worden. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

Kiel, den