Mechanisms of thermal sensitivity in rodent primary afferent neurons innervating the skin

Nisha Vastani

UCL

Abstract

The role of temperature sensations elicited from the skin, include object identification, thermoregulation and the conscious perception of pain.

Humans can differentiate at least three distinct cold sensations: innocuous cooling, cold pain and pain evoked by freezing. However, patients with painful neuropathies often suffer from cold allodynia, where normally non-painful cool stimuli begin to induce pain or cold hyperalgesia, a heightened sensitivity to a painful cold stimulus.

It is therefore vital to understand the mechanisms by which cold is signalled under normal conditions and to investigate which changes occur under pathological conditions.

The thesis will describe sets of electrophysiological recordings carried out from rodent primary afferent neurons using the in vitro skin nerve preparation in an attempt to reveal mechanisms underlying thermal sensitivity.

The discovery of thermally sensitive transient receptor potential (TRP) ion channels has given insights into the molecular mechanisms of thermal transduction. These include the heat sensitive TRPV1 ion channel and cold sensitive TRPM8 and TRPA1 ion channels. However, the role of the TRPA1 receptor in cold transduction remains controversial. Cold sensitivity of primary afferents in adult rat was studied. Selective TRP channel agonists capsaicin, menthol, and mustard oil were then applied onto the receptive field of primary afferents to determine the expression pattern of thermosensitive TRP channels. The majority of cold sensitive A and C fibre nociceptors as well as thermoreceptors were sensitive to menthol, indicating that TRPM8 is the transducer of cold on these afferents. The poor correlation of TRPA1 expression and cold sensitivity in nociceptive A and C fibres indicates that TRPA1 is unlikely to play a significant role is detecting noxious cold.

TRPV2 is another heat activated ion channel. The sensory phenotype of TRPV2 knock-out mice was studied and compared against TRPV2 wild-type

mice in both hairy and glabrous skin. Mice lacking TRPV2 had normal heat sensitive nociceptors and afferents retained mechanical sensitivity.

The involvement of potassium (K^+) channels in mediating and/or modulating thermosensation has been suggested. Based on these previous findings, the effects of the broad spectrum potassium channel blockers 4-aminopyridine (4-AP) and Tetraethylammonium (TEA) were studied on primary afferents neurons. Application of 4-AP or TEA directly on the receptive fields induced a novel cold sensitivity in a proportion of low threshold mechanoreceptors and increased the cold responses in a proportion of cold sensitive A and C fibre nociceptors. Interestingly 4-AP or TEA had no effect on the cold responses of innocuous cold thermoreceptors.

Drug induced cold sensitivity was investigated using the chemotherapeutic agent oxaliplatin, which induces a sensory neuropathy in patients. Following infusion of the drug, patients experience abnormal skin sensations (paresthesias), which are triggered or aggravated by exposures to cold. The receptive properties of afferents were investigated before and after oxaliplatin application to provide an insight into the mechanism by which this abnormal cold sensitivity develops. This study shows for the first time, that oxaliplatin applied directly on the receptive fields induces a novel cold sensitivity in half of previously cold insensitive Aβ mechanoreceptors. Just over a third of Aδ nociceptors also displayed a novel or increased sensitivity to cold after oxaliplatin application. In contrast, receptive properties of C fibres remained unchanged.

Overall, the results of the thesis provide evidence that TRPM8 is involved in the transduction of cold stimuli and that potassium and sodium conductances are involved in modulating the final response to a cold stimulus.

Declaration

I, Nisha Vastani, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Nisha Vastani

Acknowledgments

I would like to thank my principle supervisor, Professor Martin Koltzenburg for providing his help, support, guidance and time throughout my PhD. I would also like to specially thank Dr Konrad Maurer, who acted as a mentor and was always very kind, encouraging and patient towards me and the rest of my colleagues at the Neural Plasticity Unit, ICH.

I am grateful to the Medical Research Council and Novartis for providing financial support throughout my PhD.

Finally, I would like to thank my parents, brother and friends for providing their consistent support and encouragement.

Abstracts published during the PhD

N.Vastani, K.Maurer and M.Koltzenburg (2005). Sensitivity of primary afferent fibres innervating rat hairy skin to cold stimuli and transient receptor potential (TRP) channel agonists. In 11th World Congress of Pain, Sydney, Australia. International association for the Study of Pain.

N.Vastani, C.Munns, M.koltzenburg (2006). Oxaliplain induces novel cold sensitivity in sensory neurons. European Journal of Pain, Volume 10, Supplement 1, Page S92. Abstracts of Pain in Europe V 5th Congress of the European Federation of IASP chapters (EFIC), Istanbul, Turkey.

N.Vastani and M.Koltzenburg (2007). Novel cold sensitivity of cutaneous sensory neurons induced by oxaliplatin and potassium channel blockers. In Neuroscience 2007, San Diego, USA.

N.Vastani, U.Park, M.J.Caterina and M.Koltzenburg (2008). Mice lacking TRPV2 have normal heat sensitive nociceptors. In 12th World Congress of Pain, Glasgow, UK. International association for the Study of Pain.

Table of contents

List of figures and tables

Chapter 1: General Introduction

Chapter 2: Methods and Materials

Chapter 3: Mouse primary afferent properties in hairy and glabrous skin

Chapter 4: Functional expression pattern of TRPM8, TRPA1 and TRPV1 on rat primary afferent neurons

Chapter 5: Thermal and mechanical sensitivity of afferents lacking the TRPV2 receptor in mice

---

Chapter 6: The role of voltage gated potassium channels in mediating cold sensitivity

---

Chapter 7: The effect of oxaliplatin on the receptive properties in rat primary afferent neurons

---

Chapter 8: General Discussion

Chapter 1

1. Introduction

1.1. History of the study of sensation and pain

Aristotle (384-322 BC) distinguished five special senses: vision, hearing, smell, taste, and touch. These senses are the physiological methods of perception, a process by which sensory information can be acquired. The ability to perceive the surrounding environment and thereby obtain information about it is vital for the survival of any organism.

In addition to these five classical senses, humans also have the ability to sense temperature and pain. Aristotle stated that touch *'discriminates several sense qualities'*, including hard-soft, hot-cold and warm-smooth (Finger Stanley, 1994). However, he did not categorize pain as a sensation equivalent to touch, hearing or vision. He associated both pain and pleasure as emotions, as *'passions of the soul'* (Dallenbach, 1939) and attributed the heart as their source rather than the brain. Unlike the five special senses, pain did not appear to possess an obvious sense organ and since it was not restricted to a specific part of the body it was not recognised as a sensory quality. However, Aristotle did recognise that pain and touch were closely related and that touch could become painful under some conditions (Finger Stanley, 1994).

Studies of the physiology of sensation started with Johannes Müller (1801 - 1858) who devised the *'Law of Specific Nerve Energies'* in 1840. Müller proposed that the 'nerves of feeling' produced a number of different sensations including tickle, itch, pressure, pain, warmth, cold, touch and movement. He proposed that the quality of a sensation following the stimulation of a sensory nerve did not depend on the type of stimulus, but upon the pathway over which the sensory information was carried (Pearce, 2005). He developed an idea which Charles Bell had stated earlier in 1811, that there was some sort of specificity in the nerves serving the different sensations (Finger Stanley, 1994) and that the nerve of each sense was capable of one kind of sensation only.

However, evidence to support the theory that specific nerve fibres existed that corresponded with different sensory experiences was lacking. This also included pain and in 1874, the German neurologist Erb developed the *'Intensity theory of pain'* which stated that every stimulus was capable of producing pain if it reached sufficient intensity (Perl, 2007). Therefore by the late 19th century, there were three main views about the sensory nature of pain; (i) pain as an emotional state, (ii) pain resulting from all sensations if sufficiently intense and (iii) a concept first suggested by Avicenna, a Muslim philosopher and physician in the 11th century, that pain was a separate and distinct sense (Dallenbach, 1939;Perl and Kruger, 1996).

1.1.1. Early work on cutaneous sensation and structure of afferent terminals

In the 1880s three independent investigators, Magnus Blix (1849-1904) from Sweden, Alfred Goldscheider (1858-1935) from Germany and Henry Donaldson (1857-1938) from the United States described sensory spots on the skin associated with different sensations.

Blix found that electrical stimulation of different points on the skin surface evoked distinct cool or warm sensations. He then built a temperature stimulator which could be applied onto the skin. He found warm and cold spots and observed that warm spots could not be activated by cold stimuli and cold spots could not be activated by warm stimuli. He also observed skin insensitive to cold and warmth between these spots. This finding led him to believe that warm and cold receptors had different endings in the skin.

Goldscheider used cork as a stimulus for touch, ether applied on brushes or in a tube as a stimulus for cold, heated brass cylinders as a stimulus for warm and needles as a stimulus for pain. Like Blix he found warm spots to be rarer than cold spots. In 1894 he developed the theory that pain was mediated by tactile nerves and resulted from an intensive summation of their excitations (Dallenbach, 1939). This supported the *'intensity theory of pain'* which stated that pain arises from the intense stimulation of any somatosensory or visceral

organ. He was also the first to notice that temperature spots could be excited by mechanical stimulation, for example by a sharp piece of wood (Alrutz, 1897).

Donaldson in 1885 also concluded that there may be temperature sensitive spots on the skin. He attempted to identify the functional end organs of cold and heat spots in his own skin. A cold and heat spot were localised and subsequently that area of skin was cut out. He found that there were many nerve endings under both the cold and heat spots but did not observe any differences between the two (Donaldson, 1885).

Towards the end of the 19th century, Max von Frey developed the 'specificity theory of sensation' (Fig. 1.1). He discovered discrete pain spots on the human skin when probing it with fine sharp needles. He stated that each sensory modality had a specific receptor and believed that the skin was made up of four types of sensory spots; touch, cold, warmth and pain. He believed that Ruffini endings were for transmitting warmth, Krause end bulbs for cold, Meissners corpuscles for touch in glabrous skin and free nerve endings for transmitting pain (Pearce, 2005). However, this was based more on circumstantial evidence rather than anatomical. Von Frey developed ways of measuring sensory thresholds. He used hairs of different lengths and diameters which exerted different forces when pressed onto the skin until they bent. In this way he determined the threshold force required to produce the sensation of touch. Von Frey also described a phenomenon which he called *'paradox sensation of cold'*. He observed that some cold spots elicited sensations of cold when they were stimulated with warm metal points above 45 °C (Alrutz, 1897).

At the turn of the previous century, Charles Sherrington argued that those regions of the body which when stimulated elicited only sensations of pain were innervated by unmyelinated or very thin axons whose terminals lacked encapsulation. In 1906 he termed those stimuli which were capable of producing tissue damage as *'noxious'* and the neurons which signalled these events as *'nocireceptors'* (nociceptors) (Perl and Kruger, 1996).

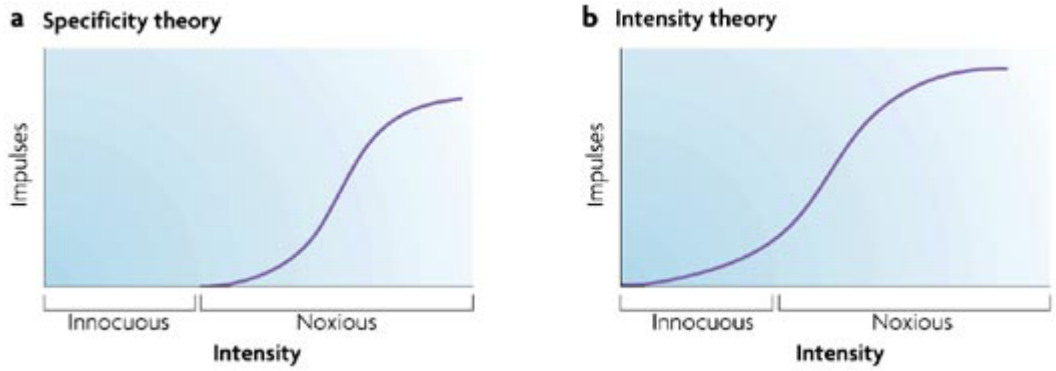

Fig.1.1 Theories of pain
a) According to the specificity theory, specialised sense organs (nociceptors) have threshold at or near noxious levels and display an increasing activity with stronger noxious stimuli. b) The intensity theory suggests that there is no difference in the thresholds of peripheral sense organs. It proposes that afferent fibres signal non-painful stimuli by a lower level of activity whereas noxious stimuli are signalled by a greater level of discharge. Taken from Perl et al, 2007.

1.1.2. Early recordings from primary afferent neurons

In the second decade of the 20th century Erlanger and Gasser in St Louis, United States succeeded in recording the compound action potential of peripheral nerve (Gasser and Erlanger, 1922). Their Nobel prize winning work provided evidence for the relationship between the diameter of the nerve fibre and conduction velocity of the compound action potential (Gasser and Erlanger, 1927). They found that pressure and local anaesthetics could differentially block components of the compound action potential (Gasser and Erlanger, 1929). Studies on the compound action potential revealed that the larger fibres required lower electrical thresholds for activation, had fast conduction velocities and were more sensitive to pressure block compared to thinner, more slowly conducting fibres. This soon lead to the conclusion that larger fibres were associated with tactile qualities and proprioception, whereas the thinner fibres were conveying temperature and pain sensations (Perl and Kruger, 1996).

A major technical advancement occurred in 1926 when Adrian & Zotterman, from Cambridge, England showed that it was possible to record electrophysiologically from single afferent nerve fibres in frog peripheral nerves

(Adrian, 1926;Adrian and Zotterman, 1926). Since then the properties of afferent nerve endings innervating mammalian skin have been explored many times and it is established that cutaneous afferent nerve fibres innervating the skin can be separated into categories based on the sensitivity of their terminals to mechanical and thermal stimuli.

However, a question still existed as to what role the cutaneous receptors played in generating sensations and whether sensations were a reflection of the pattern of activity in peripheral nerves. In 1960 Hensel and Boman, for the first time, recorded from cutaneous afferent nerve fibres from the superficial branch of the radial nerve in conscious humans (Hensel and Boman, 1960). The skin was opened under local anaesthesia and then a nerve bundle was spilt until single unit afferents could be discriminated. Using this technique, activity from, single mechanoreceptors from hairy and non-hairy (glabrous) skin as well as a cold sensitive thermoreceptor were recorded.
A further breakthrough came between the years 1965-1966 when the method of microneurography was developed by Hagbarth and Vallbo in Sweden (Vallbo et al., 2004). The technique involves percutaneous insertion of a microelectrode into nerve fascicles which enables recordings to be made from afferents in awake humans (Vallbo and Hagbarth, 1968). Microneurography therefore allows one to record simultaneously the activity of human nerve fibres and the corresponding subjective sensations.
Torebjörk and Hallin were first to record from C fibre afferents in humans (Torebjork, 1974;Torebjork and Hallin, 1970) and this preliminary report was quickly followed up by a short study from Van Hees and Gybels in 1972. Using microneurography they recorded from 25 C fibres from the superficial radial nerve which were excited by mechanical stimulation, noxious heat and histamine (Van and Gybels, 1972).

1.2. The study of thermal sensitivity

Temperature sensations elicited from the skin are used to judge the thermal states of objects and thus helps one to identify objects. Peripheral and central thermoreceptors also play an important role in thermoregulation, by detecting

temperature fluctuations in the environment. This information then allows the core internal temperature of the organism to be regulated, so that it is maintained, despite much larger variations in environmental temperatures (Hensel, 1981). Thermosensation also has a role in the conscious perception of pain.

1.2.1. Psychophysical studies of cold sensations in humans

Psychophysical experiments in humans have demonstrated that cooling the skin by just 1 ˚C from ambient temperatures elicits a non-painful 'cool' sensation (Davis, 1998;Davis and Pope, 2002;Harrison and Davis, 1999). As the temperature is lowered below 15 ˚C, a second sensation is evoked which is that of pain. The perceived cold evoked pain is commonly described as 'prickly', 'achy' and 'freezing' (Chen et al., 1996;Davis, 1998). Subzero temperatures evoke a distinct pain with a characteristic stinging quality (Davis, 1998;Simone and Kajander, 1997). Therefore in general humans can differentiate at least three different cold sensations; (i) innocuous or non-painful cooling, (ii) cold pain and (iii) pain evoked by freezing. Interestingly, noxious cooling also evokes in many subjects a burning, prickling and 'paradoxical heat' sensation (Davis, 1998;Hamalainen et al., 1982) whereas 'paradoxical cold' sensations can be elicited at the onset of an intense heat stimulus (Hensel, 1981;Konietzny, 1984).

Cooling is commonly used to achieve immediate relief from superficial wounds or burns and therefore suggests that it may interfere with the transmission of nociceptive inputs. Several studies have shown that this is the case. Firstly, in a psychophysical experiment in humans it was shown that pain sensations elicited by electrical stimulation of the nerve trunk were reduced by vibration or cooling in the area of the projected pain (Bini et al., 1984). This indicated that activity of low threshold mechanoreceptive and cold sensitive units were able to suppress pain signals at the spinal cord level. Secondly, in a microneurography study in awake humans, reducing the skin temperature below 10 ˚C led to a reduction in both A and C fibre discharges in response to mechanical stimuli (Kunesch et al., 1987). Interestingly, the suppression of C fibre responses was

more pronounced and longer lasting than in A fibres. These results suggested that the pain relieving effects of local skin cooling were due to receptor desensitisation.

Cooling therefore has an ability to inhibit pain, but conversely profound cooling itself causes pain. Psychophysical studies in humans have revealed that during compression block of A fibres, the sensation of a cold stimulus becomes more unpleasant and has a quality of 'hot burning' instead of cold (Davis, 1998;Wahren et al., 1989;Yarnitsky and Ochoa, 1990). These data suggest that the different qualities of sensations evoked by cold stimuli are mediated by different classes of cold sensitive primary afferent neurons.

1.2.2. Primary afferents and thermal sensitivity

Sensitivity to heat and cold is mediated by different sub-populations of primary afferent neurons of the dorsal root (DRG) or trigeminal ganglia (TG) which innervate the regions of the body and head, respectively. Cell bodies with the largest diameters give rise to large myelinated Aβ primary sensory neurons that typically conduct >10 m/s in rodents. There are two different fibre types; rapidly adapting (RA) and slowly adapting (SA). These fibres typically respond to light tactile stimuli. Cell bodies of medium diameter give rise to thin myelinated Aδ fibres which conduct between 1.2-10 m/s in rodents. Two groups of fibres fall into this category; down-hair (D-hair) fibres and A fibre nociceptors (AM). Small diameter cell bodies give rise to unmyelinated C fibres which conduct slowly in rodents, <1.2 m/s. There are many sub-types of C fibres. Most C fibres are polymodal in nature, responsive to noxious thermal, chemical and mechanical stimuli (Fig.1.2). Further detail about the various sub-types of fibres and their function is provided in the first results chapter (chapter 3, sections 3.1 and 3.2).

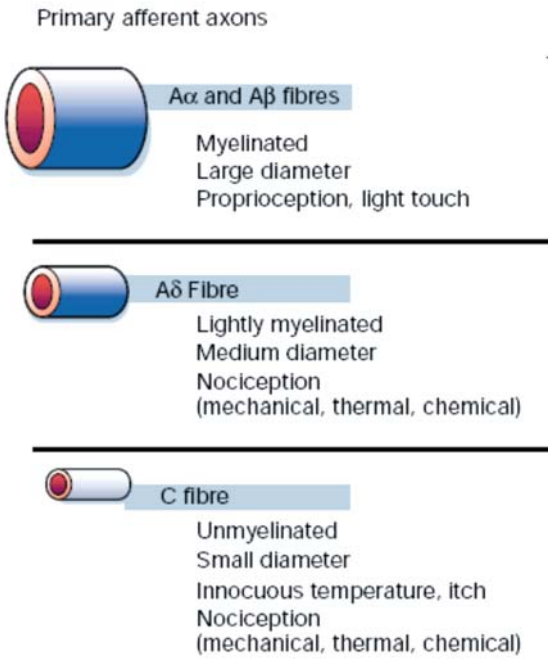

Fig.1.2. Classification of peripheral nerves according to their conduction velocity *Adapted from Julius and Basbaum, 2001.*

1.2.2.1. Cutaneous thermoreceptors

The concept of receptors specifically sensitive to only cold or warm temperatures was based on the observation that thermal sensations could be elicited from localised 'warm' or 'cold' sensory spots in the skin, a finding made by Blix, Goldscheider and Donaldson. Generally 'cold' spots seemed to be more numerous than 'warm' spots (Konietzyn, 1984). The first specific thermoreceptors identified by electrophysiological methods were those in the tongue of the cat by Zotterman in 1935 (Hensel, 1981). In the 1950s Hensel and Zotterman further investigated the firing patterns of single warm and cold fibres (Hensel and Zotterman, 1951;Zotterman, 1953) and described some of their general properties as follows; (i) warm and cold thermoreceptors displayed a regular discharge at constant temperatures (Fig.1.3), (ii) they showed a dynamic response to temperature changes (Figs.1.4 and 1.5), (iii) they were mechanically insensitive, (iv) their activity occurred in the non-painful or innocuous range. They discovered that warm fibres always increased their firing discharge in response to warming and a transient inhibition on cooling, whereas a cold fibre responded in the opposite way (Figs. 1.4 and 1.5), an

inhibition on warming and increase in activity upon cooling (Hensel et al., 1960;Hensel, 1981).

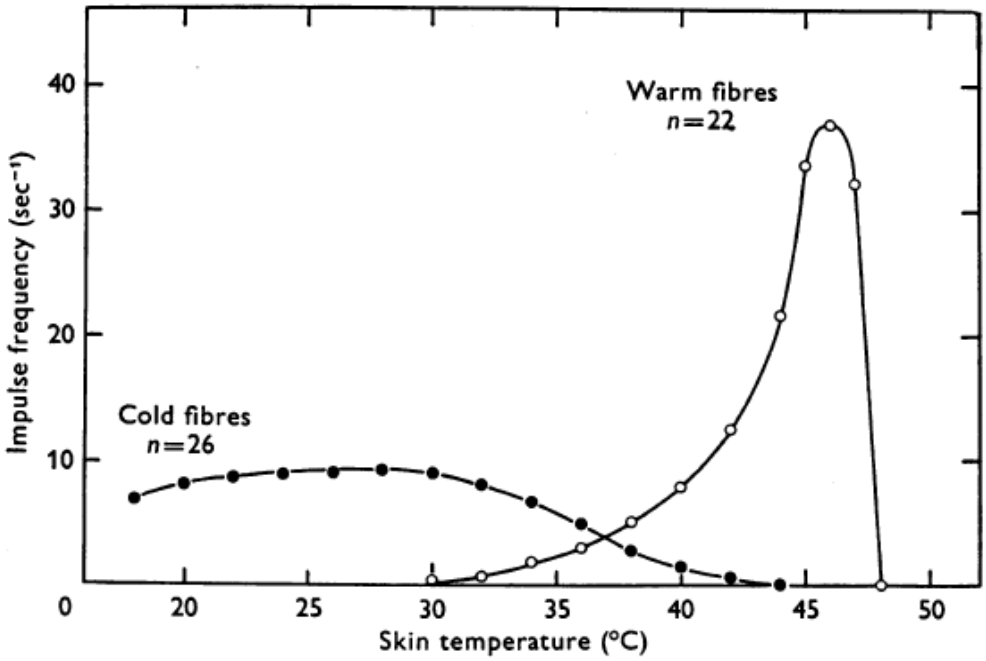

Fig.1.3. Average firing frequency of the static discharge as a function of temperature of cold and warm sensitive thermoreceptors
The average maximum of the static warm fibre discharge is at 46 °C, the maximum of cold fibre activity at 27 °C. Taken from (Hensel and Kenshalo, 1969).

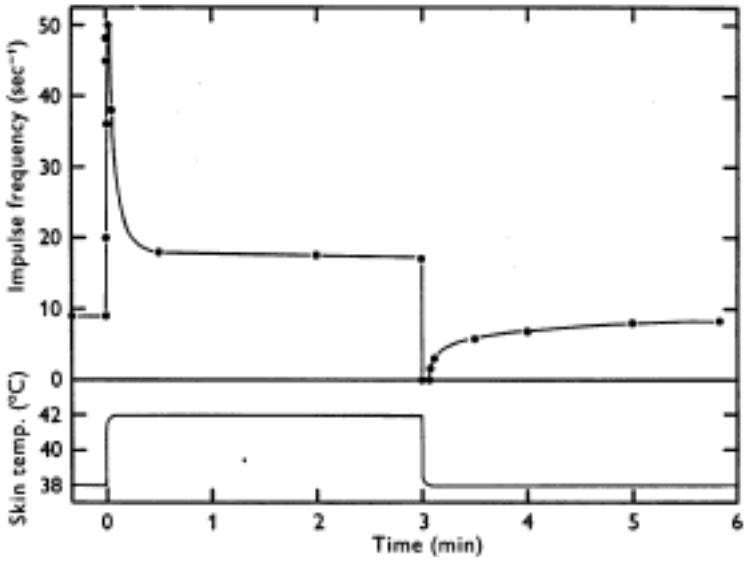

Fig.1.4. Dynamic behaviour of a warm fibre recorded from the nasal region of cats
On sudden warming, the warm receptor responds with an overshoot in firing frequency followed by a quick adaptation to a new steady state. Decreasing the temperature to the original level then causes a temporary cessation in firing, followed by a return to the original discharge rate. Taken from (Hensel and Kenshalo, 1969).

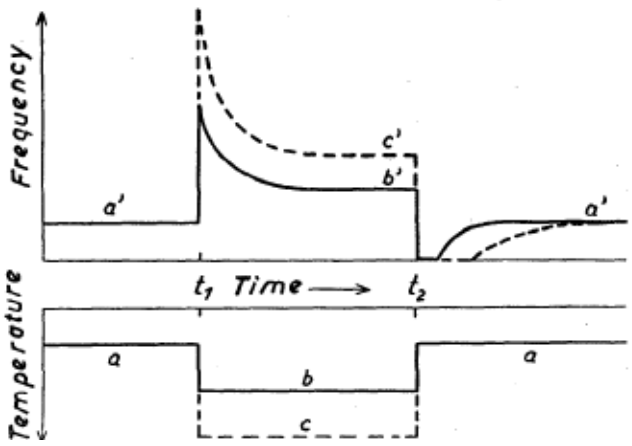

Fig.1.5. Dynamic response of a cold fibre in response to cooling
The activity of cold fibres in response to sudden cooling is essentially the opposite to that of warm fibres. At a constant temperature, a, the fibre discharges at a constant rate, a'. At time t_1, the temperature of the tongue is suddenly cooled to the constant level of b. This results in an immediate increase in cold fibre activity. The cold fibre then adapts to the temperature change and now develops a new rate of steady discharge, b', which is higher than the original frequency, a'. At time t_2, the temperature is suddenly increased to the original temperature, a. The fibre stops firing and then slowly starts firing at the original discharge rate, a. When a larger temperature drop occurs, c, the

increase in discharge is greater. When the temperature is taken back up to the original, t_2, the time for the fibre to return to its original discharge rate, a, is also greater. Taken from (Zotterman, 1953).

Activity from cold specific fibres has been recorded from the skin of human volunteers (Campero et al., 2001;Hensel and Boman, 1960) and appears to be carried in the Aδ or C fibre conduction range. These studies demonstrated that cold fibres in humans possess the same qualities as cold fibres recorded from the cat. Warm fibres have also been recorded from humans (Konietzny, 1984;Konietzny and Hensel, 1977) and the conduction velocity of these fibres suggest that they are unmyelinated C fibres. Innocuous cool and warm sensations are thought to be signalled by these fibres.

1.2.2.2. Thermal sensitivity among cutaneous mechanoreceptors and nociceptors

Among large myelinated afferents, cold sensitivity has been reported among some SA fibres (Hensel, 1974;Konietzny, 1984;Leem et al., 1993;Simone and Kajander, 1997;Spray, 1986). In vivo and vitro recordings from rodents suggest that up to 10 % of Aδ nociceptors and up to a third of C fibres respond to noxious cold (Koltzenburg, 2004). However, if the cold stimulus is lowered to temperatures below 0 ˚C, the majority of nociceptive fibres are excited (Simone and Kajander, 1997). Therefore the proportion of cold sensitive units in rodents varies depending on the cold stimulus applied.

Microneurography in the hairy skin of human volunteers has shown that up to 40 % of C fibre polymodal nociceptors are also sensitive to noxious cold temperatures (Campero et al., 1996). Cold pain is thought to be signalled by the excitation of these cold sensitive nociceptors.

Heat pain is thought to be signalled by a proportion of Aδ and C fibre nociceptors (Konietzny, 1984). Heat sensitivity has been reported in 0-21 % of Aδ nociceptors in rodent hairy skin and between 30-70 % of C fibre nociceptors (Koltzenburg, 2004). Recordings from the human peroneal nerve revealed that 45 % of mechanically sensitive C fibres were also heat sensitive C (CMH) fibres (Schmidt et al., 1995).

1.3. Afferent spinal pathways of thermal sensation

The cell bodies of primary sensory neurons are located in the DRG. Here, the axons bifurcate, and give rise to a peripheral branch that innervates various tissues and a central branch that travels through a dorsal root to enter the spinal cord. The dorsal horn of the spinal cord is the major termination zone for primary afferent axons, including those that transmit information from receptors in the skin (Fig.1.6). Nociceptive Aδ and C fibres terminate mainly in laminae I and II in the superficial dorsal horn. Some Aδ nociceptors project more deeply and terminate in lamina V. Thermoreceptors also project to the superficial dorsal horn. Low threshold mechanoreceptors and hair follicle afferents terminate in deeper laminae, IIi-V (Todd and Koerber, 2005;Woolf and Salter, 2005).

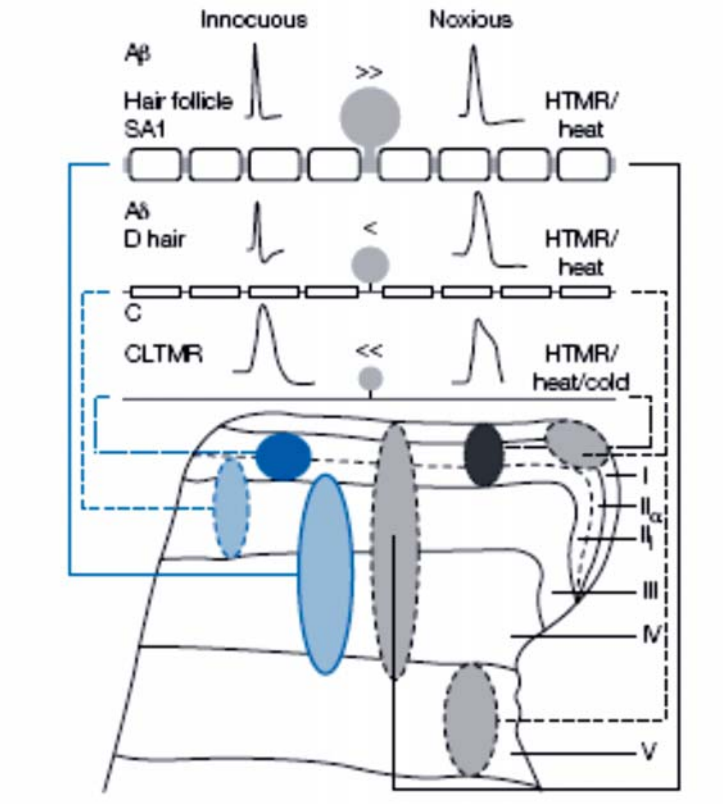

Fig.1.6. Schematic representation of the termination pattern of cutaneous afferent fibres in the dorsal horn of the spinal cord
The density of the terminals of each type of fibre is shown within each termination zone. The inequality signs represent the relative numbers of afferent fibres within a conduction velocity that respond to innocuous (blue) or noxious (grey) stimuli. CLTMR; low threshold mechanoreceptors, HTMR; high threshold mechanoreceptor, from Todd and Koerber, 2005.

The primary afferent neurons of nociceptors and thermoreceptors then synapse onto projection neurons in the spinal cord which form ascending pathways to different CNS destinations (Fig.1.7). Recordings in the dorsal horn of the monkey lumbosacral spinal cord revealed three types of neurons; cooling specific (cold) neurons, neurons responsive to noxious heat, pinch and cold (HPC) and wide-dynamic-range (WDR) neurons responsive to both innocuous and noxious cutaneous stimuli (Dostrovsky and Craig, 1996). Many cold specific neurons were found to project directly to the ventral medial nucleus (VMpo) of the thalamus, a projection known as the spinothalamic tract. Consistent with this finding, Davis et al (1999) showed that microstimulation of the VMpo region in awake humans undergoing surgery evoked cold sensations from the contralateral hand, face, leg or torso (Davis et al., 1999). However, recordings from the rat lumbar spinal cord have shown that the major targets for lamina I neurons are the caudal ventrolateral medulla (CVLM), lateral parabrachial area (LPb) and periaqueductal grey matter (PAG) (Spike et al., 2003). Furthermore, it has been shown that, 35 % of neurons recorded extracellularly in the parabrachial area of anesthetized rats responded to a peripheral noxious cold stimulus (Bester et al., 2000;Menendez et al., 1996). These findings therefore suggest that sensations of cold may be mediated by pathways ascending from the superficial dorsal horn of the spinal cord to areas in the thalamus and the parabrachial area.

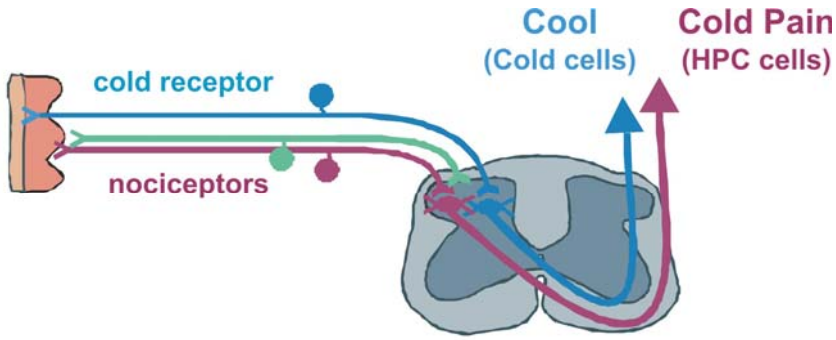

Fig.1.7. Schematic diagram representing the afferent and spinal pathways of thermal sensation
Cold sensitive afferent fibres are predominantly unmyelinated or thin myelinated and project to the superficial laminae of the dorsal horn of the spinal cord. In the peripheral nerve there are mechanically insensitive thermoreceptors (blue), nociceptors (purple) and cold insensitive units (green). Cold sensitive thermoreceptors project onto 'cold cells' and nociceptors synapse onto heat, pinch, cold (HPC) cells.

1.4. Cellular and molecular mechanisms underling cold sensitivity

Data from previous studies suggest that the sensations of innocuous cold and cold pain are mediated by separate populations of primary afferent fibres; cool sensation is signalled by activity of cold specific thermoreceptors and cold pain by activity of nociceptors. However the peripheral mechanisms that contribute to the sensation of cold are not fully understood. It is unclear whether cold depolarises sensory nerve fibres by activating an excitatory 'cold receptor' or by modulating a combination of ion channels and pumps that together alter the membrane potential (Julius and McCleskey, 2005).

Early work on the Na^+/K^+ ATPase pump indicated that it may be involved in cold transduction. It was proposed that cooling would decrease the pump activity and thereby depolarise the cell (Spray, 1986). However more recent work failed to support this possibility (Reid and Flonta, 2001). Cooling has also been found to increase the activity in members of the degerin/epithelial sodium channels (DEG/ENac) (Askwith et al., 2001).

1.4.1. Voltage gated potassium (K^+) channels

Several studies have shown the involvement of potassium currents in mediating cold sensitivity in DRG neurons. Reid and Flonta predicted that cold would either activate an inward current or inhibit an outward one (Reid and Flonta, 2001). They showed that cooling inhibited an outward current in all neurons which was active at the resting membrane potential. Interestingly, upon cooling there was a greater inhibition of this current (named I_{COLD}) in cold sensitive neurons compared to cold insensitive neurons. This indicated that during cooling, inhibition of this current in cold sensitive neurons brought them closer to their firing threshold. Viana et al (2002) discovered that 30 % of cold insensitive trigeminal neurons expressed a slowly inactivating outward K^+ current, which could be fully inhibited by 4-aminopyridine (4-AP). They went on to demonstrate that blockade of this current (named I_{KD}) by 4-AP induced a novel cold response in 40 % of previously cold insensitive neurons (Viana et al., 2002). Thus, expression of I_{KD} in a proportion of cold insensitive neurons

effectively acts to counteract the inhibition of I_{COLD} during cooling, preventing these neurons from reaching firing threshold. Further detail about voltage gated potassium channels can be found in the introduction section to chapter 6.

1.4.2. TRP channels

An important insight into the molecular mechanism of thermal transduction has come from the cloning and characterisation of temperature activated transient receptor potential (TRP) ion channels. Whereas the activity of many ion channels is modulated by temperature, thermo-TRP channels are different in that temperature alone can activate these channels (Patapoutian et al., 2003). Temperatures from noxious heat to noxious cold can activate several members of the TRP ion channel family which are expressed in the sensory nervous system (Fig.1.8.).

Capsaicin, an ingredient of red hot chilli peppers, played a key role in identifying the heat sensitive TRPV1 (previously known as VR1) receptor in 1997 (Caterina et al., 1997). The activation threshold was found to be near 43 °C, a temperature which most subjects report as 'hot', a special quality distinguished from 'warmth' (Konietzny, 1984). The TRPV1 receptor therefore became a perfect candidate for the detection of noxious heat. This was soon followed by the cloning and characterisation of other heat activated transient receptor potential (TRP) ion channels; TRPV2 (previously known as VRL1), TRPV3 and TRPV4 (Patapoutian et al., 2003;Tominaga and Caterina, 2004). Functional analysis in heterologous expression system of mammalian cells and xenopus oocytes showed that TRPV2 is insensitive to capsaicin and has a much higher threshold for activation by heat, >52 ˚C (Caterina et al., 1999). TRPV3 and TRPV4 have been shown to have activation thresholds between 34-38 °C and 27-34 °C, respectively (Patapoutian et al., 2003), making them ideal candidates for the detection of warm temperatures. In addition to these, two cold activated TRP channels have also been identified: the menthol sensitive TRPM8 receptor (formerly known as CMR1) and TRPA1 (formerly known as ANKTM1). In heterlogous systems, the TRPM8 receptor has an activation threshold between 25-28 °C, similar to the temperature range at which innocuous cool fibres are activated. TRPA1 on the other hand is

activated by colder temperatures, near 17 °C (Patapoutian et al., 2003). Non-painful cool stimuli are therefore thought to be transduced by TRPM8, whereas painful cold stimuli are thought to be transduced by TRPA1. Further detail on TRP channels can be found in chapters 4 and 5.

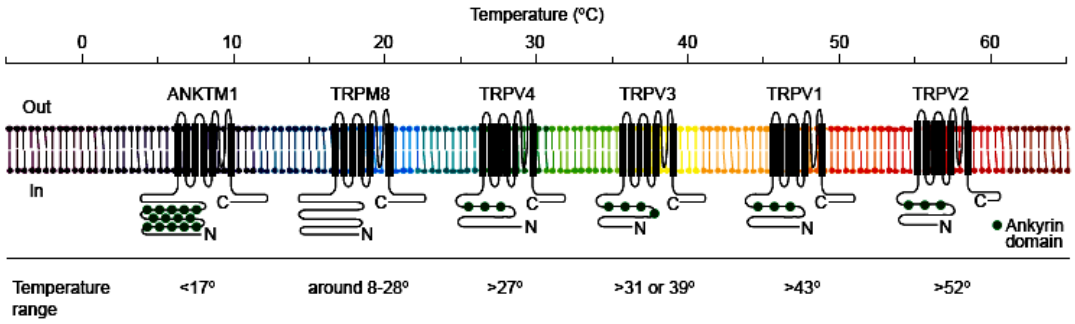

Fig.1.8. Thermosensitive TRP channels
Listed below each channel are their reported thermal thresholds and the range of temperature to which they respond when examined in heterologous systems. Theoretically these channels can account for the detection of all commonly encountered thermal stimuli, from noxious cold to noxious heat. Taken from (Jordt et al., 2003).

1.5. Abnormal cold sensitivity in humans

Abnormal cold perception is common in patients with painful neuropathy and after peripheral nerve injury (Atherton et al., 2008;Scadding and Koltzenburg, 2005). Patients often suffer from cold allodynia, where normally non-painful cool stimuli begin to induce pain or cold hyperalgesia, a heightened sensitivity to a painful cold stimulus (Scadding and Koltzenburg, 2005). Cold hyperalgesia and impaired cold perception were described in a group of patients with painful polyneuropathic conditions (Ochoa and Yarnitsky, 1994) and cold allodynia was described in patients suffering with post-traumatic neuralgia and postherpetic neuralgia (Jorum et al., 2003). 30 % of fibromyalgia syndrome patients experience cold intolerance and have lower thresholds for detecting non-painful cold and painful cold stimuli (Berglund et al., 2002).

1.5.1. Chemotherapy induced abnormal sensitivity to cold

Another clinically important condition of acute cold intolerance has been described in a group of patients receiving certain antineoplastic chemotherapy. Systemic injection of oxaliplatin is associated with the development of an acute peripheral neuropathy in 90 % of patients. Patients commonly develop paraesthesias, defined as non-painful but abnormal sensations such as numbness, tingling and 'pins and needles' in the fingers, hands, feet and lips. Patients also experience dyesthesias which are defined as painful or distressing sensations in the forearms, legs, trunk, mouth and throat (Extra et al., 1990). Interestingly, all of these symptoms are aggravated by exposure to cold temperatures, such as by eating, drinking or handling a cold object or even breathing in cold air (Lehky et al., 2004;Leonard et al., 2005;Wilson et al., 2002). As well as the development of a sensory neuropathy a motor neuropathy also exists. Painful involuntary masticatory spasms has been observed in a group of patients after oxaliplatin administration (Santini et al., 2003).

Oxaliplatin induced neuropathy is detrimental to patients in terms of unpleasant symptoms and the need to reduce the dose or discontinue treatment. However, currently there are no therapies available to treat or prevent the chemotherapy induced neuropathies.
Needle electromyography (EMG) carried out in patients post oxaliplatin infusion revealed repetitive discharges in motor units (Fig.1.9), whereas before only a single discharge occurred during voluntary activation (Wilson et al., 2002). This indicates that neuronal hyperexcitability is the underlying cause of the neuropathy. However, little is known about the mechanisms underling oxaliplatin induced hyperexcitability and more importantly why exposure to cold exacerbates the abnormal sensory symptoms in patients. Further detail is provided in chapter 7.

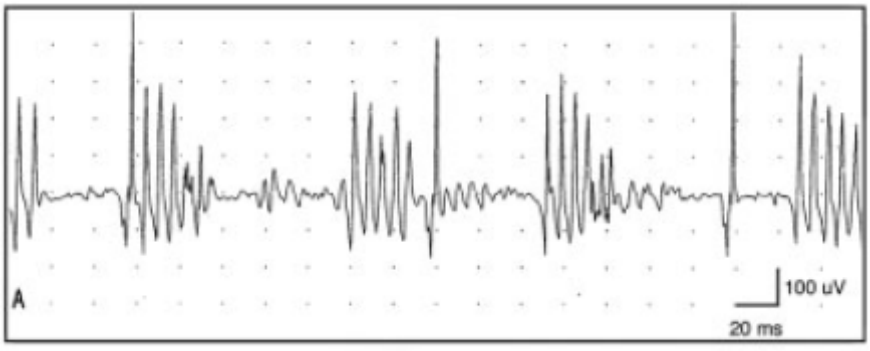

Fig.1.9. Example of needle EMG findings within 48 hours of oxaliplatin infusion
During voluntary contractions, individual motor fibre fired as multiplets. Taken from Lehky et al (2004).

1.6. Aims of the study

The cellular and molecular mechanisms that contribute to the signalling of cold stimuli are currently not fully understood. A heightened sensitivity to cold is a common symptom in patients with neuropathic pain and acute cold intolerance has been described in patients receiving the chemotherapeutic agent oxaliplatin. The aim of the study was to therefore investigate the molecular mechanisms by which cold is signalled, in the periphery, at the receptive terminals of primary afferent neurons innervating the skin of rodents.

1. Firstly, thermal sensitivity among primary afferent neurons innervating the hairy and glabrous skin were investigated in adult mice (chapter 3), using the *skin-nerve preparation*, an electrophysiological technique involving single fibre recordings in vitro, developed by Peter Reeh in the 1980's (see chapter 2 for more detail). The aim of this chapter (chapter 3) was to identify and characterise the different sub-populations of fibres found in this preparation and to investigate in detail their response properties to controlled mechanical and thermal stimulation. The receptive properties of mouse hairy skin have been investigated previously (see section 3.2). However, this is the first time that the receptive properties of primary afferents innervating mouse glabrous skin have been studied in vitro and this is important since many behavioural studies investigate the response to mechanical and thermal stimuli applied to glabrous skin in mice. In addition to this, chapter 5 investigated the properties of afferents from hairy and glabrous skin from mice lacking the heat activated

TRPV2 ion channel for the first time. Therefore, recordings from normal wild-type mice were carried in the current chapter prior to those of chapter 5 to perfect the recording technique from glabrous skin.

2. The second results chapter (chapter 4), investigated the functional expression pattern of cold and heat sensitive TRP ion channels on the various sub-types of fibres found in the hairy skin of the adult rat. Fibres were characterised using electrical, mechanical and thermal stimuli. This was then followed by application of capsaicin, a selective agonist of TRPV1, mustard oil, a selective agonist of TRPA1 and menthol, a selective agonist of TRPM8. Many previous studies have investigated the expression pattern of TRP channels using cultured DRG neurons from rodents. However few studies exist which have investigated the functional expression of TRP channels in single identified afferent fibres.

3. The third results chapter (chapter 5) investigated the role of the TRPV2 receptor, which has shown to be activated by noxious temperatures above 52 ˚C in heterologous systems. However, the role of this ion channel in the native system is currently unknown. For the first time, the receptive properties of fibres from mice lacking the TRPV2 receptor were studied in both the hairy and glabrous skin.

4. The fourth results chapter (chapter 6) aimed to investigate the role of voltage gated potassium channels potassium in mediating cold sensitivity in rat primary afferent neurons. Blockade of voltage gated potassium channels has previously been shown to induce a novel cold sensitivity in a proportion of cultured mice DRG neurons. However, there has been no study to date which has systematically investigated the effect of potassium channel blockade and thermosensitivity in identified single primary afferent neurons. Following fibre characterisation, the wide spectrum voltage gated potassium channel blockers 4-aminopyridine or tetraethylammonium were applied directly onto the receptive fields of fibres.

5. The fifth and final results chapter (chapter 7) aimed to investigate the effects of the chemotherapeutic agent oxaliplatin on the thermal sensitivity of fibres found in rat hairy skin, since at present no study has reported the effects of oxaliplatin on individually characterised sensory neurons.

Chapter 2

2. Methods and Materials

In this chapter a general description of the neurophysiological data acquisition and analysis is provided. More detailed information is given in additional method sections of the result chapters.

2.1 The skin nerve in vitro preparation

The mammalian skin nerve preparation was developed by Peter Reeh in the 1980's (Reeh, 1986) and later adapted for mice (Koltzenburg et al. 1997). In comparison to other widely used techniques, such as recording from single units in vivo or human microneurography, the technique allows experimental control over the external variables which could interfere with the physiology of the receptors. For example it is difficult to control the effective concentration of drugs applied at the receptive field site in vivo. Also the method of drug administration (e.g. by intracutaneous injection) could damage the skin and this could alter the properties of the receptors under investigation (Reeh, 1986). Administration of drugs in vivo via close arterial injection is dependent on the transport of the drug via blood vessels, and therefore some of the drugs may interfere with their own distribution by causing dilation or constriction of blood vessels. Using the in vitro skin nerve preparation, however, allows the receptive fields of individual units to be directly stimulated by electrical, mechanical, thermal and chemical stimuli in a non-invasive, controlled manner. In addition to this, there are no obvious time related changes in respect to excitability and spontaneous activity and the preparation can be used for recordings up to 8 hours from the time of dissection.
However, care must to be taken during the dissection of the saphenous nerve, since damage caused during the dissection (for example by cutting, stretching or drying out the nerve) can affect the quality of the preparation. Many fibres are spontaneously active immediately after the dissection and therefore the preparation should be left in the organ bath for at least one hour before carrying out recordings, by which time no spontaneous activity remains.

2.1.1. Animals

All animals were killed by cervical dislocation and the dissection started subsequently.
The hairy skin of female Sprague Dawley rats was dissected for use in experiments which involved the application of drugs to the receptive fields of individual fibres (chapters 4, 6 and 7). These investigations were carried out in rats rather than mice since the area of skin was much larger and thus allowed recording of a larger number of fibres in an individual experiment. The rats were aged between five and six months, weighing between 300-350 g. The hairy and glabrous skin of male C57/blk6 mice was dissected for use in experiments carried out in chapter 3. The hairy and glabrous skin of a hybrid strain of male and female adult mice was dissected for use in experiments carried out in chapter 5 (see chapter 5.2 for more detail).

2.2. Saphenous and Tibial nerve dissection

2.2.1. Saphenous nerve dissection

Firstly, the hair of the foot and leg of the animal was shaved off. An incision was then made across the toes and followed around the ankle area and up to the thigh region from both sides of the leg. The skin was then freed from the underlying connective tissue down to the ankle region where the nerve enters the skin. This exposed the saphenous nerve, which was then dissected free from the surrounding connective tissue and muscle up to the ankle region. The nerve was dissected from the lumbosacral plexus to ensure a sufficient length of the nerve for recording. The hairy skin with the nerve attached was then carefully dissected away from the underlying muscle and placed in an organ bath.

2.2.2. Tibial nerve dissection

After removing the surrounding hair of the foot and leg, an incision was made across the foot pad and followed around the ankle area. After removing the skin

from the underlying connective tissue the tibial nerve was cut at the branch point with the sciatic nerve and dissected free from the surrounding muscle down to the ankle joint. Since the area of glabrous skin of the mouse foot pad was very small, the incision was made so that some of hairy skin bordering the glabrous skin was also removed. When the preparation was placed into the organ bath, this area of hairy skin was used to pin down the preparation so that the area of glabrous skin could be maximised for carrying out recordings.

2.3. Experimental set-up

The skin with the nerve attached was placed in an organ bath and superfused at a rate of 15 ml/min with oxygenated synthetic interstitial fluid [SIF, consisting of (in mM) 108 NaCl, 3.5 KCL, 3.5 $MgSO_4$, 26 $NaHCO_3$, 1.7 NaH_2PO_4, 1.5 $CaCl_2$, 9.6 sodium gluconate. 5.55 glucose and 7.6 sucrose and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)] at a temperature of 32 °C and a pH of 7.4 (Kress et al., 1992;Reeh, 1986). The skin was mounted inside-up (corium side up) and pinned down. Extra connective tissue on the preparation such as muscle and tendons were carefully removed. The nerve was then taken through a small gap into a small recording chamber which was isolated from the main organ bath and was filled with paraffin oil. The nerve was carefully placed on a little mirror which acted as a platform, where it could then be desheathed (see Fig.2.1).

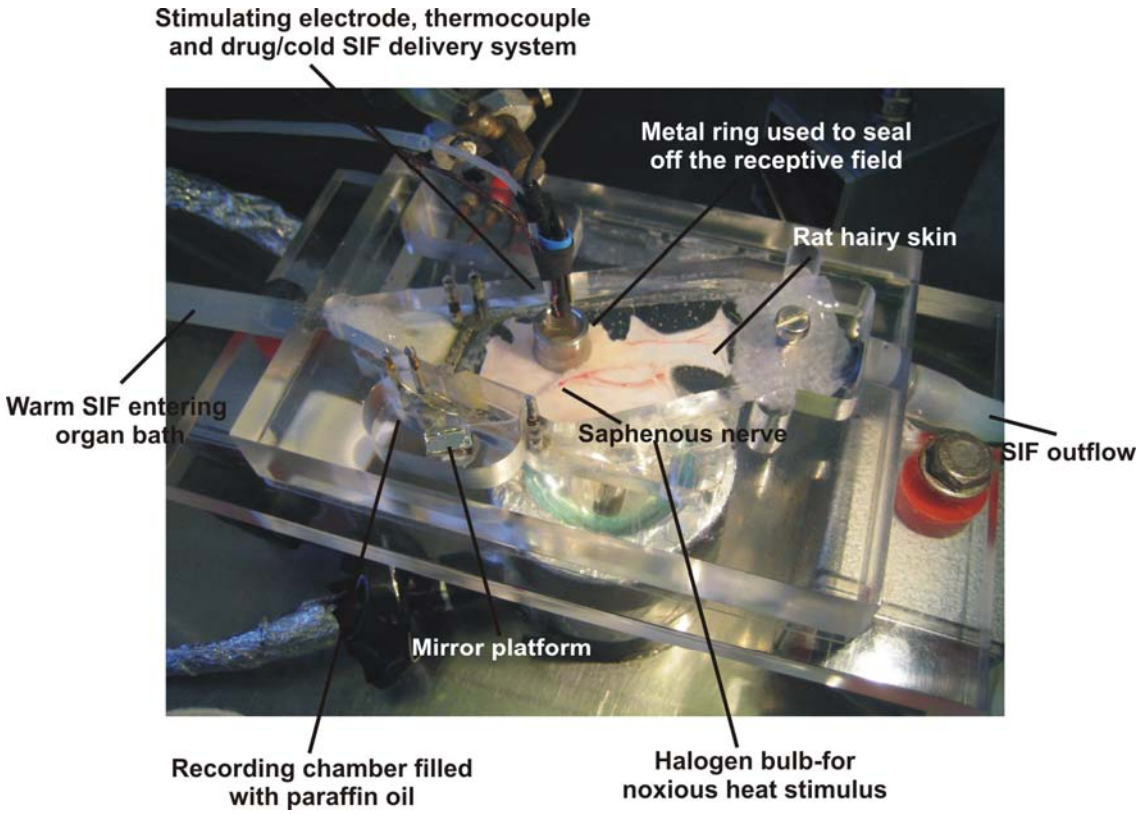

Fig.2.1 Experimental set up. *See text for further detail.*

2.4. Recording technique

With the use of sharpened watchmakers' forceps, filaments were teased from the desheathed nerve and functionally single sensory neurones were recorded extracellularly with a low-noise differential amplifier.
Receptive fields of primary afferent fibres were identified with a mechanical search stimulus (manual probing of the skin with a glass rod, which exerts forces >500 mN) which is known to activate >90 % of the rat cutaneous fibres in this preparation (Kress et al., 1992).
In addition to this mechanical search procedure a cold search stimulus was also used in a number of experiments to find mechanically insensitive cold thermoreceptors. The stimulus was administered using a hollow pen like device made out of brass that could be filled with dry ice. The device was moved above the skin without touching it.

The conduction velocity of each axon was determined by electrically stimulating the receptive field with supramaximal square-wave pulses (duration 0.1-1.0 ms, interstimulus interval 1-5 s) using a silver-silver chloride electrode with a

reference electrode placed nearby in the organ bath. The identity of the action potentials evoked by electrical or adequate natural stimulation was determined on the basis of the shape of the action potential and whether the electrical stimulation of a skin region excited only a single unit. Fibres conducting more quickly than 10 m/s were considered to be large myelinated Aβ fibres, those that conducting between 1.2-10 m/s were considered to be thin myelinated fibres and those conducting below 1.2 m/s were considered to by unmyelinated C fibres (Kress et al., 1992).
After determining the conduction velocity, fibres were subjected to a standard protocol consisting of mechanical, cold and heat stimuli.
All mechanical, chemical and cold stimuli were applied to the corium side of the skin, whereas radiant heat was directed to the hairy side through the translucent bottom of the organ bath (see Fig.2.1).

2.4.1. Mechanical stimulation

The mechanical threshold of each unit was determined with a calibrated von Frey bristle with a uniform tip diameter of 0.8 mm. The bristles covered a force ranging from 0.25 to 362 mN.
To further characterise the unit, mechanical adaptation properties of the fibres were investigated using a custom made feedback controlled mechanical stimulator with a probe tip diameter of 0.8 mm and two different protocols of supramaximal stimuli. The stimulator was placed perpendicularly onto the most sensitive spot of the receptive field. Each stimulus began with an adaptive baseline period of 5 s at a force of 1 mN. At the end of each stimulus the force returned to 1 mN for 5 s before the probe was lifted off the skin.
In the ramp and hold mode the stimulus rose within 200 ms to a preset force plateau that varied between 5 and 300 mN and lasted for 10 s. This stimulus configuration has been found to clearly distinguish between adaptation properties of the different afferent fibres (Koltzenburg et al. 1997). Stimuli were delivered in ascending order, with an interstimulus interval (from start to start of a stimulus) of 60 s. In the ramp only mode the force rose from the adaptation baseline to a preset force of 100 to 400 mN in 20 s. This was usually carried

out on Aδ and C fibres and was a useful way to see whether they encoded the stimulus intensity.

2.4.2. Thermal Stimulation

For further stimulation, the area around the electrically located nerve ending was isolated from the surrounding tissue with a small self-sealing metal ring which had an inner diameter of 8 mm and which could be perfused separately. A thermocouple was gently pressed into the corium after evacuation of the fluid within the ring to measure the intracutaneous temperature. For cold stimulation, intracutaneous temperature was lowered to approximately 4 °C. This was done by instillation of cold SIF by a roller pump into the small separate chamber formed by the metal ring. By manually adapting the rate of instillation an almost linear cold ramp was achieved over 30 seconds from 32 °C to 4 °C. In some experiments the cold stimulus was applied for an additional 30 s at 4 °C. Recovery to the ambient temperature of the organ bath was achieved by passive re-warming or by instillation of warm SIF.
For controlled heat stimulation, a halogen lamp with a reflector was placed below the organ bath and focused on the receptive field. The receptive field was isolated with a self sealing metal ring and the fluid within the ring was removed before application of the heat. Units were tested with a standard feedback controlled heat ramp rising linearly at a rate of 1 °C/s from an adapting temperature of 32 °C to an intracutaneous peak temperature of 47 °C within 15 s. Reeh (1986) showed that increasing the corium temperature from 32 to 47 °C as measured on the corium side corresponds to a rise of temperature from 32 to 52 °C as measured on the epidermal side. In some experiments the heat stimulus was raised from 32 to 50 °C as measured on the corium side and then held at 50 °C for a total of 30 seconds (see chapter 5 for more detail).

2.4.3. Chemical Stimulation

Details of how individual receptive fields were stimulated with chemical stimuli are provided in the method section of the individual result chapters.

2.5. Data acquisition

Unitary action potentials were recorded with a low-noise differential amplifier and monitored on an oscilloscope and loud speaker. A preamplifier with an amplification factor of 100 and a main amplifier with an amplification factor of 1000 were used. A high (100 Hz) and low pass (1 kHz) filters were used. An additional 50 Hz notch filter was used as this further improved the signal to noise ratio. The signal was then monitored on an oscilloscope and recorded using the SPIKE 2 version 5 (CED, Cambridge) programme. A noise cut filter was used before the signal was transmitted to the loud speaker.

2.6. Data Analysis and statistical tests

Only fibres which could be stimulated naturally or electrically at the end of a stimulation block were analysed. All quantitative analysis was carried out offline.

Firstly, a template of the fibre of interest was created, using a template-matching function on the programme. This was carried out by manually setting a set of trigger windows, within which the peak or the trough of the desired action potential would lie (Fig.2.2).

The programme then created a template of the action potential which satisfied the above criteria. Other fibres in the recording which had action potential peaks that did not lie within the set limits were excluded, and templates of these fibres were not created. In cases were more than one fibre fell into the trigger windows (i.e. the action potential peak of other fibres fell within the same limits of the fibre of interest) the programme could discriminate between different fibres and their action potential shapes using a template matching algorithm. In this case, more than 1 template would be created, and the desired template could then be selected for quantitative analysis.

A new channel was then created above the original recording, which displayed the selected action potentials (Fig.2.3). This served as quality control to ensure that all and only the action potentials of a unit of interest were captured for further analysis. If this was not the case, the process described above was repeated, until the template matching corresponded to the original recording.

Once the unit discrimination had been accomplished a custom made software script was then used to analyse the data of individual units in more detail. The script enabled the quantitative analysis of mechanical, thermal and chemically evoked responses.

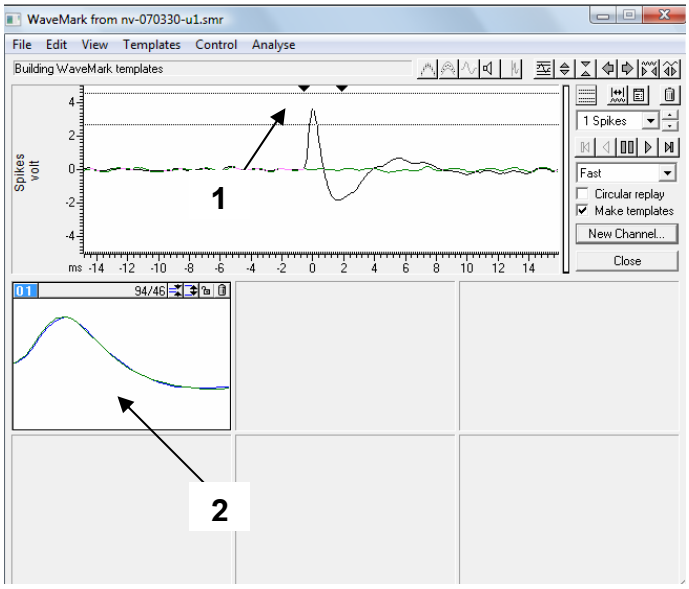

Fig.2.2 Illustration of the discrimination process between different units using the SPIKE programme. *The trigger windows used to capture the peak of the desired action potential are shown in (1) and (2) (using different time scales) represents the template created by the programme of action potentials whose peak lie within the area of the cursors.*

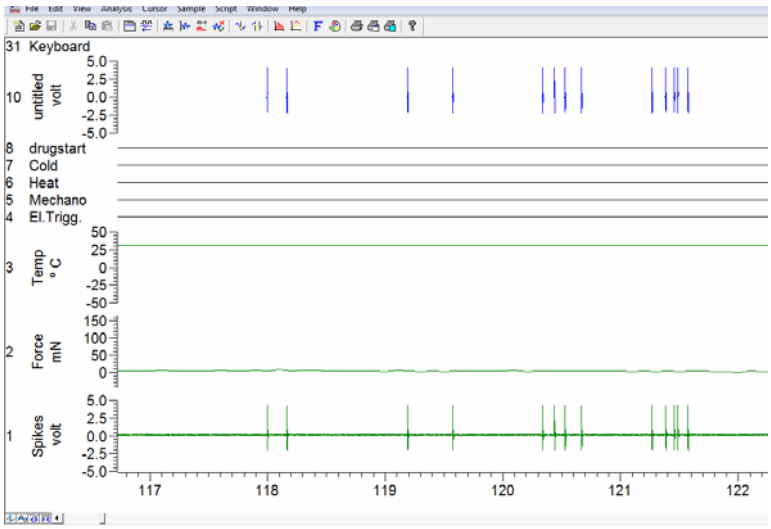

Fig.2.3 Data analysis using the SPIKE programme. *The original recording is displayed in the channel numbered '1' at the bottom in green. The template created by the programme of the original action potential to be analysed is shown in the channel numbered '10' in blue.*

2.6.1. Analyses of mechanical responses

A ‘start’ cursor was placed in line with the mechanical trigger pulse (see Fig.2.3 (1) below), which represented the exact moment at which the force was applied onto the skin. The TTL trigger impulse which lasted for 1 ms, was provided by the control of the mechanical stimulator at the onset of each stimulus ramp. A further trigger impulse was generated once the end of the preset ramp (200 ms for ramp and hold stimuli and 20 s for ramp only stimuli) had been reached). The script then generated an output window, representing the number of impulses which occurred in 1 second bins during the stimulus, before the stimulus and after the stimulus (Fig.2.4 (2)). The time windows chosen for the quantitative analysis were 11 s for the ramp and hold mode and 21 s for the ramp only configuration. Time windows of identical (i.e. 11 or 21 s) preceding or following the stimulus window were also available for analysis. Although the total mechanical stimulus duration was 10.2 or 20 s, respectively, an analysis window of 11 and 21 windows was chosen to capture all action potentials that were generated during the ramp as well as at the end of a stimulus. Figure 2.4 illustrates that action potentials could be generated before the force plateau of a ramp and hold stimulus was reached and it could outlast the end of the plateau of the stimulus. The data were then exported to an Excel spreadsheet for further analysis. Mean mechanical responses of the discharge were plotted by averaging the responses in 1-s bins. The total number of impulses generated during the mechanical stimulation was also obtained using these data.

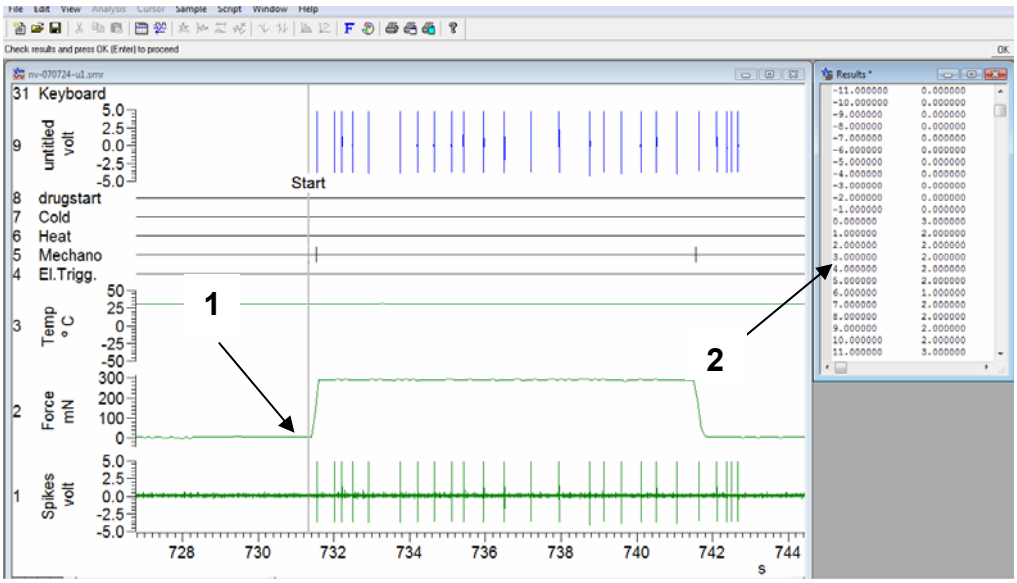

Fig.2.4 Analysis of mechanical responses using SPIKE. *Note that the trigger in trace 5 is obscured by the cursor and only the trigger at the end of the onset ramp is visible*

2.6.2. Analyses of thermal responses

To analyse heat evoked responses, a 'start' cursor was placed at the onset of the heat ramp indicated by the recorded TTL trigger pulse (Fig.2.5 (1)). The software script then generated an output window which represented the number of impulses which occurred in 1-s bins, 16 s during the stimulus and 16 s before and after the stimulus (Fig.2.5 (2)). Although the stimulus duration of the heat ramp was 15 seconds, a 16-second-long analysis interval was used, as described previously (Koltzenburg et al. 1997) to capture those action potentials that were generated a the very end of a stimulus.

Cold evoked responses were analysed in the same way except that the 'start' trigger was placed manually at the beginning of each stimulus (Fig.2.6 (1)). Since the application period of a cold stimulus varied in different experiments (30 s to 60 s), a 'stop' cursor was also placed manually at the end of each cold stimulus (Fig.2.6 (2)). Mean thermal responses of the discharge were plotted by averaging the responses in 1-s bins. The total number of impulses and peak discharge rate evoked by each thermal stimulus were determined for each afferent fibre in this way.

The heat and cold thresholds of units were defined as the temperature (in °C) that elicited an instantaneous frequency of >1.0 imp/s. This approach has been

previously shown to be a reliable criterion for threshold definition in units with or without ongoing activity (Koltzenburg et al., 1997). In units without ongoing activity this threshold definition corresponds generally to the second action potential generated by a stimulus.

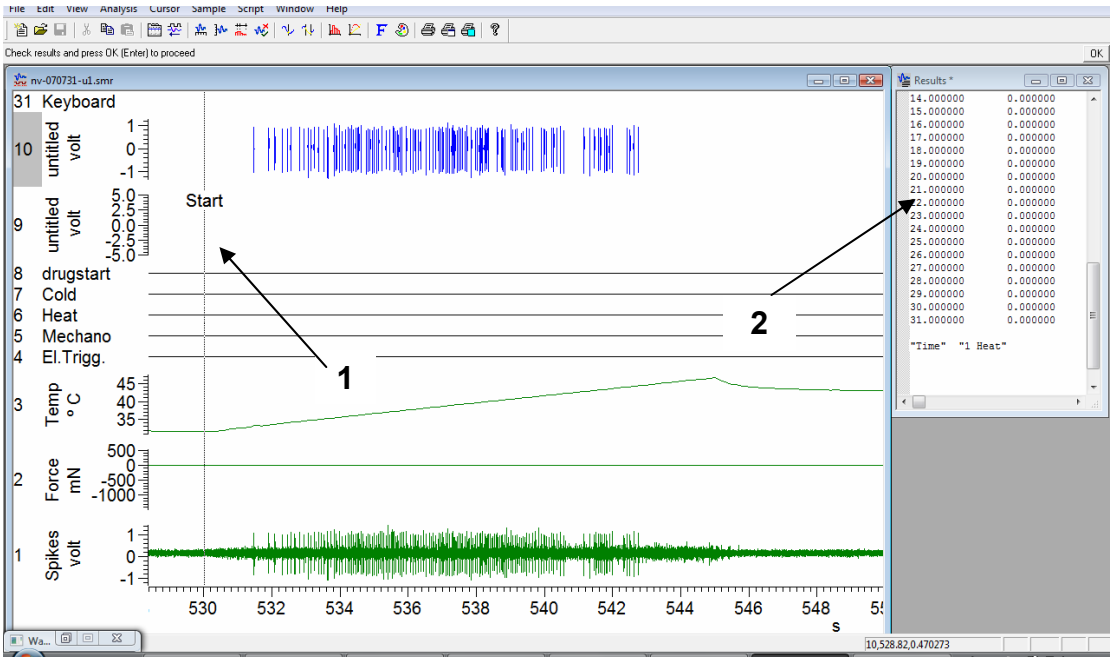

Fig.2.5 Analysis of heat responses using SPIKE. *Note that the trigger in trace 6 is obscured by the cursor.*

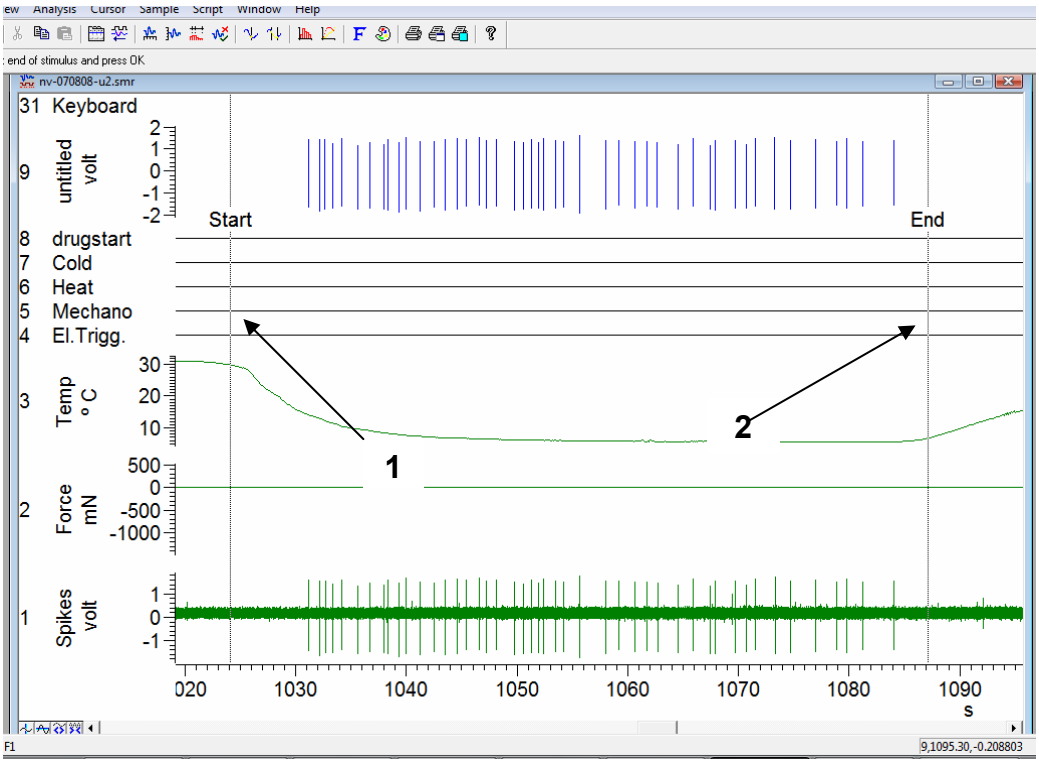

Fig.2.6 Analysis of cold responses using SPIKE

2.6.3. Analyses of chemical responses

A 'start' and 'stop' cursor were placed manually at the beginning and end of a drug challenge. The script then generated an output window which represented the number of impulses which occurred in 1-s bins during the stimulus, and a designated time before and after the drug challenge.

For units with spontaneous activity, the difference between instant frequency before the first drug challenge (spontaneous activity) and the instant frequency during the drug stimulus (*actual response*) was considered. A response was considered only if the *actual response* was at least double the instant frequency of the spontaneous activity.

2.6.4. Statistical Tests

All statistical tests were carried out using the STATISTICA 6.1 software package (Statsoft, Tulsa, OK, USA).

All values are given as mean ± standard error of the mean (SEM) except for the von Frey hair thresholds, which are given as median and first (Q1) and third

(Q3) quartiles. Depending on the data to be analysed parametric and non-parametric procedures were used.

To statistically compare mechanical and thermal responses before and after chemical application the non-parametric Wilcoxon matched pairs signed rank test or paired students t-test was used. Comparisons were made between the mean response thresholds to cold and heat using the Student's t-test (paired and unpaired).

The Mann-Whitney U test was used to compare differences between von Frey thresholds. The χ^2 test was used to evaluate whether the proportion of fibres which displayed a novel or increased sensitivity to stimuli where significantly different between different subpopulations of fibres. The response of Aβ fibres to graded increase in constant force stimuli was evaluated by ANOVA. Differences were considered to be significant if $P<0.05$.

Chapter 3

Mouse primary afferent properties in hairy and glabrous skin

3.1 Introduction

The properties of primary afferent neurons innervating the skin have been previously studied in vivo in rabbit, cat, monkey and humans as described in Chapter 1. Considering that rodents are routinely used to investigate the behavioural mechanisms of pain, few studies have comprehensively investigated the properties of primary afferent neurons innervating the glabrous skin of rats and mice. The most commonly studied area in behavioural experiments is the hind paw of the rodent where mechanical and thermal stimuli are applied to the glabrous skin and withdrawal responses to these stimuli are then measured. The results of these studies have improved our knowledge of nociceptive mechanisms and contribute to our knowledge on how chronic pain states can develop after nerve or tissue injury. It is therefore astonishing how little information exists about the functional properties of primary sensory neurons innervating the plantar surface of the paws. Furthermore, behavioural experiments carried out on transgenic mice lacking particular receptors or ion channels have given an important insight into the function of these receptors and ion channels in the pain pathway (LaCroix-Fralish et al., 2007).

A detailed investigation of the primary afferent sensory neurons innervating the hairy skin of the hind leg in mice has already been carried out in vitro by Koltzenburg et al (1997). The properties of mouse glabrous skin have also been studied by Cain et al (2001) in vivo. A very recent study by Banik and Brennan (2008) have investigated the properties of Aδ and C fibre nociceptors innervating the glabrous skin of the hind paw in mice in vitro.

Aim of the present study

The properties of primary afferent sensory neurons from both the hairy and glabrous skin in mice have not been investigated in the same study so far. The

aim of the present study was to characterise and compare the properties of fibres innervating both the hairy and glabrous skin in mice.

3.2. Methods

4.2.1. Skin-nerve in vitro preparation

Adult male C57/Blk6 mice were used for the in vitro electrophysiological analysis. To investigate the properties of fibres innervating the hairy skin, the saphenous nerve attached with the skin was dissected as described in Chapter 2. The tibial nerve with skin attached was dissected to study the properties innervating the glabrous skin. Fibres were characterised using electrical, mechanical and thermal stimuli and the data was analysed as described in detail in Chapter 2.

3.3. Results

A total of 84 units were recorded in the present study. Of those, 53 fibres were studied from the hairy skin of 20 C57/BLK6 adult mice. 31 fibres were studied from the glabrous skin of 9 C57/BLK6 adult mice.

3.3.1. Functional types of mechanically sensitive units

In hairy skin, four types of myelinated receptor types were distinguished on the basis of their conduction velocity, their adaptation properties in response to a controlled mechanical stimulus and size of the receptive field. Among the units with large myelinated (Aβ) axons, two receptor types were found; rapidly adapting (RA) and slowly adapting (SA) fibres. RA fibres typically discharged only at the beginning and end of a constant force stimulus, thereby adapting rapidly to the mechanical stimulus. In contrast, SA fibres showed a slowly adapting response in response to constant force stimulation (Fig.3.3.1). Most of the fibres had small, punctate receptive fields and the majority responded readily to stimulation with a von Frey hair exerting a bending force of 1.0 mN. Among units with thin (Aδ) axons, two receptors were found; D-hair (DH) receptors or high threshold A (AM) fibres. DH units had extremely low mechanical thresholds and always responded to von Frey hairs with a bending force of 1.0 mN. They usually showed a brisk rapidly adapting (RA) discharge at the onset and offset of a supramaximal constant force stimulus. AM units had thresholds that usually exceeded 1.0 mN and all had a slowly adapting discharge to suprathreshold constant force stimuli (Fig.3.3.1). The receptive fields of both units were usually larger than those of Aβ fibres and had sensitive spots of higher mechanical sensitivity than the rest of the receptive field.
In glabrous skin, only RA, SA, AM and CM fibres were present. No D-hair receptors were found. Mechanically sensitive unmyelinated C (CM) fibres were recorded from both hairy and glabrous skin. They generally displayed a slowly adapting response to constant force stimulation, similar to that of AM fibres and are thought to have a nociceptive function.
Two other types of functionally distinct C fibres are also present in the hairy skin of rodents; low-threshold mechanically sensitive (CLTM) fibres and

mechanically insensitive cold responsive (CC) fibres. CLTM fibres are thought to have a non-nociceptive function and CC fibres are thermoreceptors which respond to innocuous cold temperatures. None of the C fibres recorded form glabrous skin were classified as CLTM in this present sample. However, the properties of these fibres have been investigated in another sample of units recorded from rat hairy skin (see chapter 4.2). They have lower mechanical thresholds than CM fibres and display an irregular discharge during constant force stimulation, firing mostly to the onset and offset of the stimulus. Since CC fibres are mechanically insensitive, their receptive fields have to be searched for using a cold stimulus. This was not carried out in this sample of units. However the properties of these units have been studied in the hairy skin of rats (see chapter 4.2).

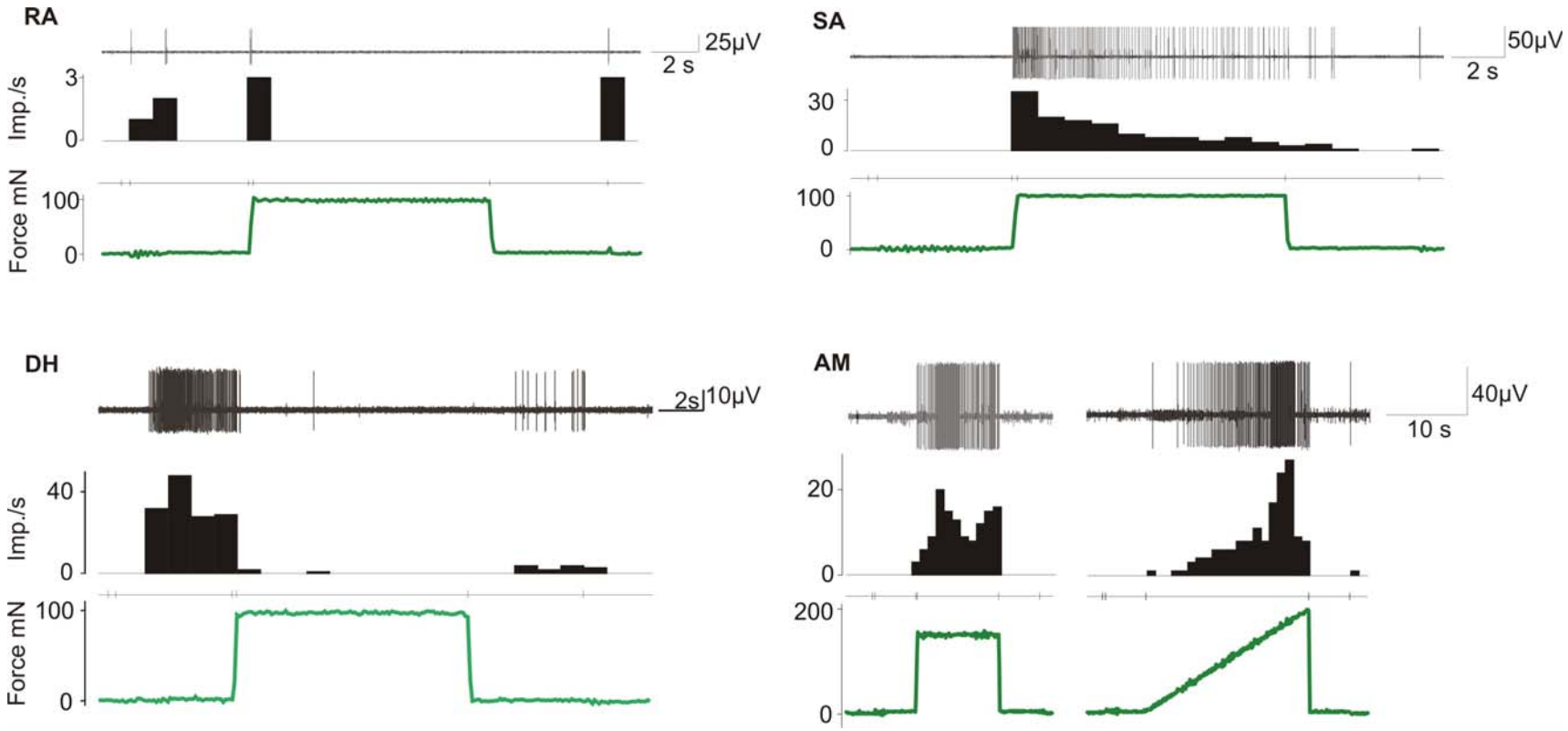

Fig.3.3.1 Examples of the types of A fibre mechanoreceptors responding to constant force stimuli
Top*: Rapidly adapting (RA) fibres typically respond at the beginning and end of the mechanical stimulus. Slowly adapting (SA) fibres typically fire throughout the stimulus, displaying a higher firing frequency at the start of the stimulus, followed by a lower level of firing.* ***Bottom****: D-hair (DH) mechanoreceptors usually display a rapidly adapting response, firing mostly during the onset and offset of the stimulus. High threshold mechanically sensitive A (AM) fibres display a slowly adapting response to a constant force stimulus. AM fibres encode the intensity of the mechanical stimulus, increasing firing frequency with increasing force.*

3.3.2. Conduction velocity of mechanoreceptors

Table.3.3.1 shows the mean conduction velocities of fibres found in the hairy and glabrous skin. Fibres which conducted over 10 m/s were considered to be large myelinated Aβ fibres. Those which conducted between 1.2-10 m/s were considered to be Aδ fibres and those which conducted under 1.2 m/s unmyelinated C fibres. The mean conduction velocity of Aβ fibres recorded from the hairy skin was 16.2 ± 2.1 m/s (n=7) which did not differ significantly ($p>0.4$, unpaired students t-test) from the mean conduction velocity of Aβ fibres recorded from the glabrous skin (18.9 ± 2.0 m/s, n=13). The mean conduction velocity of Aδ fibres recorded from the hairy skin was 7.6 ± 0.76 m/s (n=29) which did not differ significantly ($p>0.9$, unpaired students t-test) from the mean conduction velocity of Aδ fibres recorded from the glabrous skin (7.6 ± 4.5 m/s, n=4). In each of the samples recorded from the hairy or glabrous skin, there was one AM fibre which conducted above 10 m/s. It is unlikely that these were SA Aβ fibres because of their different mechanical thresholds and response profiles to supramaximal mechanical stimulation (see below). The mean conduction velocity of C fibres recorded from the hairy skin was 0.53 ± 0.04 m/s (n=17) which did not differ significantly ($p>0.1$, unpaired students t-test) from the mean conduction velocity of C fibres recorded from the glabrous skin (0.64 ± 0.05 m/s, n=14).

Table.3.3.1 Conduction velocity and von Frey hair thresholds

Preparation	Fibre Type	n	Conduction velocity, m/s		Von Frey threshold, mN	
Saphenous nerve	RA	2	14.2	(12.2-16.1)	0.63	(0.25,1)
	SA	5	17.0 ± 2.9	(11.1-26.3)	0.25	(0.25, 0.25)
	DH	2	8.0	(7.9-8.1)	1	(1,1)
	AM	16	7.6 ± 0.8	(1.6-12.9)	8	(2,22.6)
	CM	17	0.53 ± 0.04	(0.37-0.98)	11.2	(8,16)
Tibial nerve	RA	9	21.0 ± 2.2	(14.2-36.0)	1	(1,1)
	SA	4	14.2 ± 3.5	(10-24.4)	1	(1,2.5)
	AM	4	7.6 ± 4.5	(1.3-13.4)	8	(4.5, 15.3)
	CM	14	0.64 ± 0.05	(0.3-0.95)	11.3	(8,16)

All values are means ± SE; except for von Frey hair thresholds which are median and first (Q_1) and third (Q_3) quartiles. There was no significant difference in von Frey hair thresholds between RA, DH and CM fibres recorded from hairy and glabrous skin (RA; p>0.4, AM; p>0.7, CM; p>0.6, Mann Whitney U test). SA fibres from hairy skin had significantly lower mechanical thresholds than those recorded from the glabrous skin (p<0.05, Mann Whitney U test). RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; DH, D-hair receptors; CM, C mechano-sensitive nociceptors.

3.3.3. Quantitative analysis of mechanical sensitivity

Measurements of the mechanical thresholds with the use of von Frey hairs revealed that there was no significant difference in the mechanical thresholds between RA, AM and C fibres from hairy and glabrous skin (see Table.3.3.1). SA fibres from hairy skin had significantly lower mechanical thresholds than those recorded from the glabrous skin (p<0.05, Mann Whitney U test).

A feedback-controlled mechanical stimulator was used to investigate stimulus-response functions at suprathreshold stimulus intensities for the different receptor types from both hairy and glabrous skin. Constant forces ranging from 5-150 mN were commonly used to study the mechanical adaptation properties of RA, SA and DH fibres. A mechanical ramp stimulus from 0-200 mN was applied on the receptive fields of high threshold A and C fibres.

RA units from both hairy and glabrous skin displayed similar discharge frequencies in response to constant force stimuli of different forces (Fig.3.3.2). RA fibres from both hairy and glabrous skin discharged on average a maximum of 6 impulses per second during the phasic period of a 100 mN stimulus. Plotting the average number of spikes elicited during the mechanical stimuli revealed that there was no significant difference in total response between RA fibres from hairy or glabrous skin (Fig.3.3.3). However, a statistical analysis was limited since only 2 RA fibres were recorded from the hairy skin.

SA fibres from hairy skin appeared to respond greater between forces of 5-20 mN compared to SA fibres from glabrous skin (Fig.3.3.4). Fibres from hairy skin also appeared to be more responsive during the onset of the stimulus compared to those from glabrous skin. This is consistent with the finding that SA fibres from hairy skin had lower mechanical thresholds than those from glabrous skin. Plotting the mean response during each force application revealed that there was a trend of SA fibres from hairy skin to respond with more action potentials than SA fibres from glabrous skin between forces of 5-100 mN. However, statistically this difference was not significant (Fig.3.3.5). As well as an obvious difference in mechanical adaptation property, SA fibres were always clearly distinguishable from RA fibres since they displayed a higher peak and total discharge at the lowest stimulus intensity applied (in this case 5 mN). RA fibres discharged on average <10 imp/s at the highest stimulus intensity (Fig.3.3.3), whereas SA fibres discharges on average >20 imp/s at the lowest stimulus intensity (Fig.3.3.5).

The average response of 2 DH fibres to constant force stimulation is shown in Fig.3.3.6. Fibres typically responded during the beginning and end of the stimulus, as well as during the onset and offset of the stimulus. High mechanical sensitivity of these fibres caused them to discharge during the small fluctuations caused by the feedback-controlled force adjustments mechanical probe at low stimulus intensities. Difference in their conduction velocity, receptive field size and mechanical sensitivity always distinguished them from RA fibres.

High threshold A fibres from both hairy and glabrous skin displayed a very similar response pattern in response to a mechanical ramp stimulus

(Fig.3.3.7). AM fibres encoded the stimulus intensity; displaying an increase in firing frequency in response to increasing force, therefore consistent with a role in nociception. AM fibres from hairy skin displayed a slightly higher mean total response than those from glabrous skin, but statistically this difference was insignificant ($p>0.4$, unpaired t-test). AM fibres were generally distinguishable from SA fibres on the basis of their conduction velocity and mechanical thresholds. In addition to this, during suprathreshold stimulation SA fibres displayed a typical initial burst of activity followed by a lower level of firing, whereas AM fibres did not and often increased their discharge frequency during the stimulus plateau (see Fig.3.3.1).

On average, CM fibres from glabrous skin were much better at encoding the stimulus intensity than those recorded from hairy skin (Fig.3.3.8). Although the mean total number of spikes elicited from CM fibres from glabrous skin was greater than those from hairy skin, statistically this difference was insignificant ($p>0.1$, unpaired t-test). A clear difference was observed between the mean AM and CM fibre response to mechanical stimulation in the hairy skin. In comparison to AM fibres, CM fibres from the hairy skin displayed a lower peak discharge rate (<5 imp/s compared to >10 imp/s for AM fibres) and total response (<70 spikes compared to >110 spikes for AM fibres). This difference did not exist in the glabrous skin. Both AM and CM fibres displayed a similar peak discharge rate and mean total response to mechanical stimulation (Fig.3.3.9).

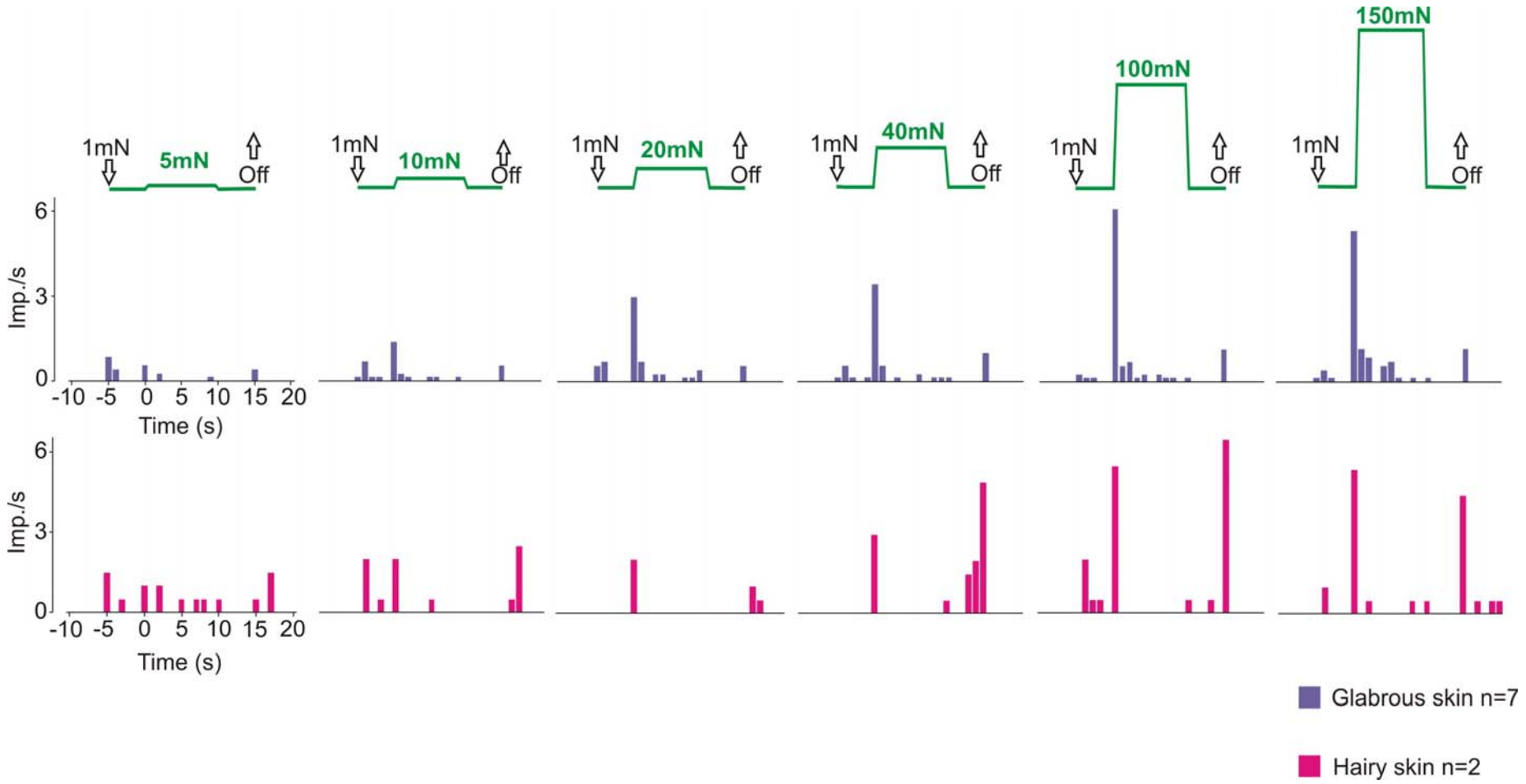

Fig.3.3.2 Mean response of rapidly adapting (RA) units from hairy and glabrous skin to constant force stimulation
There did not appear to be any striking differences in response to constant force stimulation between RA fibres recorded from hairy or glabrous skin.

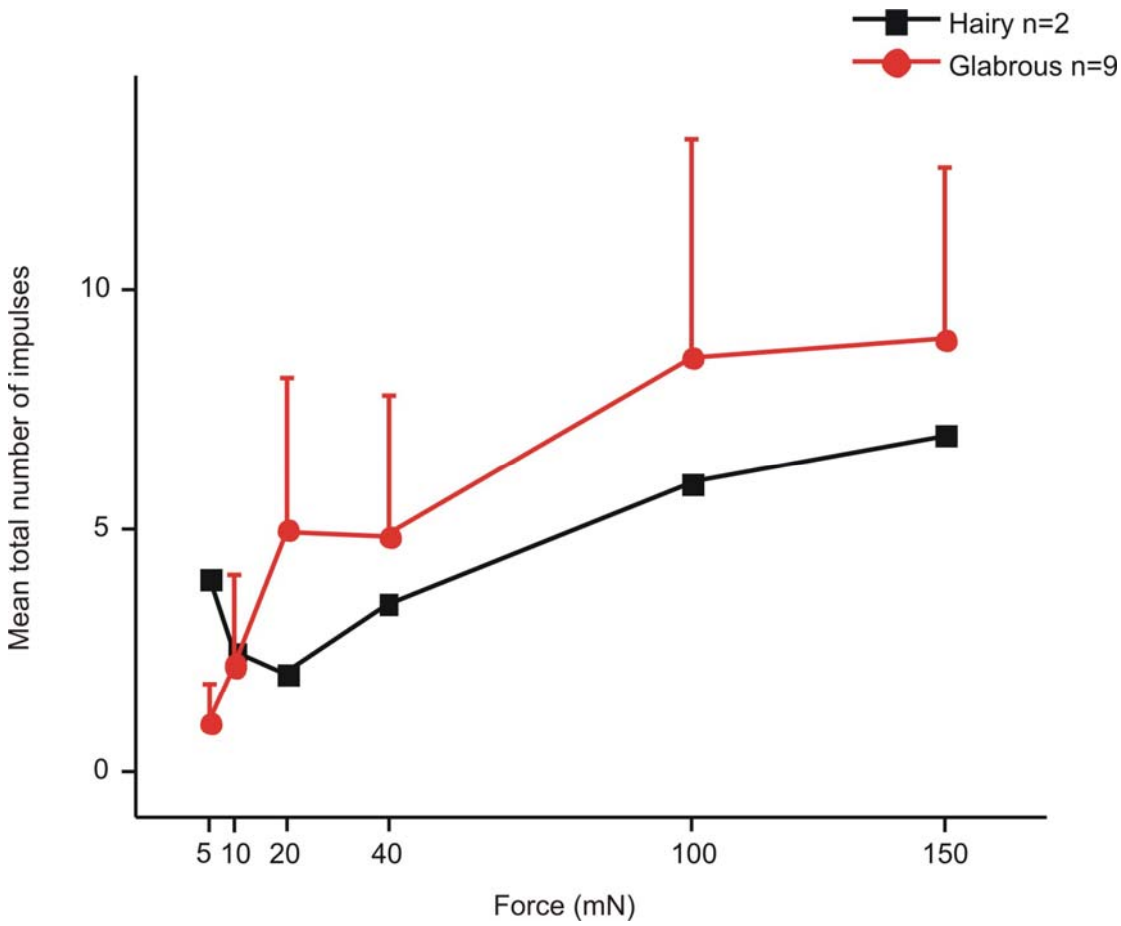

Fig.3.3.3 Mean response of rapidly adapting fibres recorded from hairy and glabrous skin

There appeared to be no significant difference between the total response to constant force stimulation between fibres recorded from hairy or glabrous skin. Values are mean ± SEM.

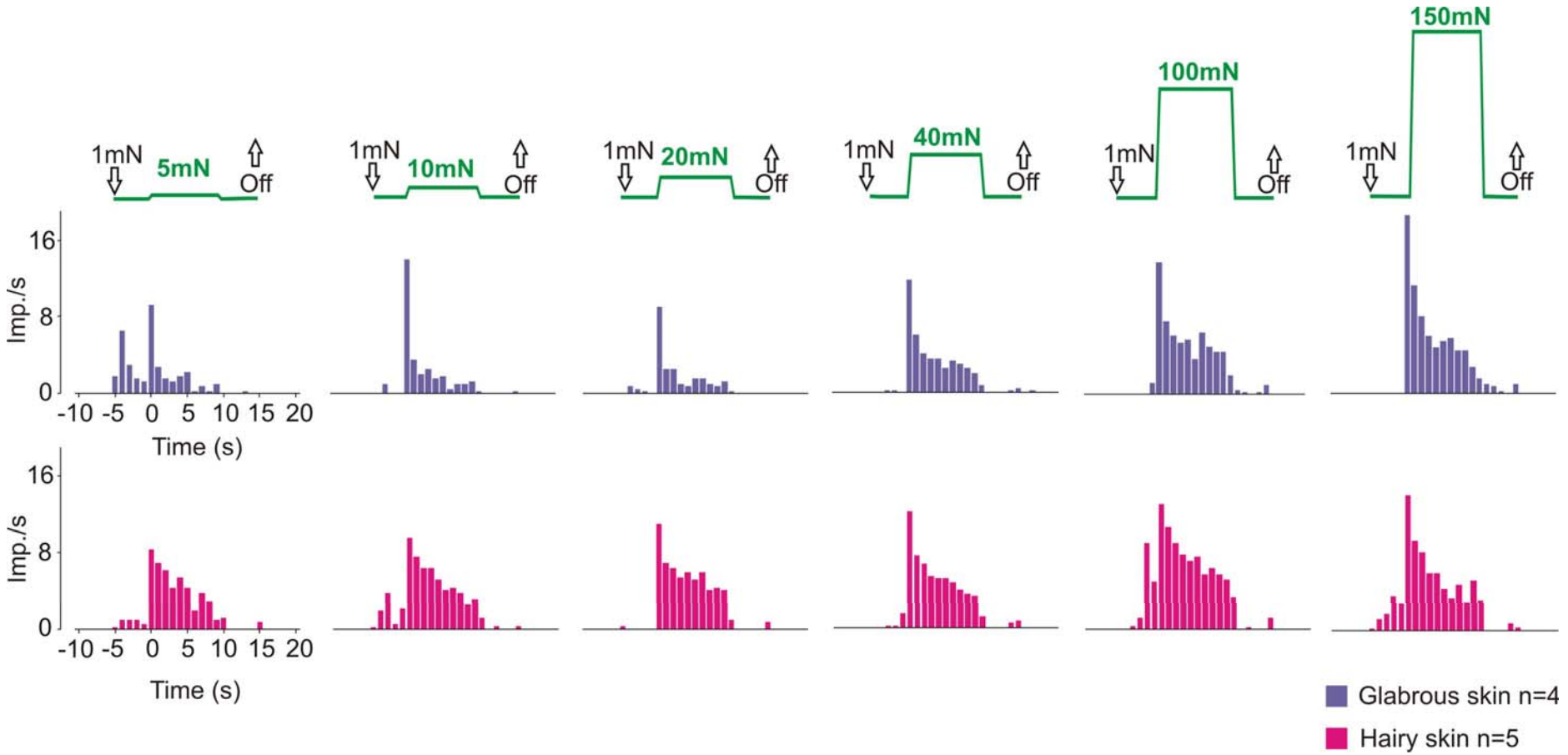

Fig.3.3.4 Mean response of slowly adapting (SA) fibres from hairy and glabrous skin to constant force stimulation
SA fibres from hairy skin were more responsive to constant force stimuli between 5-20 mN compared to fibres recorded from glabrous skin.

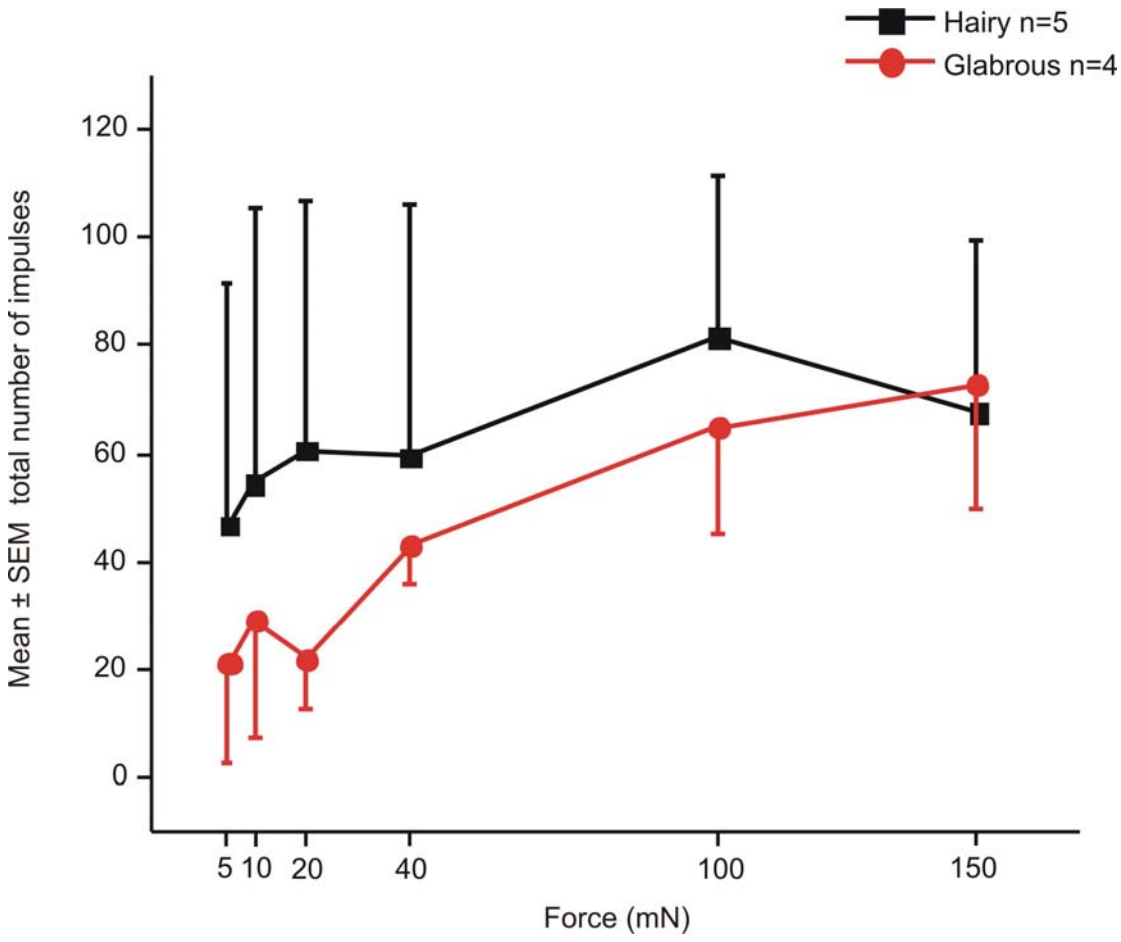

Fig.3.3.5 Mean ± SEM total response of slowly adapting (SA) fibres recorded from hairy and glabrous skin

SA fibres recorded from hairy skin displayed a greater response to constant force stimuli from 5-100 mN. However, statistically these differences were insignificant (F (1, 61)=0.94, p>0.3, 1 way ANOVA).

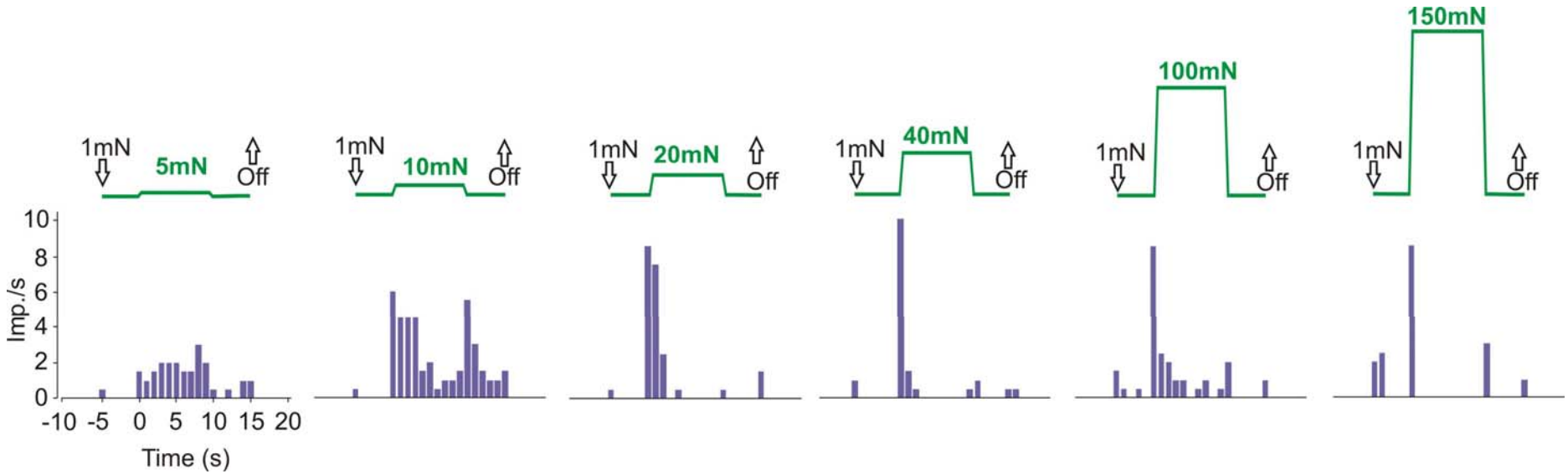

Fig.3.3.6 Average response of D-hair fibres responding to constant force stimulation
D-hair fibres responded mainly at the beginning and end of the mechanical stimulus. This was more evident at the higher forces between 20-150 mN. At lower forces, fibres discharged throughout the stimulus. This is probably due to the small fluctuations of the feedback control caused by the mechanical probe on the receptive fields of the fibres.

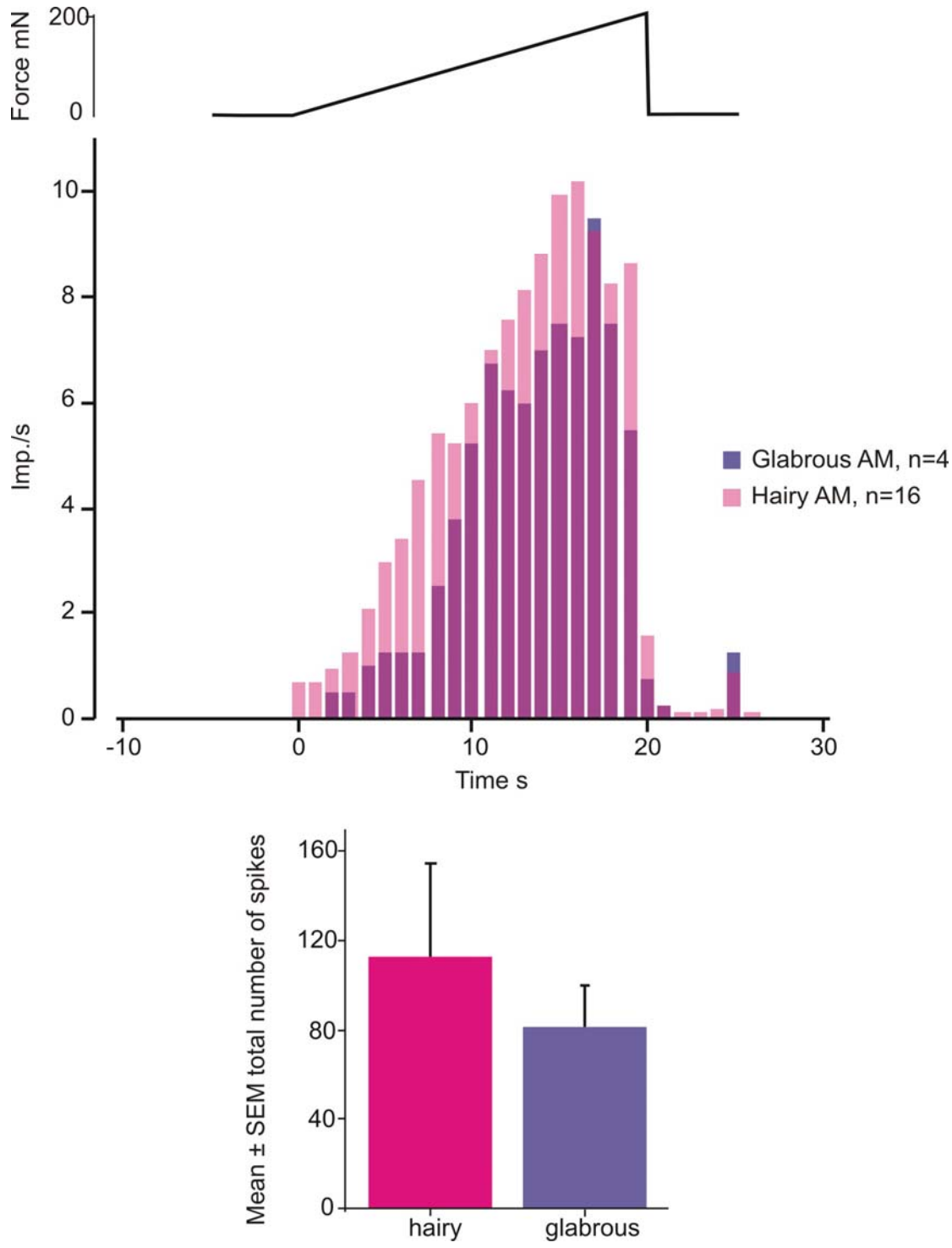

Fig.3.3.7 Mean response of high threshold mechanically sensitive A (AM) fibres from hairy and glabrous skin to a mechanical ramp stimulus *AM fibres from hairy skin appeared to be slightly more responsive during a ramp stimulus compared to those recorded from glabrous skin. However, this difference was not statistically significant (p>0.4, unpaired t-test).*

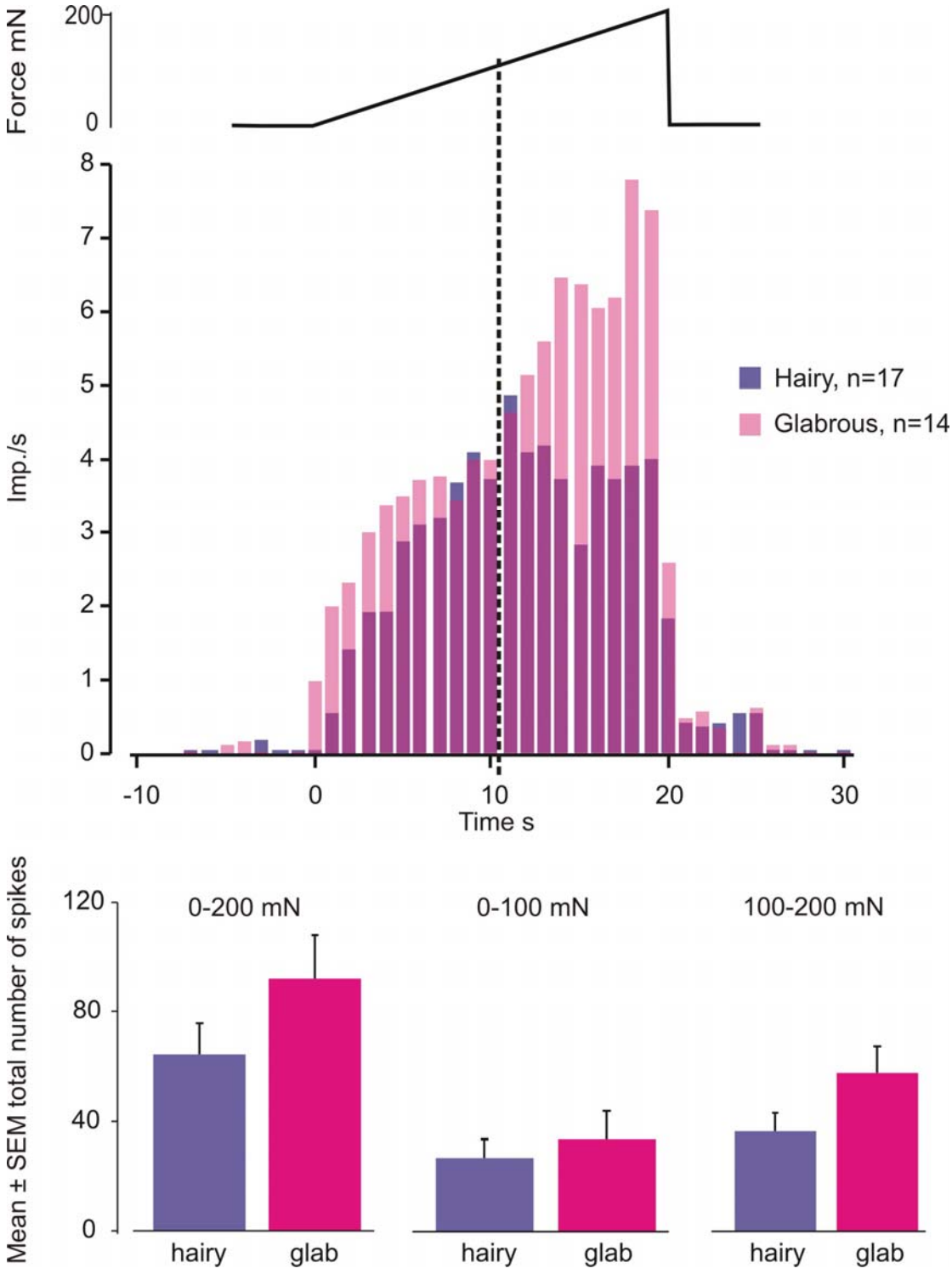

Fig.3.3.8 Mean response of high threshold mechanically sensitive C (CM) fibres from hairy and glabrous skin to a ramp stimulus
CM fibres recorded from glabrous skin were better at encoding the intensity of the mechanical stimulus above 100 mN than those recorded from hairy skin. The mean total response was also greater, but statistically the difference in the total number of action potentials did not reach significance ($p>0.1$, unpaired t-test).

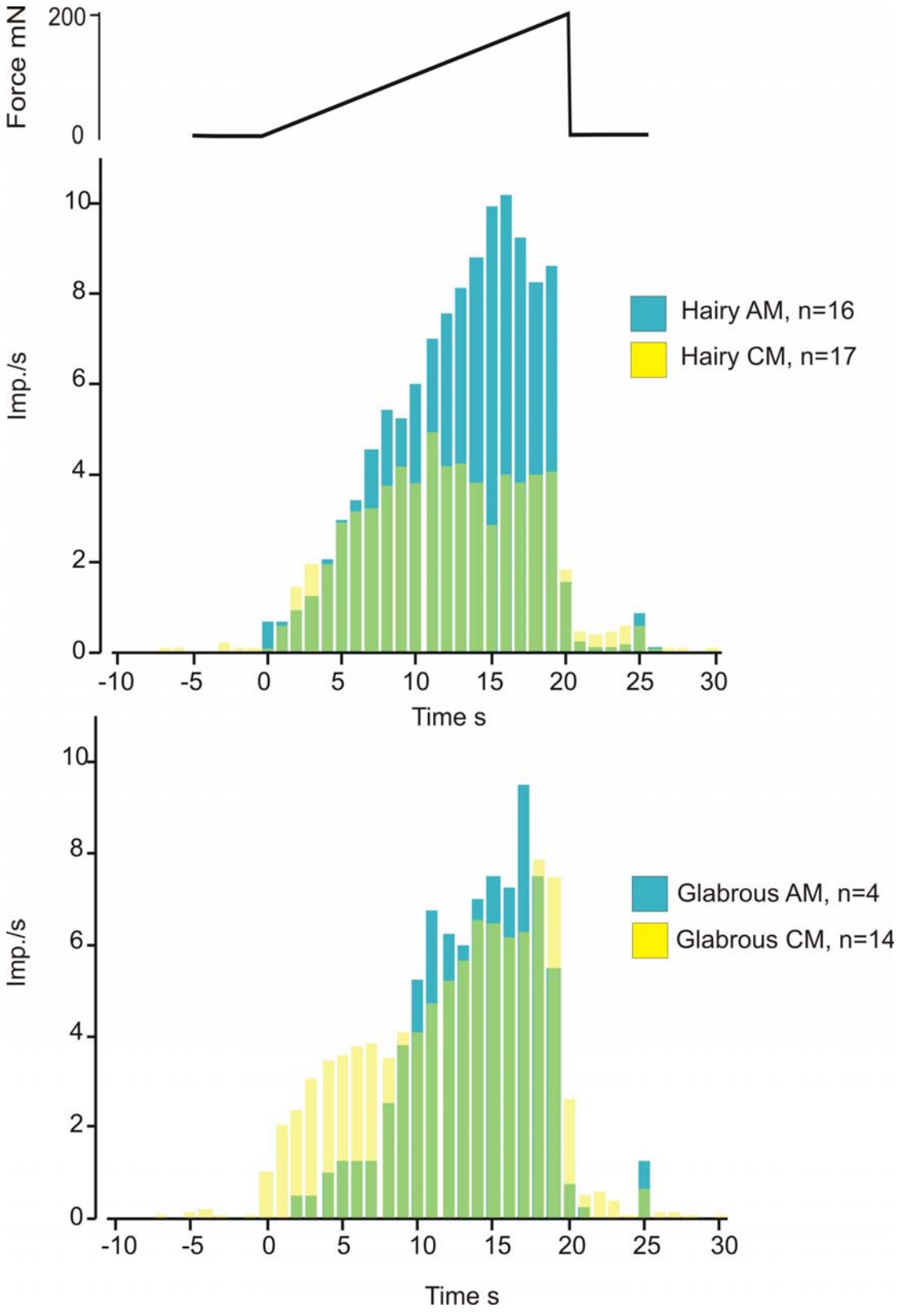

Fig.3.3.9 Comparison of the mechanical response pattern between AM and CM fibres recorded from hairy and glabrous skin

In the hairy skin, AM fibres encoded the intensity of the mechanical stimulus and responded greater compared to CM fibres at stimulus intensities above 100 mN. Interestingly, this was not observed in the glabrous skin where both AM and CM fibres had a similar response profile to a mechanical ramp stimulus.

3.3.4. Response of mechanically sensitive afferent fibres to thermal stimuli

Fibres were tested for their response to noxious cold and heat stimuli. Because the search procedure identified only the receptive fields of mechanically sensitive units, pure thermoreceptors were not included in the present analysis.

In this sample of units, Aβ fibres were unresponsive to cold and heat stimuli. However, a small proportion of Aβ fibres have been shown to respond briefly to cooling and we have also observed this in other samples of units recorded from the hairy skin of rats (see chapters 4.2, 6.2 and 7.2 for examples).
In the hairy skin, of 16 AM fibres tested, 2 (12.5 %) were activated by noxious heat (AMH), with an average heat threshold of 35.9 ˚C as measured on the corium side which corresponds to approximately 41 ˚C as measured on the epidermal surface (see chapter 2 for details). In the glabrous skin, none of the AM fibres (0/4) responded to noxious heat. None of the AM units responded to noxious cold. However, a large sample of these units has been studied in the rat hairy skin and the response properties of these fibres will be shown in chapter 4.2.
Sensitivity to thermal stimuli tended to be higher among unmyelinated compared to myelinated nociceptors. In the hairy skin, of 17 C fibres tested, 7 (41 %) were activated by heat (CMH), with average heat threshold of 36.4 ± 1.0 ˚C. In the glabrous skin, of 14 C fibres tested, 3 (21 %) were activated by heat, with an average heat threshold of 35.8 ± 0.9 ˚C. There was no significant difference in the proportion of heat sensitive C fibres between hairy and glabrous skin ($p>0.2$, χ^2 test). There was no significant difference in the heat thresholds between CMH fibres from hairy and glabrous skin ($p>0.7$, unpaired t-test). CMH fibres from hairy skin appeared to signal the intensity of the stimulus better than those from glabrous skin (Fig.3.3.10). However, CMH fibres from glabrous skin displayed a higher peak discharge rate and responded more robustly, although statistically this difference was insignificant ($p>0.3$, unpaired t-test).
In comparison to AMH fibres, CMH fibres from hairy skin displayed a greater response than AMH fibres (Fig.3.3.11).

In the hairy skin, of 17 C fibres, 3 (~18 %) responded to cold (CMC) with an average cold threshold of 16.2 ± 3.2 ˚C. One of these fibres was also responsive to noxious heat (CMCH fibre). In the glabrous skin of 14 C fibres, 2 (~11 %) responded to cold with an average response threshold of 19.8 ˚C. The mean response to a cold stimulus is shown in Fig.3.3.12. Fibres generally displayed an increase in firing rate as the temperature decreased. The mean total response during a cold stimulus was greater in CMC fibres from glabrous skin but a statistical analysis was limited.

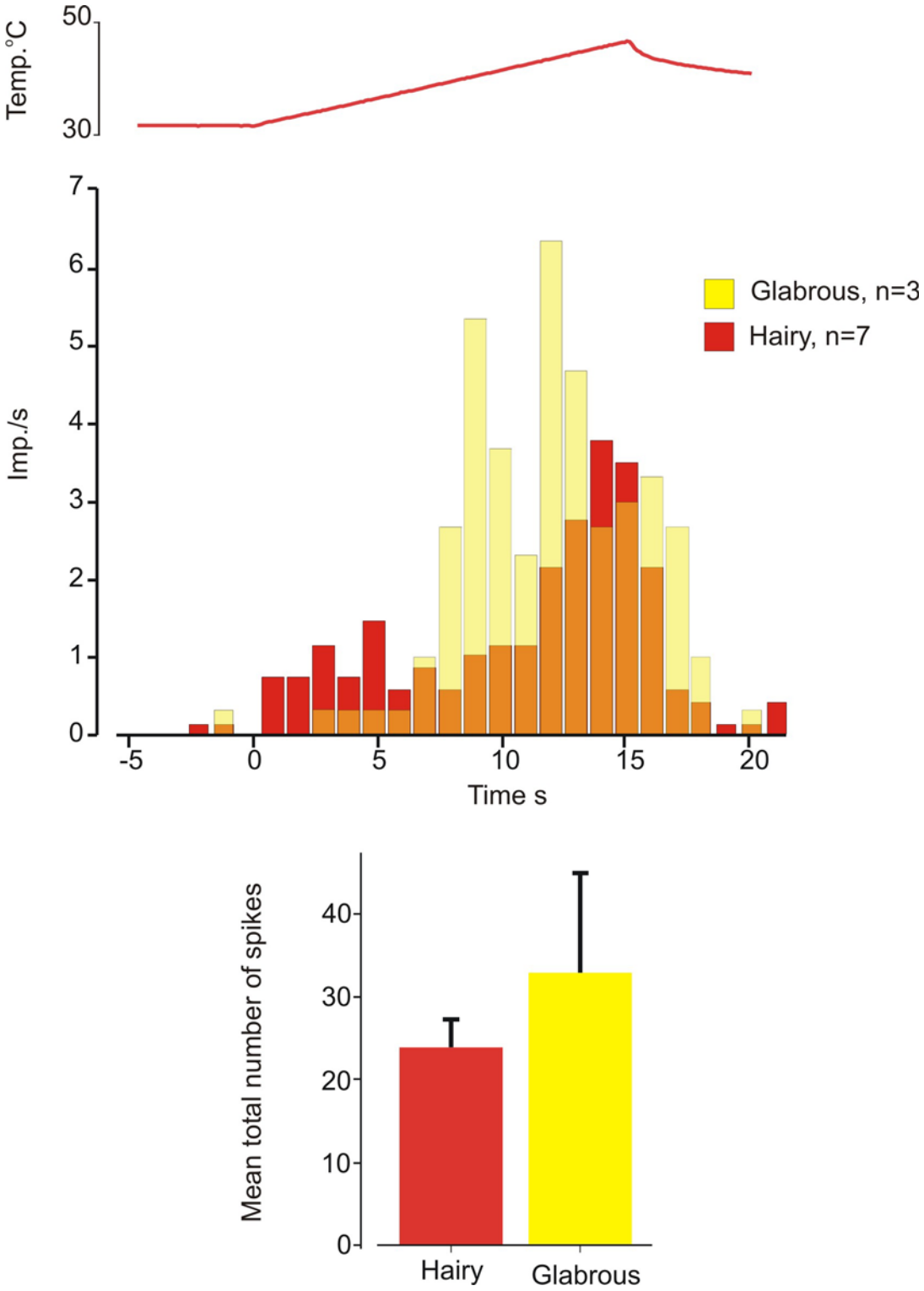

Fig.3.3.10 Mean heat response of CMH fibres recorded from hairy and glabrous skin

CMH fibres recorded from hairy skin appeared better at encoding the heat stimulus than those recorded from glabrous skin. However, CMH fibres from glabrous skin responded more vigorously (33 ± 12 spikes) to heat than did CMH fibres from hairy skin (24 ± 3 spikes), although statistically this difference did not reach significance (p>0.3, unpaired t-test).

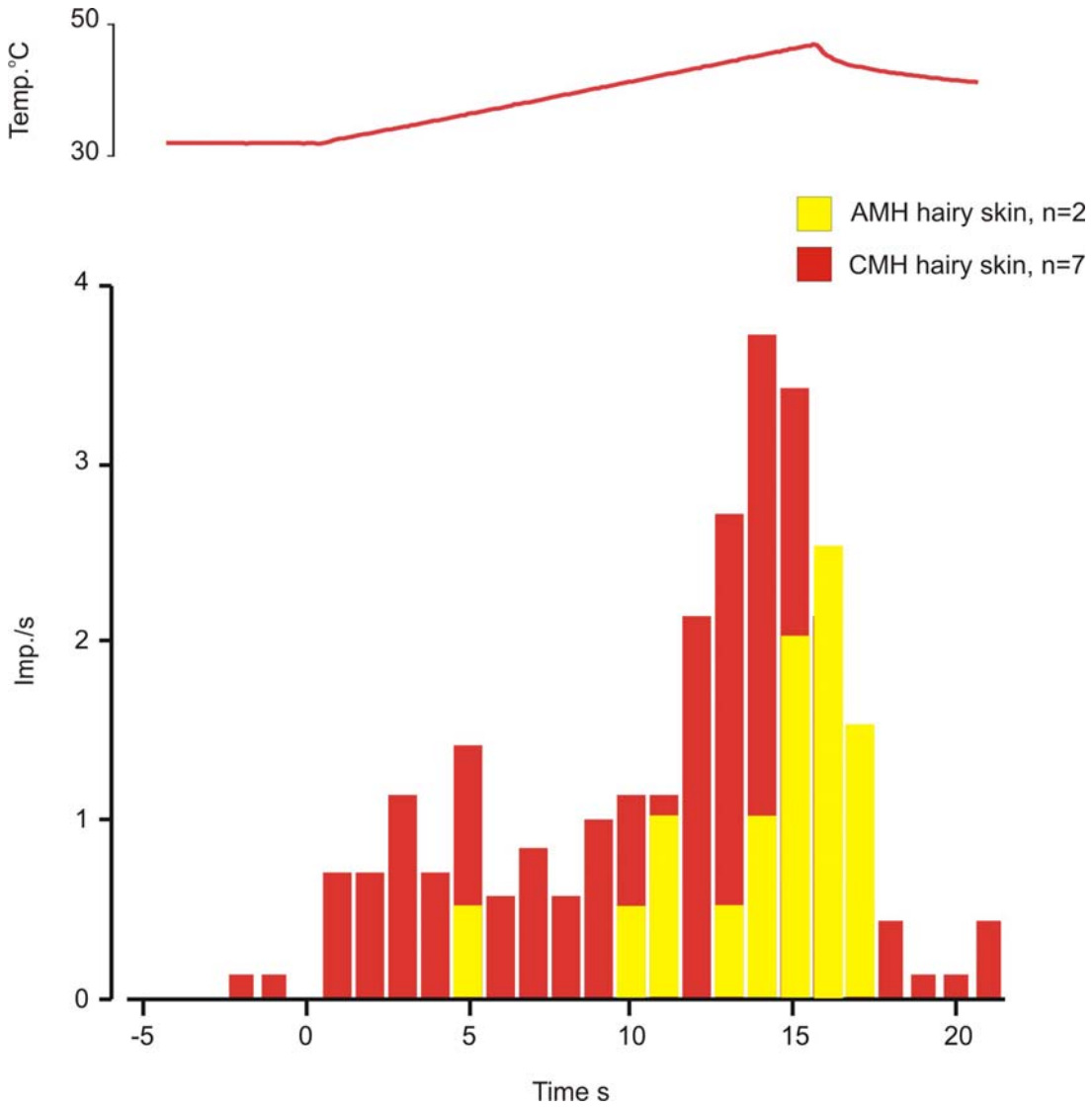

Fig.3.3.11 Average heat response of A and C fibre nociceptors recorded from hairy skin

C fibre nociceptors responded more vigorously (24 ± 3 spikes) than did A fibre nociceptors (8 spikes, range 4-12 spikes). There was no difference in the mean heat threshold between A (35.9 ˚C) and C (36.4 ˚C) fibre nociceptors.

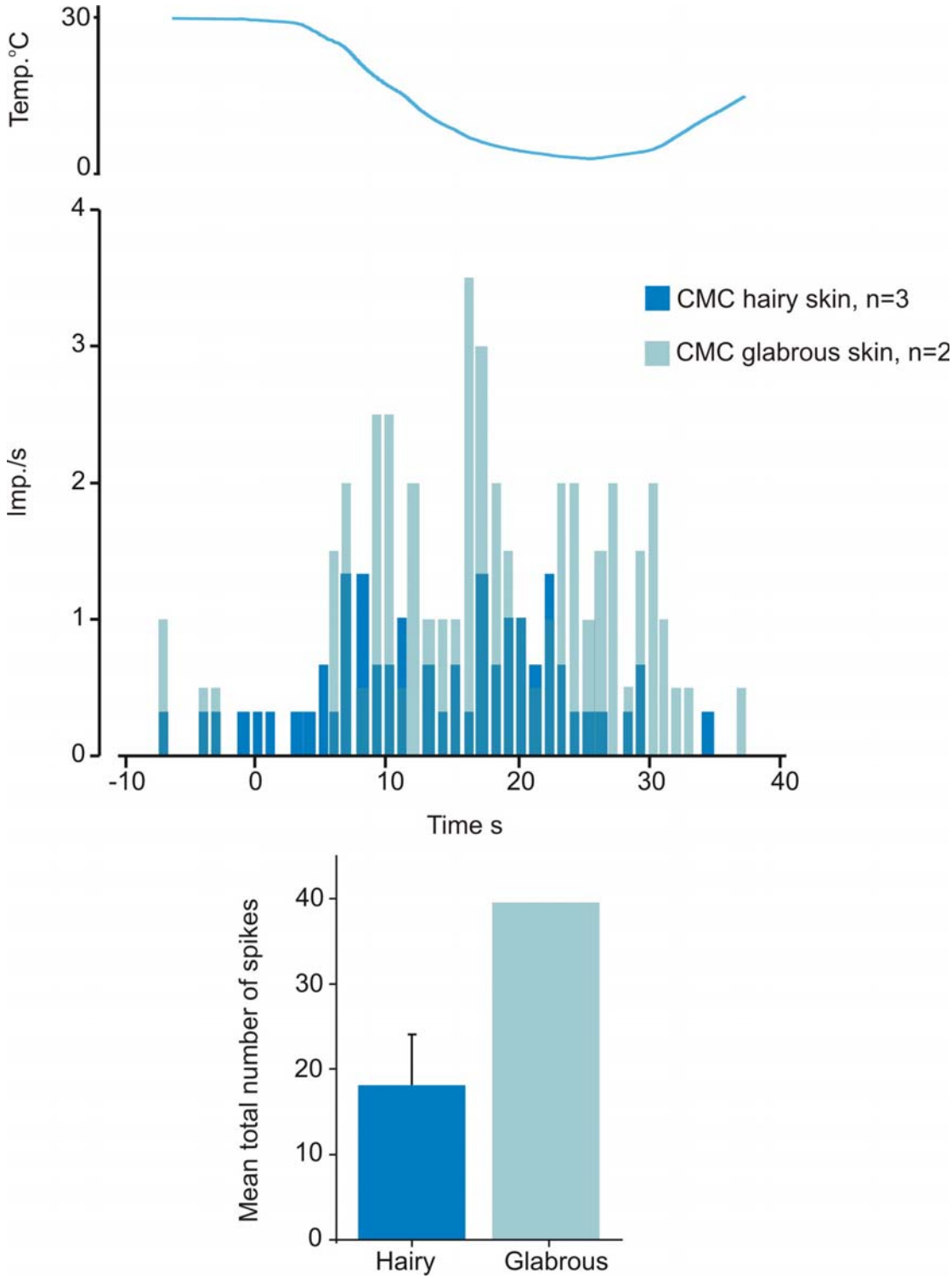

Fig.3.3.12 Mean response of C fibre nociceptors to cold in hairy and glabrous skin

C fibre nociceptors from both hairy and glabrous skin displayed an increasing response to cold during the dynamic phase of the ramp. On average CMC fibres from glabrous skin were more responsive during cooling, but a statistical analysis was limited.

3.4. Discussion

The present study investigated in detail the receptive properties of myelinated A fibres and unmyelinated C fibres innervating the hairy and glabrous skin of the mouse.

3.4.1. Aβ-fibres

Aβ fibres from both hairy and glabrous skin displayed either rapidly adapting (RA) or slowly adapting (SA) properties in response to constant force stimulation of their receptive fields. This is in accordance with the study by Koltzenburg et al (1997), who investigated in detail the receptive properties of fibres found in mouse hairy skin, in vitro (Koltzenburg et al., 1997). It also agrees with the findings from Cain et al (2001) who investigated the fibres innervating the glabrous skin of mice in vivo (Cain et al., 2001).

Rapidly adapting mechanoreceptors

Previous studies in the cat and primate hairy skin have shown that low threshold rapidly adapting Aβ units can be divided into three types of receptors; hair follicle receptors, field receptors and Pacinian corpuscles (Burgess et al., 1968;Perl, 1968). Hairs with thick and straight shafts were named ‘guard’ hairs and displayed a rapidly adapting response to hair displacement. Field receptors were activated by skin movement alone and not hair movement. Pacinian corpuscles were found to be highly sensitive to vibratory stimuli (Burgess et al., 1968). Single fibre recordings from conscious human subjects have also revealed that gently pulling or bending a hair follicle elicited a few impulses which were associated with a sensation of touch (Hensel and Boman, 1960a;Jarvilehto et al., 1981). In non-hairy glabrous skin, the rapidly adapting fibre group is made up of Pacinian corpuscles located in the subcutaneous tissue and Meissner corpuscles which are located close to the skin surface in the ridges of the dermis (Vallbo and Johansson, 1984). In the present study, it is unlikely that the RA fibres recorded from both hairy and glabrous skin are Pacinian corpuscles since these are located deep in subcutaneous tissue and therefore not preserved in this preparation. In the hairy skin it was not possible

to distinguish between field and hair receptors, as individual hair follicles were not accessible for individual stimulation due to the inside-up mounting of the skin.

Slowly adapting mechanoreceptors

In cat hairy skin, direct stimulation of the skin was found to activate two types of receptors which adapted slowly to mechanical stimuli and were classified as SA type 1 (SA I) and type 2 receptors (SA II). SA I receptors were excited from dome like elevations on the skin, displayed an irregular discharge during mechanical indentation of the skin and were insensitive to skin stretch. In contrast SA II receptors often displayed a resting discharge in the absence of stimulation, were sensitive to skin stretch and displayed a more regular discharge in response to mechanical stimulation (Burgess et al., 1968). The presence of SA I and SA II receptors have also been reported in human hairy skin (Jarvilehto et al., 1981). It has been shown that the endings of SA I units are associated with the Merkel cell complexes of the touch domes (Iggo and Muir, 1969), which are present in hairy and glabrous skin located at the tip of the epidermal ridges (Vallbo and Johansson, 1984). The end organs of SA II fibres are thought to be Ruffini endings located deeper in the dermis (Chambers et al., 1972;Vallbo and Johansson, 1984). In the present study, properties of SA fibres recorded from the hairy and glabrous skin resemble those of SA I fibres rather than SA II fibres.

Properties of RA and SA mechanoreceptors in hairy and glabrous skin

In the present study, Aβ fibres recorded from hairy skin by Koltzenburg et al (1997) conducted on average more slowly (14.5 m/s) than those recorded from glabrous skin (22.2 m/s) recorded by Cain et al (2001). In the present study, no difference in conduction velocity of Aβ fibres between hairy and glabrous skin was observed.

In response to constant force stimulation, SA fibres from hairy skin displayed greater firing than fibres from glabrous skin, consistent with the observation that SA fibres from hairy skin had lower mechanical thresholds than those in

glabrous skin. We failed to observe this difference for RA fibres. Von Frey hair thresholds were reported to be higher for both RA and SA fibres in glabrous skin (Cain et al., 2001) compared to hairy skin (Koltzenburg et al., 1997), consistent with the present findings. In the primate, mechanical thresholds of glabrous SA fibres were also reported to be higher than those of hairy skin (Perl, 1968). Glabrous skin is thicker than hairy skin (Harrison and Davis, 1999) and therefore the higher reported mechanical thresholds reported in glabrous skin may be due to the structural differences between the skin types.

3.4.2. Aδ-fibres

In the mouse hairy skin two types of afferents were found with conduction velocities in the Aδ range; Down-hair receptors (D-hair) and high threshold A mechanoreceptors (AM). DH fibres were not found in the glabrous skin. These findings are in accordance with those of Koltzenburg et al (1997) and Cain et al (2001) (Cain et al., 2001;Koltzenburg et al., 1997).

D-hair receptors

DH receptors were first identified in cat hairy skin (Burgess et al., 1968) and were distinguishable from guard hairs in several ways; they had thin and wavy hair shafts, were shorter than guard hairs and conducted slower in the Aδ conduction velocity range. They were found to be extremely sensitive to mechanical stimulation and adapted rapidly to mechanical displacement of the hair (Burgess et al., 1968). However, in the primate DH receptors appeared to be slowly adapting when stimulated using a hand held stimulator (Perl, 1968). Single unit recordings have revealed a population of low threshold Aδ fibres with similar properties to DH receptors in healthy human volunteers (Adriaensen et al., 1983). In the present study, DH fibres were easily distinguishable from RA and SA fibres on the basis of their mechanical response properties and conduction velocity. However, due to the inside up mounting of the skin, the down-hairs were not accessible for stimulation.

High threshold mechanically sensitive Aδ (AM) fibres

High threshold mechanical fibres with myelinated axons responsive to noxious stimuli have been previously described in the hairy skin of cats, monkey and humans (Adriaensen et al., 1983;Burgess and Perl, 1967;Perl, 1968). The receptive fields of these fibres have been described as areas of mechanically sensitive spots surrounded by unresponsive areas (Burgess and Perl, 1967;Perl, 1968). In primate hairy and glabrous skin, high threshold mechanoreceptors which were specifically activated by high pressure applied to the skin and not to cold or heat (Perl, 1968) have been described and these correspond to the AM fibres recorded in the present study. Primate high threshold mechanoreceptors from both hairy and glabrous skin displayed a slow adaptation to maintained deformation of the skin. In cat hairy skin the receptive terminals of nociceptive A fibres have been shown to be non specialised nerve endings which penetrate the epidermal-dermal border where they lose their myelin sheath (Kruger et al., 1981). In the present study no difference was observed in the conduction velocity or mechanical von Frey hair thresholds between hairy and glabrous AM fibres. AM fibres from both skin types were able to encode the intensity of the mechanical stimulus. This is in accordance with the recent findings from Banik and Brennan (2008) who also showed that Aδ nociceptors recorded in vitro from mice glabrous skin displayed increasing response to greater mechanical forces (Banik and Brennan, 2008). In the present study some A-fibres recorded from both hairy and glabrous skin had high mechanical thresholds and encoded stimulus intensity in the noxious range, but had a conduction velocity in the range of large myelinated fibres. The fibres could be classified as Aβ nociceptors (Koerber et al., 1988;Lawson, 1992).

Thermal sensitivity of AM fibre nociceptors

Heat sensitivity among myelinated A fibre nociceptors has been previously reported in the hairy and glabrous skin of humans and primates (Adriaensen et al., 1983;Campbell et al., 1979;Treede et al., 1995).

In the present study 12.5 % of AM fibres in hairy skin responded to noxious heating with an average heat threshold of 35.9 ˚C, whereas none responded from glabrous skin. Interestingly, Banik and Brennan (2008) also found that none of the Aδ nociceptors recorded from uninjured mice glabrous skin were heat sensitive (Banik and Brennan, 2008).
Previously in mouse hairy skin 26 % of AM fibres were activated by noxious heat with a mean threshold of 42.5 ˚C (Koltzenburg et al., 1997), similar to the proportion of heat sensitive A fibre nociceptors reported (21 %) in the hairy skin of humans (Adriaensen et al., 1983). In mouse glabrous skin 12 % of AM fibre responded to noxious heat (Cain et al., 2001). In the present study a lack of heat sensitivity among AM fibres from glabrous skin may be due to the small number of AM fibres sampled. Heat sensitivity of A fibre nociceptors is discussed further in Chapter 5. Cold sensitivity among AM fibres from hairy and glabrous skin has also been reported (Cain et al., 2001;Koltzenburg et al., 1997). Although no cold sensitive AM fibres were recorded in the present sample, these fibres have been recorded from rat hairy skin and their properties are described in Chapter 3.

3.4.3. C fibres

Unmyelinated C fibres have been classified according to their mechanical and thermal sensitivity. There are two main groups of C fibres; mechanically sensitive C fibres (CM) and mechanically insensitive C fibres. Mechanically sensitive C fibres are further divided into those with low mechanical thresholds (CLTM) which may have a non-nociceptive function and those with high mechanical thresholds (CM), thought to be nociceptors. Mechanically insensitive C fibres are thermoreceptors, commonly referred to as warm and cool fibres, which signal non-painful warm and cool temperatures. Their receptive endings have been located in the epidermal layer of the skin (Meyer et al., 2005).
In the present study, only CM fibres were encountered in the hairy and glabrous skin. This observation would agree with the findings of Cain et al (2001) who found that mechanically sensitive C fibres in the glabrous skin did not have thresholds lower than 6 mN. The lack of CLTM units in the glabrous

skin is supported by findings from human microneurography studies which showed that CLTM units are found in the forearm, but not in the hand of humans (Vallbo et al., 1999).

Unmyelinated C fibres have been previously recorded from the hairy skin of adult cats (Bessou and Perl, 1969). Over 30 % of fibres were found to be mechanically low threshold C fibres and displayed rapid adaptation during mechanical stimulation. Some of these fibres were also sensitive to cooling. Although no CLTM fibres were recorded in the present study, they have been recorded in another sample from rat hairy skin (see chapter 4) and many were found to be responsive to cold, in agreement with the above finding. Bessou and Perl (1969) reported that most cold sensitive thermoreceptors displayed a background discharge, were highly sensitive to changes in temperature and relatively insensitive to mechanical stimulation. In the present investigation, receptive fields of fibres were searched for using a mechanical stimulus and therefore mechanically insensitive cold thermoreceptors were not found. However, these thermoreceptors are present in the rat hairy skin (see chapter 4) and have properties as described above. The most frequently encountered unmyelinated fibres in the study by Bessou and Perl (1969) were described as polymodal nociceptors which were activated by intense mechanical stimuli, noxious heat and sometimes also low skin temperatures. C fibres activated by only strong mechanical stimuli were also found. The findings of the present study are in agreement with the above observation. Interestingly, CM fibres from glabrous skin were better at encoding the intensity of a mechanical ramp stimulus than CM fibres from hairy skin. This suggests that C fibre nociceptors from glabrous skin are better at signaling the intensity of noxious mechanical stimuli than C fibre nociceptors from hairy skin.

Thermal sensitivity of C fibre nociceptors

In the present study 41 % and 21 % of CM fibres were activated by noxious heat in hairy and glabrous skin, respectively. The proportion of CM fibres sensitive to heat in mouse hairy skin is identical to that found by Koltzenburg et al (1997). However, Cain et al (2001) reported a much higher percentage of heat sensitive CM fibres in mouse glabrous skin (82 %) as did Banik and

Brennan (2008) (75 %). This difference may be partly due to the heat stimulus employed by Cain et al (2001) which was applied at a much faster rate and ranged from 35-51 ˚C compared to the stimulus used in the present study. In the present study, there was no difference in the proportion of CMH fibres and no significant difference in the mean heat thresholds. CMH fibres from both hairy and glabrous skin were able to encode the intensity of the noxious heat stimulus. These results are in agreement with those of Treede et al (1995) who reported no difference in heat responses of CMH fibres recorded in hairy and glabrous skin of the monkey (Treede et al., 1995).

In the present study, 18 % and 11 % of CM fibres were found to be cold sensitive in hairy and glabrous skin, respectively. Koltzenburg et al (1997) reported that 3/10 CM fibres were cold sensitive, while Cain et al (2001) reported that the majority of C fibres (77 %) were cold sensitive. Banik and Brennan (2008) found that none of the C fibres (0/9) recorded from glabrous skin in vitro were sensitive to cold.

The cold stimulus applied by Cain et al ranged from 28˚ C to minus 12˚ C and it has been shown previously that the majority of fibres are activated at temperatures below 0˚ C (Simone and Kajander, 1997). Therefore the difference in cold stimuli applied may account for the high proportion of cold sensitive C fibres reported by Cain et al (2001).

Comparison of thermal and mechanical responses between A and C fibre nociceptors in hairy skin

In the hairy skin, A fibre nociceptors were much better at encoding the intensity of the mechanical ramp stimulus compared to C fibre nociceptors. As the force increased, AM fibres displayed an increase in firing rate whereas the response of the CM fibres reached a plateau during higher forces. This finding is identical to that of Slugg et al (2000) who reported that A fibre nociceptors were better at providing more discriminative information regarding the intensity of noxious mechanical stimuli than C fibre nociceptors in primate hairy skin (Slugg et al., 2000). Interestingly, this trend was not observed in the glabrous skin and therefore suggests that both A and C fibre nociceptors are capable of signaling the intensity of noxious mechanical stimuli in glabrous skin.

In the hairy skin, heat responses of A fibre nociceptors were much less vigorous compared to those of C fibre nociceptors, in agreement with previous findings from Koltzenburg et al (1997). However, no significant difference in heat threshold was observed, whereas Koltzenburg et al (1997) reported that A fibre nociceptors had a higher mean threshold than C fibre nociceptors.

Summary

A detailed investigation of the primary afferent sensory neurons innervating the hairy skin of the hind leg in mice has already been carried out in vitro by Koltzenburg et al (1997). However, this is the first study which has systematically investigated the receptive properties of Aβ, Aδ and C fibres innvervating the glabrous skin in mouse. The very small area of the mouse foot pad introduced a technical difficulty in recording from afferents innervating the glabrous skin and therefore fewer units were recorded per experiment compared to recordings from the hairy skin.

The same fibre types were encountered in both hairy and glabrous skin, except D-hair fibres which were found only in the hairy skin. Aβ mechanoreceptors appeared to be mechanically more sensitive in the hairy compared to glabrous skin. The mechanical properties of Aδ nociceptors (AM fibres) were similar in both skin types. Heat sensitivity among these fibres was found to be low in both skin types. Heat and cold sensitive C fibres were recorded from both skin types. The mechanical encoding properties of CM fibres were much better in the glabrous compared to hairy skin.

The next result chapter (chapter 4) investigates the functional expression pattern of cold activated TRPM8 and TRPA1 and heat activated TRPV1 ion channels on primary afferents innervating rat hairy skin. The following chapter (chapter 5) investigates the role of the heat activated TRPV2 receptor in mice. This was done by comparing the receptive properties of fibres recorded from wild-type and TRPV2 deficient mice in both hairy and glabrous. Recordings from wild-type mice in this chapter were therefore carried out to establish the recording technique from the glabrous skin in mice, before carrying out recordings from TRPV2 deficient mice.

Chapter 4

Functional expression pattern of TRPM8, TRPA1 and TRPV1 on rat primary afferent neurons

4.1 Introduction

Insights into the molecular mechanisms of thermal sensation have come from usage of natural compounds which elicit the sensation of heat or cold when applied to the skin.

One such compound is capsaicin, an ingredient of red hot chilli peppers which evokes the sensation of burning pain, and has been shown to excite polymodal CMH nociceptors in the rat skin (Szolcsanyi et al., 1988). The pungent compound allylisothiocyanate (mustard oil) has also been previously shown to excite CMH fibres in vivo from the rat saphenous nerve (Reeh et al., 1986).

Another such compound is menthol, which elicits a cooling sensation (Wasner et al., 2004) and has been shown to excite themosensitive cold afferents. Hensel and Zotterman (1951) showed that menthol could sensitise cold receptors and Schäfer et al (1986) described how menthol induced increases in firing frequencies among feline nasal and lingual cold receptors (Hensel and Zotterman, 1951;Schäfer et al., 1986). However, the molecular targets of these compounds were not discovered until later.

In 1997, capsaicin played a key role in the identification of the heat and proton activated vanilloid receptor subtype 1, VR1 (Caterina et al., 1997). The channel was identified as a non-selective cation channel structurally related to members of the transient receptor potential (TRP) family of ion channels, found in drosophila. The VR1 receptor, later renamed as TRPV1, has an activation threshold near 43 °C and is expressed in both trigeminal and sensory root ganglion on predominantly medium and small diameter sized neurons (Caterina et al., 1997).

This was soon followed by the cloning and characterisation of other heat activated TRP ion channels TRPV2 (formerly known as vanilloid receptor like protein 1 (VRL-1)), TRPV3 and TRPV4. In 1999, TRPV2, a capsaicin insensitive, heat activated ion channel with an activation threshold of ~52 °C was discovered (Caterina et al., 1999). In the DRG and trigeminal ganglia TRPV2 is predominantly expressed in medium to large sized neurons. The role of the TRPV2 receptor shall be discussed in detail in Chapter 5. In 2002, TRPV3 was cloned and characterised as a channel activated by warm temperatures between 34 to 38 °C and was found to be specifically expressed in keratinocytes (Peier et al., 2002b). Shortly after, the TRPV4 receptor which was originally identified as an osmosensor (Patapoutian et al., 2003) was shown to be also activated by heat. Warm temperatures, between 27 to 34 °C evoked responses in xenopus oocytes and HEK cells expressing TRPV4 (Guler et al., 2002). TRPV4 immunoreactivity was found in structures in the hypothalamus suggesting that it could function as a transducer of warm stimuli in the hypothalamus.

In addition to these, two cold activated TRP channels have also been identified: TRPM8 (formerly known as CMR1) and TRPA1 (formerly known as ANKTM1). Two groups independently cloned and characterised TRPM8 which is activated by cold and the cooling compound menthol (McKemy et al., 2002;Peier et al., 2002). When TRPM8 is expressed in heterologous expression systems, it is activated at temperatures between ~25 to 28 °C suggesting that TRPM8 is involved in innocuous thermal sensation. This notion was further supported by the lack of co-expression of TRPM8 with TRPV1, calcitonin gene-related peptide (CGRP) and isolectin B4 (IB4), all of which are thought to mark a subset of nociceptors (Peier et al., 2002;Story et al., 2003). However, other studies have found conflicting results. McKemy (2002) using calcium imaging showed that 54 % of cold and menthol sensitive trigeminal neurons in culture were also activated by capsaicin. Using whole cell recordings, Okazawa (2004) reported, that TRPV1 was expressed in 29 % of TRPM8 positive cells in the rat DRG. Because TRPV1 expression is generally thought to be restricted to nociceptive neurons these results suggest that TRPM8 may also be expressed on nociceptors.

TRPA1 was originally described as a cold responsive, menthol insensitive TRP channel (Story et al., 2003). When mouse TRPA1 was expressed in heterologous systems, cells exhibited an average activation threshold of 17.5 °C (lower than that of TRPM8) ranging from 12 to 24 °C. Expression patterns of TRPA1 mRNA in DRG revealed that it was most likely to be expressed in non-myelinated C fibres or thin myelinated Aδ fibres. TRPA1 mRNA was also found to be expressed in CGRP and Substance P (SP) positive neurons. In addition to this, 97 % of TRPA1 positive neurons expressed TRPV1 (Story et al., 2003). This suggested that TRPA1 mRNA was found in a small population of neurons that also expressed TRPV1 but not TRPM8. Its expression pattern and lower activation threshold than TRPM8 suggested that TRPA1 expressing neurons were a class of polymodal nociceptor which responded to both noxious cold and heat. TRPA1 has been shown to be activated by natural compounds such as mustard oil and cinnamaldehyde (Jordt et al., 2004;Bandell et al., 2004) which are known to elicit burning sensations. This further suggested that TRPA1-positive primary afferent neurons could be involved in the “burning” component of noxious cold.

However, doubts about the cold sensitivity of TRPA1 arose when only 4 % of mustard oil sensitive TG neurons in the rat responded to cold (Jordt et al., 2004). Furthermore, these 4 % of neurons also responded to menthol, suggesting that TRPM8 accounted for cold sensitivity in these neurons. Native rat DRG neurons have been shown to be activated very slowly, with delays of up to 1 minute in response to cold and TRPA1 agonists (Bandell et al., 2004) again raising doubts into TRPA1s’ actions as a receptor for noxious cold sensation.

Aim of the present study

Since the identification of thermosensitive TRP ion channels, many studies have used DRG in culture as a model of primary afferent sensory neurons to investigate the functional expression pattern of these channels. However, few have attempted to investigate TRP channel expression using single fibre recordings. The present study aimed to investigate the thermal and TRP channel expression in characterised primary afferent neurons innervating the

hairy skin of the rat hind leg using the selective agonists capsaicin, menthol and mustard oil.

4.2 Methods

4.2.1. Skin-nerve in vitro preparation

The saphenous nerve with the skin of the of the hind paw attached was dissected from adult female Sprague Dawley rats as described in chapter 2. The skin was mounted corium inside up in an organ bath and superfused with warm oxygenated SIF. Receptive fields of individual primary afferent neurons were then identified with a mechanical (glass rod) or cold (cold pen) search stimulus and subsequently characterised using electrical, mechanical and thermal stimuli as described in chapter 2.

4.2.2 Chemical stimulation

Fibres were tested with application of TRP channel agonists menthol (250 μM, 2.5 mM), mustard oil (250 μM, 2.5 mM) and capsaicin (2 μM) in this order of application. Solutions were made by diluting stock solutions (2.5 M menthol, 2.5 M mustard oil, 20 mM capsaicin) of the TRP channel agonists into warm SIF. All stock solutions were made up using 100 % ethanol. The final concentration of ethanol was therefore 0.1 and 0.01 v/v %.

Each agonist was delivered in ascending order of concentration to determine a dose-response relationship. Application of each concentration on the receptive field of the fibre lasted for 60 s and was administered at a flow rate of 5 ml/min to assure rapid replenishment of each solution. All chemicals were delivered at 32 ^{o}C to avoid any confounding effect of cooling on the units that were cold sensitive. A wash out period of variable duration of 1-10 minutes was used between each application of the TRP channel agonist. 8 fibres (6 C fibres and 2 AM fibres) were also tested with application of vehicle (0.1 % ethanol dissolved in SIF). None of these responded.

The same skin area in subsequent recordings, of the same preparation was avoided to avoid re-exposure to noxious or chemical stimuli in order to prevent possible sensitisation or desensitisation of units.

For quantitative analysis all fibres were required to either be activated by TRP channel agonists or to be activated by adequate mechanical, thermal or

electrical stimuli at the end of the chemical stimulation to determine the proportion of TRP channel agonist responsive fibres. This precaution was necessary to obtain a percentage that was not artificially lowered because fibres were lost due to deterioration of the recording conditions.

4.3. Results

Recordings were made from a total of 234 afferent fibres of which 50 were classified as Aβ fibres, 58 as Aδ fibres and 126 as C fibres.
The mean conduction velocity of Aβ fibres was 15.6 ± 0.6 m/s. The mean conduction velocity of Aδ fibres was 4.8 ± 0.4 m/s. The mean conduction velocity of C fibres was 0.49 ± 0.02 m/s. The general properties of A and C fibres are displayed in Table 1 and 2, respectively.
Fibres recorded in this study were not a representative sample of the units in the saphenous nerve, because many experiments focused on the study of afferents that were cold sensitive.

4.3.1. Aβ fibres

A total of 30 rapidly adapting (RA) and 20 slowly adapting (SA) fibres were recorded. None of the fibres displayed any spontaneous activity.
1 out of 30 (3 %) of RA fibres responded with a brief non-sustained excitation at the beginning of a cold stimulus (Fig.4.3.1). The response threshold of this fibre was 16.3 °C. None of the RA fibres responded to a noxious heat stimulus.
3 out of 20 (15 %) of SA fibres responded with a brief, non sustained excitation to a cold stimulus. An example of one such unit is shown in Fig.4.3.2. The average response threshold to cold was 20.2 ± 1.9 °C ranging from 16.5-23 °C. None were responsive to a noxious heat stimulus.
11 RA and 20 SA fibres were tested for their sensitivity to the TRP channel agonists menthol, mustard oil and capsaicin. None of the units were activated by any of the TRP channel agonists (Table.3.4.4).

TABLE.4.3.1 Conduction velocity, von Frey hair thresholds, heat thresholds and cold thresholds of A fibres

Type	n	Conduction velocity, m/s	Von Frey hair threshold, mN	Cold sensitive	Cold threshold, °C	Heat sensitive	Heat threshold, °C
RA	30	15.1 ± 0.7	2 (0.3, 5.6)	1	16.3	-	-
SA	20	15.8 ± 1.0	1.2 (0.7, 8.4)	3	20.2 ± 1.9	-	-
DH	17	5.5 ± 0.6	0.3 (0.3, 1)	-	-	-	-
AM	41	3.8 ± 0.4	16 (11.2, 32)	10	15.7 ± 2.7	4	36.7 ± 2.8

All values are means ± SE; except those for von Frey thresholds which are median and first (Q1) and third (Q3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; DH, D-hair receptors; AM, high threshold mechanosensitive A fibre.

Table.4.3.2 Conduction velocity, von Frey hair thresholds, heat thresholds and cold thresholds of C fibres

Type	n	Conduction velocity, m/s	Von Frey hair threshold, mN	Cold sensitive	Cold threshold, °C	Heat sensitive	Heat threshold, °C
CM	93	0.49 ± 0.02	22.4 (11.2, 32)	41	17.2 ± 1.3	40	37.0 ± 0.6
CLTM	12	0.42 ± 0.02	0.85 (0.5, 1)	7	18.9 ± 3.1	6	34.3 ± 1.9
CC	21	0.51 ± 0.03	-	21	26.9 ± 1.3	0	-

All values are means ± SE; except those for von Frey thresholds which are median and first (Q1) and third (Q3) quartiles. CM, C mechano-sensitive nociceptor; CLTM, C low threshold mechanosensitive fibre; CC, mechanically insensitive cold fibre.

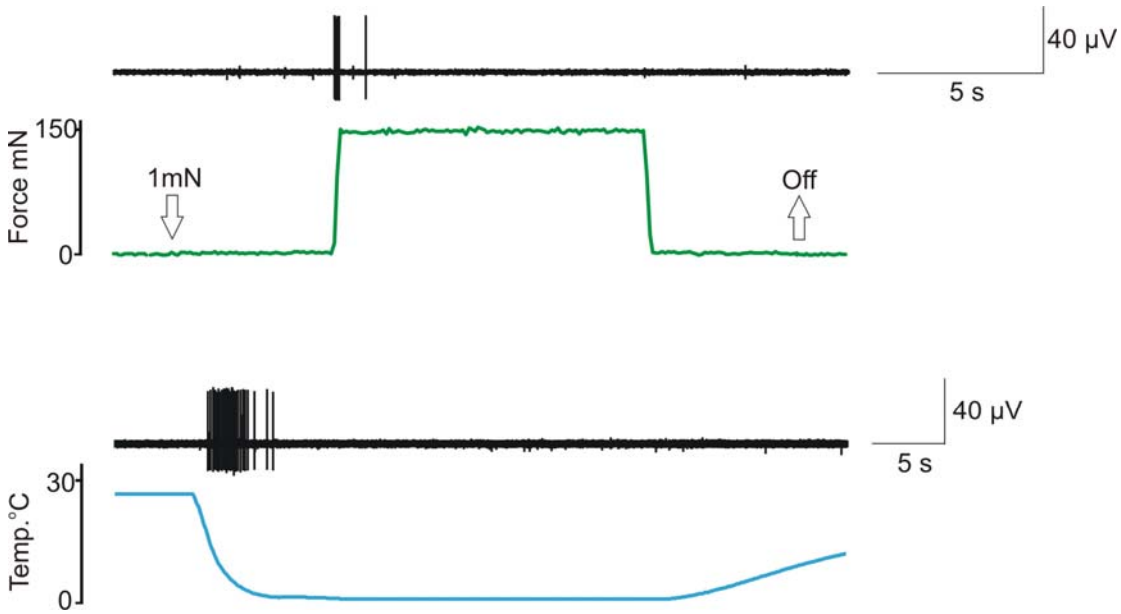

Fig.4.3.1 Example of a rapidly adapting Aβ fibre responding to a mechanical and cold stimulus
The fibre adapted rapidly to a constant force stimulus of 150 mN and went on to respond briefly during the application of cold.

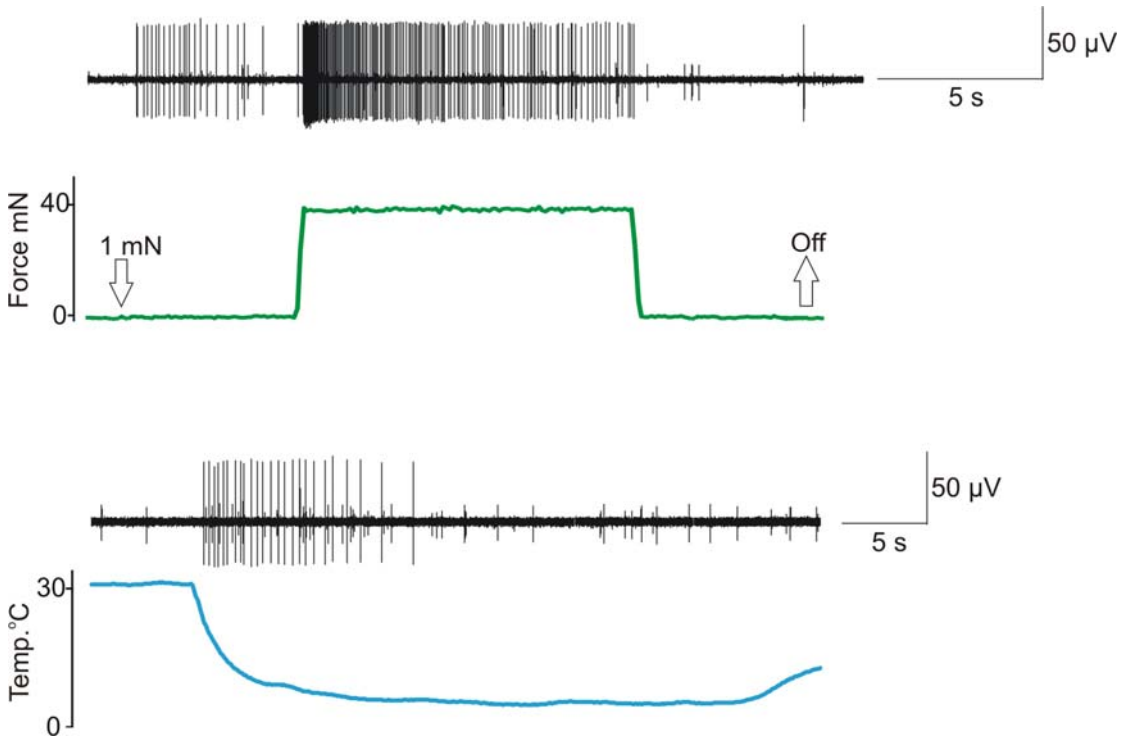

Fig.4.3.2. Example of a slowly adapting Aβ fibre responding to a mechanical and cold stimulus
The fibre adapted slowly during a constant force stimulus of 40 mN. The fibre then responded briefly during a cold stimulus.

TABLE.4.3.3 A fibre sensitivity to TRP channel agonists menthol, mustard oil and capsaicin

Type	Menthol tested, n	Total menthol sensitive, (%)	Mustard oil tested, n	Total Mustard oil sensitive, (%)	Capsaicin tested, n	Total Capsaicin sensitive, (%)	Total Camphor sensitive, (%)
RA	11	0/11, (0)	11	0/11, (0)	11	0/11, (0)	-
SA	20	0/20, (0)	20	0/20,(0)	20	0/20, (0)	0/1, (0)
DH	8	0/8, (0)	8	0/8, (0)	8	0/8, (0)	-
AM	20	0/20, (0)	20	0/20, (0)	20	0/20, (0)	0/1, (0)
AMC	6	5/6, (83)	5	3/5, (60)	5	0/5 (0)	0/1, (0)
AMH	3	0/3 (0)	3	1/3 (33)	3	3/3 (100)	0/3, (0)

All values are given as the proportion of fibres which were responsive to that particular TRP agonist. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; DH, D-hair receptors; AM, high threshold mechanosensitive A fibre. AMC, high threshold mechano-cold A fibre; AMH, high threshold mechano-heat A fibre.

4.3.2. Aδ fibres

A total of 17 D-hair (DH) and 41 high threshold mechanically sensitive A (AM) fibres were recorded. None were initially spontaneously active. None of the DH units was responsive to either cold or heat stimuli. 8 out of 17 DH fibres were tested for their sensitivity to TRP channel agonists. None responded.

10 out of 41 (24 %) AM units responded to cold (AMC, n=9, AMCH, n=1), an example is illustrated in Fig.4.3.3A. The average response threshold to cold was 15.7 ± 2.7 °C, ranging from 7-31.5 °C. Encoding of cold intensity by AMC units was studied by plotting the mean discharge rate as a function of stimulus temperature (Fig.4.3.4). Mean afferent discharge increased during the dynamic phase of the cold ramp, peaking at 1.4 impulses per second at approximately 9 °C. Using a cold search stimulus, no mechanically insensitive thermoreceptors with thin myelinated axons were found.
4 out of 41 (10 %) AM units were responsive to heat (AMH, n=3, AMCH, n=1). The average response threshold to heat, measured on the corium side, was 36.7 ± 2.8 °C. An example of a heat response is shown in Fig.4.3.6A.
28 units (68 %) were neither sensitive to cold nor heat and were therefore classified as thermo-insensitive (AM) units.

In total, 7 AMC units were tested with TRP channel agonists (see Table.4.3.3). From these, 6 were tested with menthol of which, 4 (67 %) responded to the lower concentration (250 μM) while 5 (83 %) units were excited by the higher concentration (2.5 mM). An example of an AMC fibre responding to menthol is shown in Fig.4.3.3B. Plotting the mean discharge to menthol application as a function of time revealed that these fibres were excited in a dose dependant manner (Fig.4.3.5A). The mean response to 2.5 mM menthol application was significantly greater ($p<0.05$, Wilcoxon matched pairs test, n=5) than to 250 μM menthol (Fig.4.3.5B). 5 AMC fibres were tested with mustard oil and capsaicin application. Low levels of activity were observed in 3 out of 5 (60 %) units during the application of mustard oil. During the washout phases, the fibre discharge often increased. During capsaicin application, fibres continued to discharge at the same rate as they

had done in the wash out phase. Based on this observation it was assumed that these fibres were not sensitive to capsaicin.

All the AMH units (n=3) were subjected to TRP channel agonist application (see Table.4.3.3). None responded to menthol. A weak response to mustard oil was seen in 1 unit. All three units (100 %) were excited by capsaicin (an example of an AMH fibre responding to capsaicin application is shown in Fig.4.3.6B).

20 out of 28 AM units were tested for their sensitivity to TRP channel agonists. None were responsive.

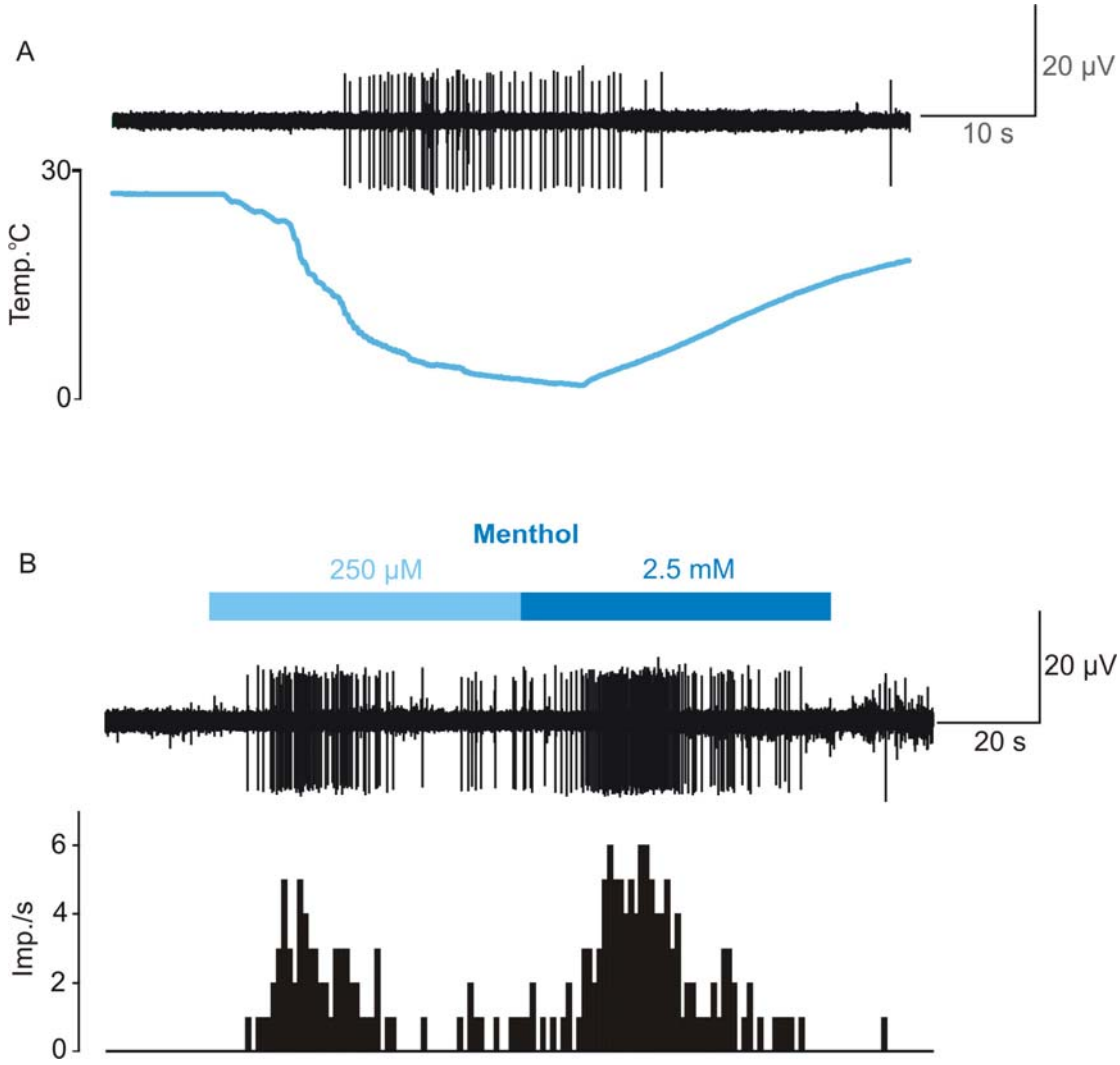

Fig.4.3.3. High threshold mechanically sensitive cold (AMC) fibre
(A) Example of an AMC responding to a 30 second cold stimulus. (B) The same fibre went on to respond to 250 µM and 2.5 mM menthol application (60 s).

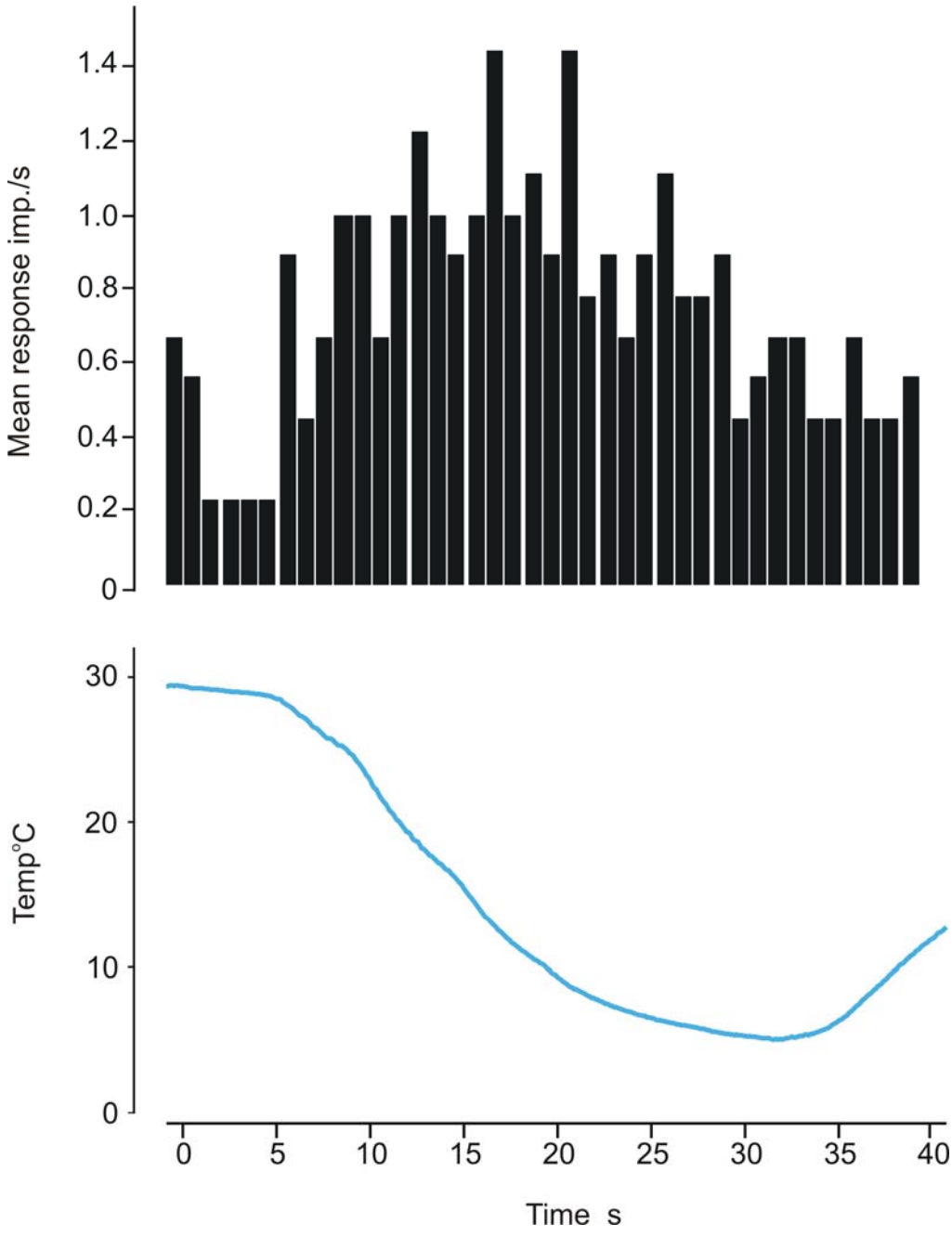

Fig.4.3.4. Average response of high threshold mechano-cold A fibers (AMC) to a 30 s cold stimulus (n=10)

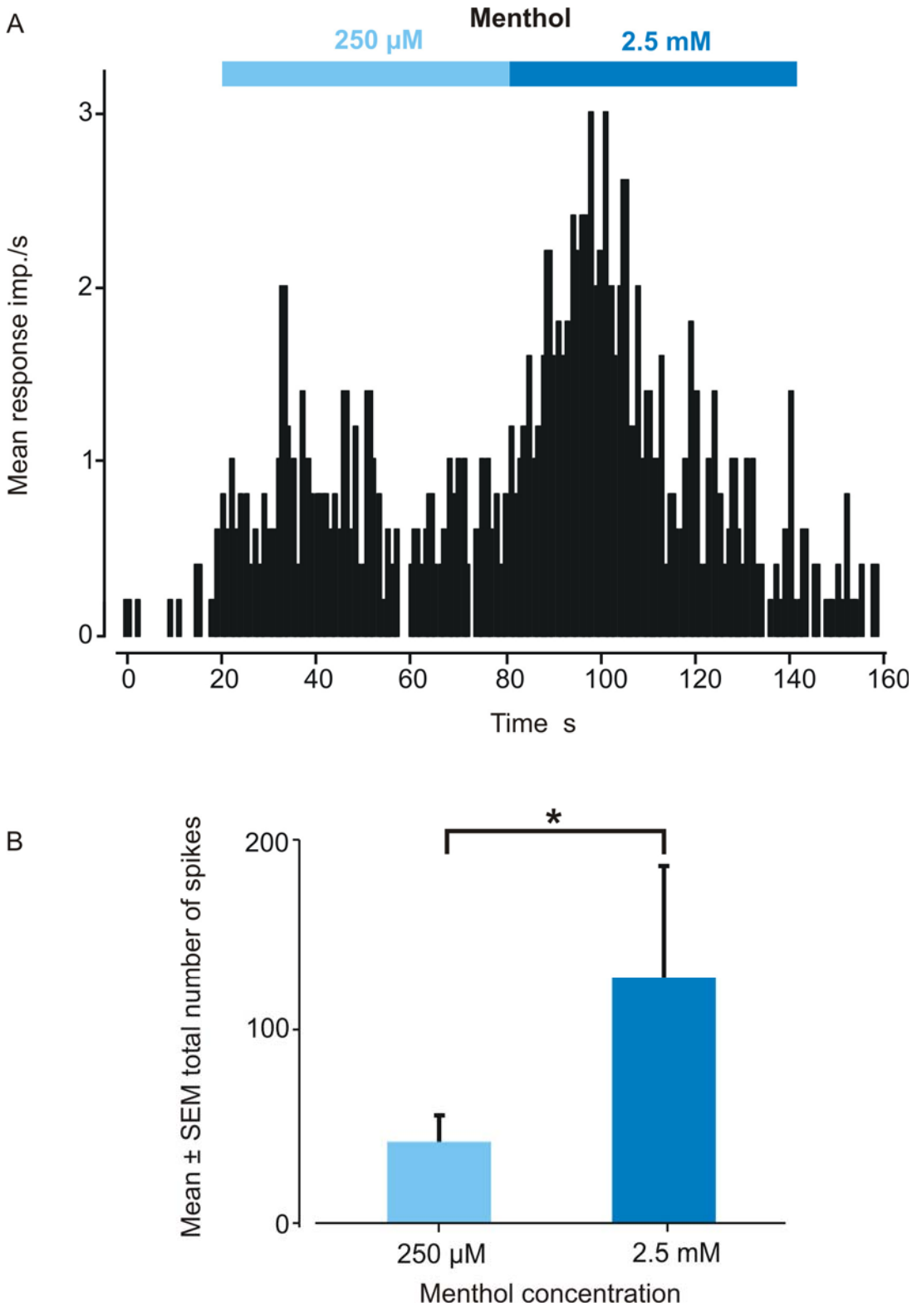

Fig.4.3.5. Average response of AMC fibres to menthol application
(A) The mean response of AMC fibres (n=5) to 60 s application of 250 µM and 2.5 mM menthol is shown. (B) The total response during application of 2.5 mM menthol application was significantly greater ($p<0.05$, Wilcoxon matched pairs test, n=5) compared to 250 µM menthol.

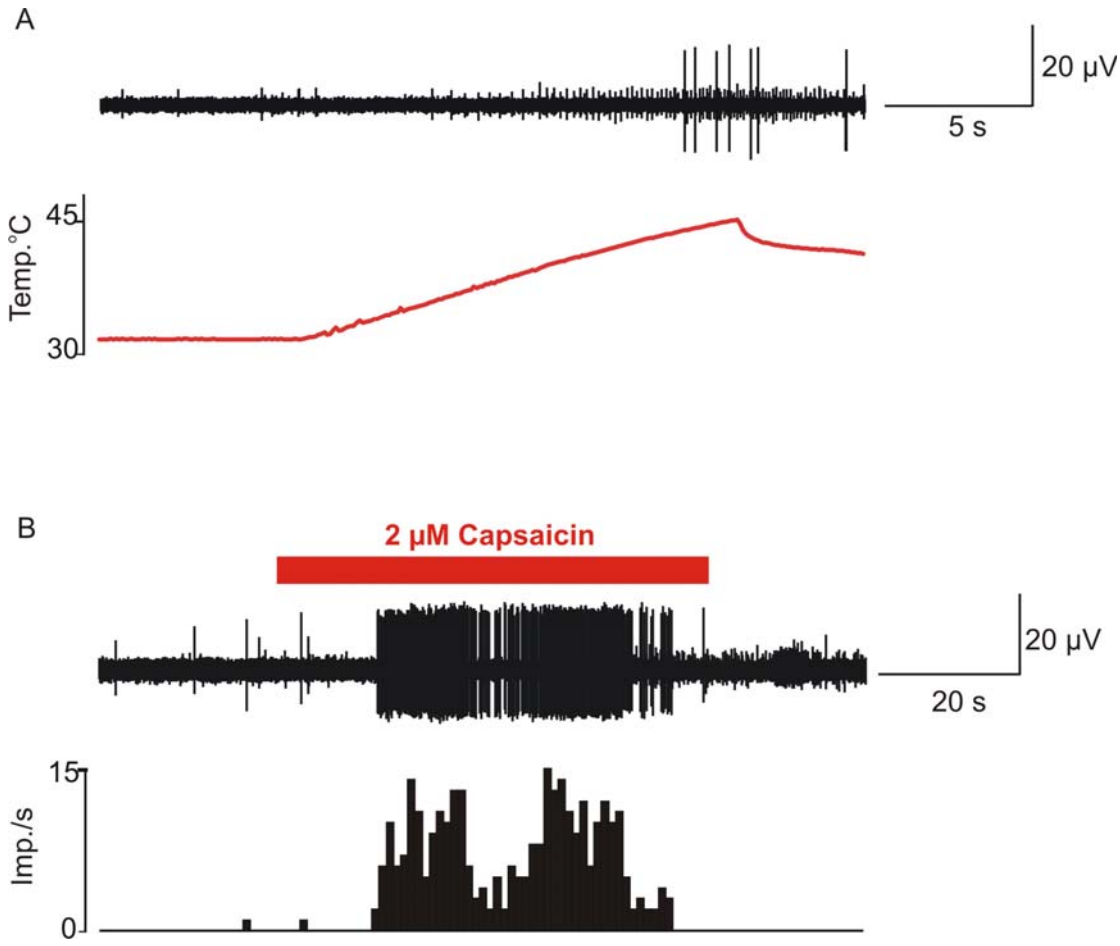

Fig.4.3.6. Example of a mechanically sensitive Aδ heat nociceptor (AMH) *(A) Example of an AMH fibre responding to a 15 s noxious heat stimulus (32-47 °C). (B) The same fibre went on to respond to application of 2 µM Capsaicin (60 s).*

4.3.3. C fibres

A total of 126 unmyelinated C fibres were recorded. Of these, 105 units were mechanically sensitive (CM), while 21 units were mechanically insensitive cold fibres (CC). None of the mechanically sensitive C fibres were initially spontaneously active.

Fibres were classified into 3 main subpopulations as shown in Table.4.3.2. The receptive field of mechano-sensitive fibres was searched by manual probing of the skin using glass rod stimulation. The receptive field of mechano-insensitive cold fibres were first identified by a cold stimulus and subsequently tested with a glass rod stimulus to determine their mechanical sensitivity. Some CMC fibres were also initially identified by the cold stimulus.

Low threshold sensitive mechanosensitive C-fibres (C-LTM)

Overall, 7 out of 12 (58 %) CLTM units responded to a cold stimulus with an average response threshold of 18.9 ± 3.1 °C. 6 out of 12 (50 %) CLTM units responded to heat, with an average response threshold of 34.3 ± 1.9 °C. 4 units responded to cold (33 %) but not to heat (CLTM-C), 3 units (25 %) responded to heat but not to cold (CLTM-H), 3 units (25 %) responded to both cold and heat stimuli (CLTM-CH) and only 2 units (17 %) were thermally insensitive (CLTM). 10 CLTM units (9 which were thermally sensitive and 1 insensitive) were tested with TRP channel agonists. However, none responded (see Table.4.3.4).

TABLE.4.3.4 C fibre sensitivity to TRP channel agonists menthol, mustard oil, capsaicin and camphor

Type	Menthol tested, n	Total menthol sensitive, (%)	Mustard oil tested, n	Total Mustard oil sensitive, (%)	Capsaicin tested, n	Total Capsaicin sensitive, (%)	Camphor tested, n	Total Camphor sensitive, (%)
CM	12	0/12, (0)	12	0/12, (0)	12	0/12, (0)	0	-
CMC	29	27/29, (93)	27	8/27, (30)	27	1/27, (4)	4	0/4, (0)
CMH	17	0/17, (0)	17	5/17, (29)	17	11/17, (65)	5	0/5, (0)
CMCH	8	3/8, (37)	7	2/7, (29)	8	3/8, (37)	1	0/1, (0)
CLTM	1	0/1, (0)	1	0/1, (0)	1	0/1 (0)	1	0/1, (0)
CLTM-C	4	0/4, (0)	4	0/4, (0)	4	0/4, (0)	0	-
CLTM-H	3	0/3, (0)	3	0/3, (0)	3	0/3, (0)	1	0/1, (0)
CLTM-CH	2	0/2, (0)	2	0/2, (0)	2	0/2, (0)	1	0/1, (0)
CC	12	8/12, (67)	12	0/12, (0)	12	0/12, (0)	0	-

All values are given as the proportion of fibres which were responsive to that particular TRP agonist. CM, C mechano-sensitive nociceptor; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM, C low threshold mechanosensitive fibre; CLTM-C, C low-threshold mechanosensitive cold fibre, CLTM-H, C low-threshold mechanosensitive heat fibre; CLTMCH, C low-threshold mechano-sensitive heat-cold fibre; CC, C mechano-insensitive cold fibre.

CM-fibres

93 high threshold mechanically sensitive C fibres (CM) were recorded. 41 out of 93 (44 %) CM fibres responded to cold (CMC) with an average response threshold of 17.2 ± 1.3 °C. 40 out of 93 (43 %) CM fibres responded to heat with an average response threshold of 37.0 ± 0.6 °C (Table.4.3.2).

A total of 20 thermally insensitive C mechano units (CM) were recorded. From these, 12 units were tested with TRP channel agonists and none responded to any of the TRP channel agonists. All but one unit were still mechanically responsive after the chemical stimulation.

Mechano- and heat-sensitive C-fibres (CMH)

A total of 33 CMH fibres were recorded. Their average response threshold to a noxious heat stimulus was 36.6 ± 0.7 °C (an example is shown in Fig.4.3.8A). This did not differ significantly ($p>0.9$, unpaired t-test) from the mean response threshold of AMH fibres (n=4, 36.7 ± 2.8 °C). Encoding of heat intensity by CMH units was studied by plotting the mean discharge rate as a function of stimulus temperature (Fig.4.3.7). As the intensity of the heat stimulus increased, the mean discharge rate also increased. The mean maximal discharge rate was 4.8 impulses per second, at 45 °C. On average, there was a trend of CMH fibres to respond more during a heat stimulus (44 ± 8 spikes, n=33) compared to AMH fibres (20 ± 8 spikes, n=4). However, the difference did not reach statistical significance ($p>0.3$, unpaired t-test).

27 CMH fibres were tested with TRP channel agonists (see Table.4.3.4). Of these 17 units were activated mechanically at the end of the chemical protocol or were excited by the chemicals and have been included in the following analysis.
None of the units were responsive to menthol. 5 out of 17 units (29 %) were excited by mustard oil. 2 units (12 %) weakly responded to the lower concentration (250 µM) of mustard oil, whereas 5 units (29 %) responded to the higher concentration (2.5 mM) of mustard oil. A common finding in these fibres was that during the washout phase following the mustard oil application, there

was a gradual increase in fibre activity, often appearing as a delayed response. Time locked responses, such as the one shown in Fig.4.3.8B were less commonly observed. 11 units (65 %) responded to 2 μM capsaicin (an example is shown in Fig.4.3.8C). The mean response of these fibres to capsaicin application is illustrated in Fig.4.3.9. Within seconds of the application there was an immediate increase in fibre discharge, which peaked at 10 s, followed by a lower rate of sustained discharge for the remaining application period. Fibre activity then gradually decreased during the washout phase following application. On average AMH fibres displayed a greater response to capsaicin (229 ± 51 spikes, n=4) compared to CMH fibres (90 ± 51 spikes, n=11) ($p=0.05$, Mann-Whitney U test).

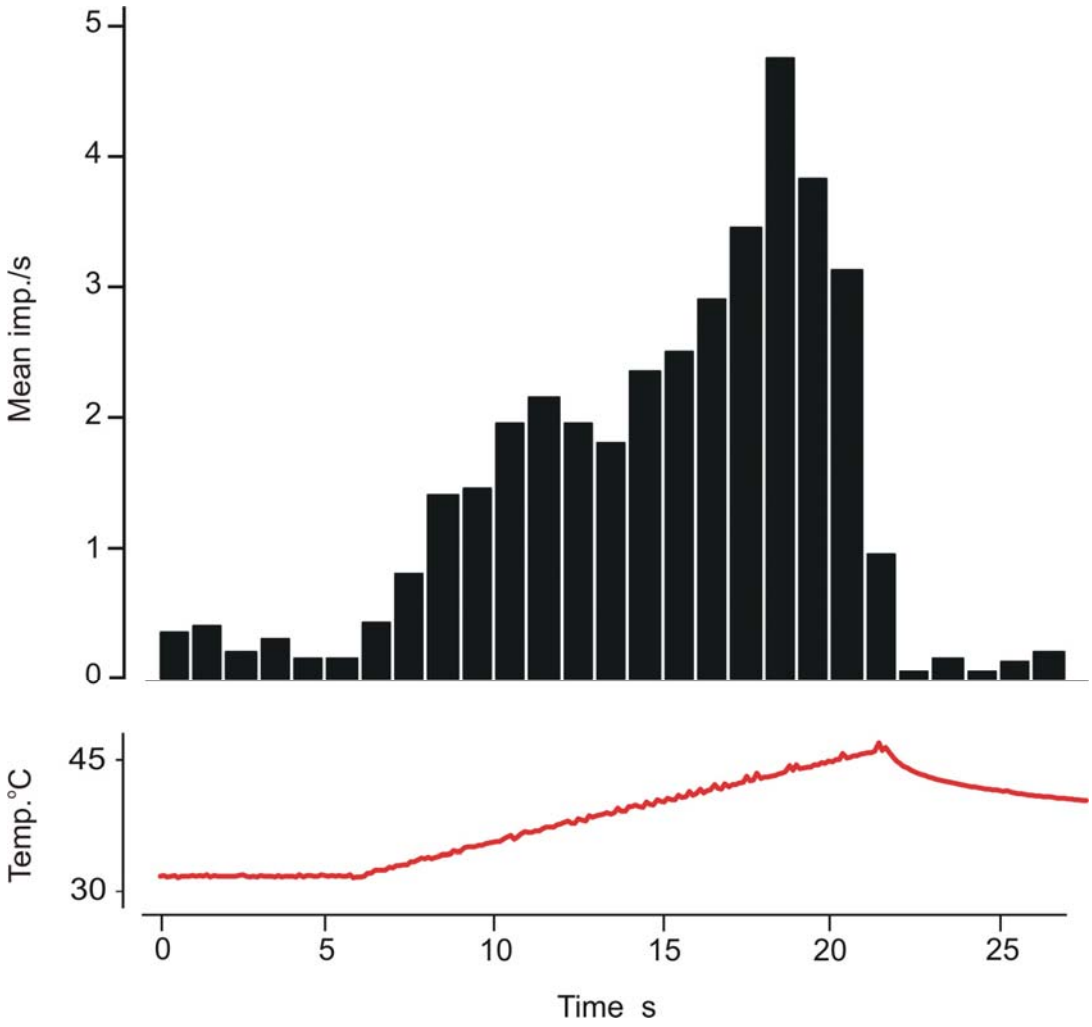

Fig.4.3.7. Average heat response of a population of mechano-heat sensitive C nociceptive fibres (CMH, n=30)

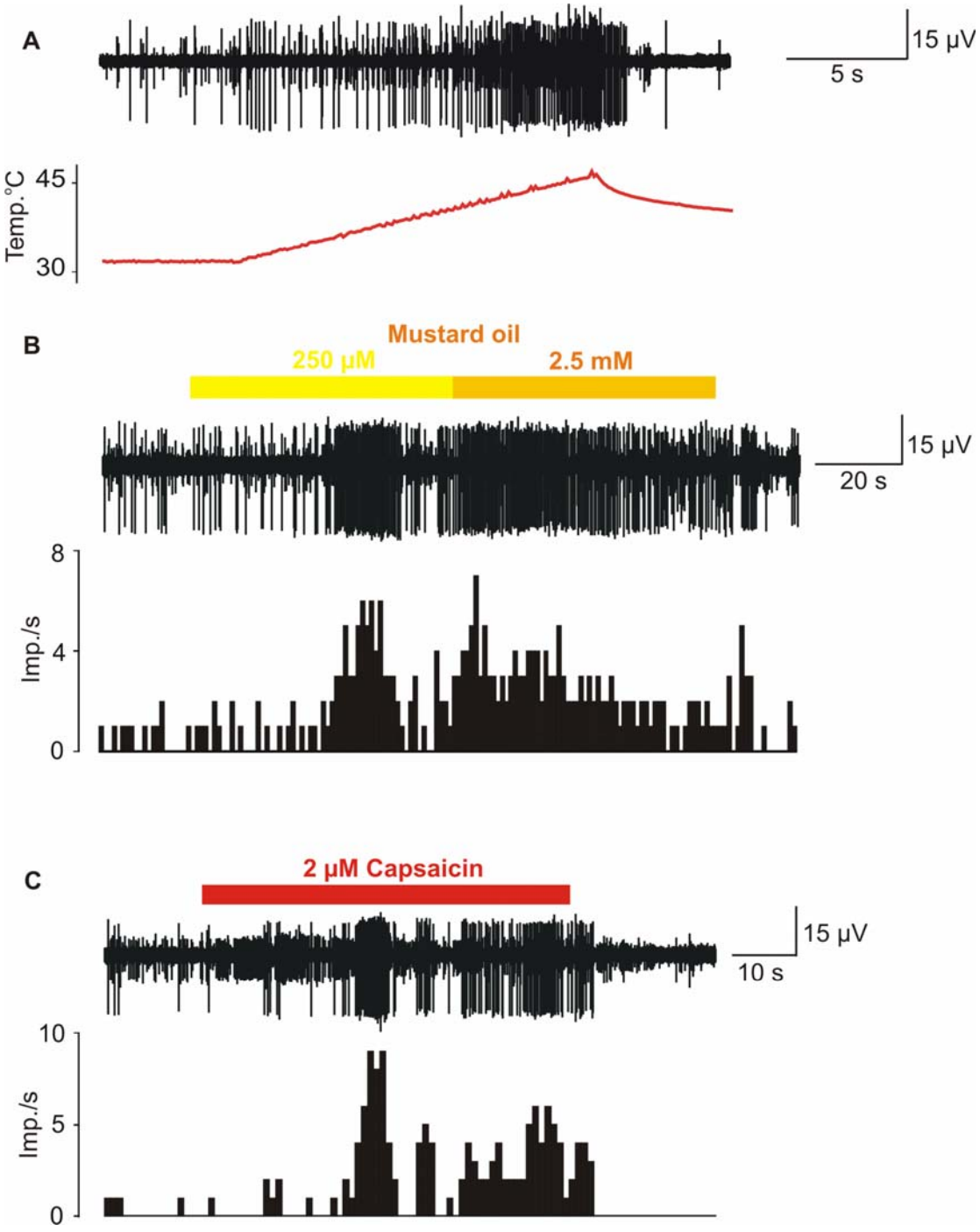

Fig.4.3.8. Example of mechanically heat sensitive C fibre responding to noxious heat and chemical stimuli

(A) Example of a CMH fibre responding to a 15 s noxious heat stimulus (32-47 °C). (B) The same fibre was then recruited by both concentrations of Mustard Oil and (C) 2 µM Capsaicin.

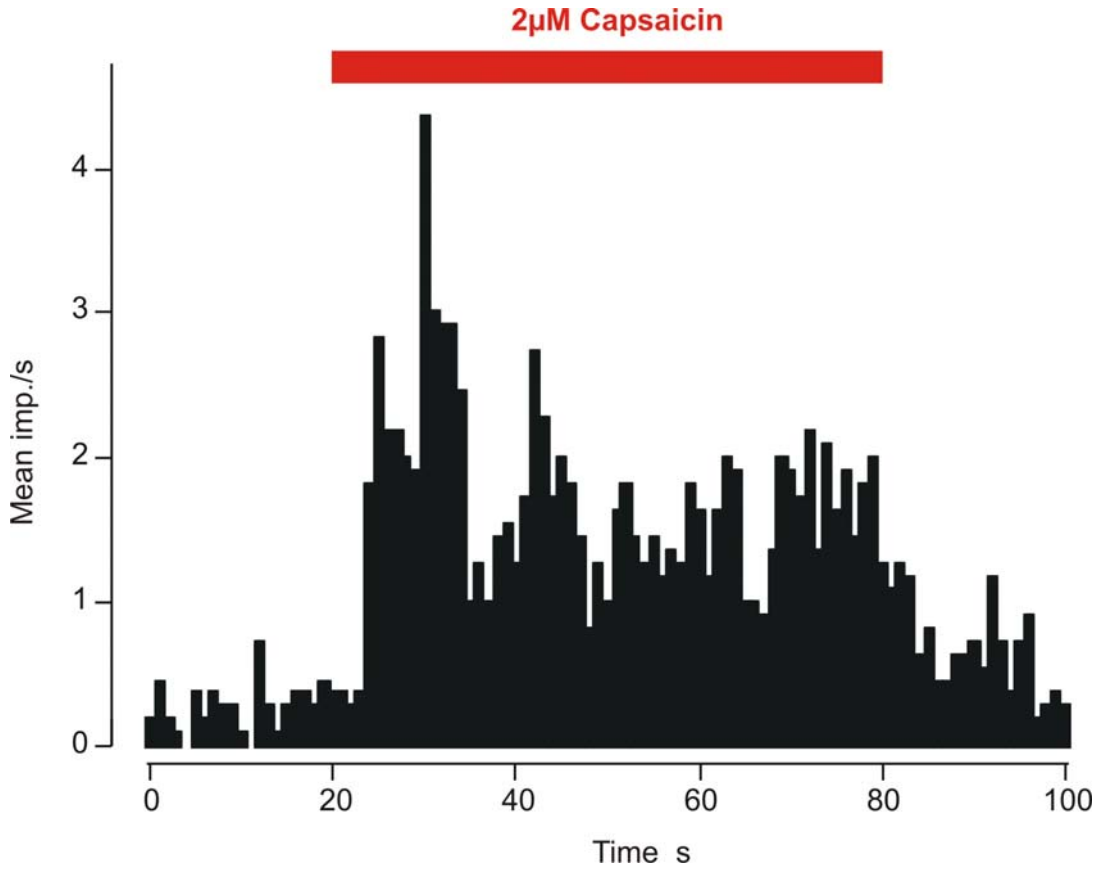

Fig.4.3.9. Average response of CMH fibres to 2 μM Capsaicin application (n=11)

Mechano- and cold-sensitive C-fibres (CMC)

32 CMC fibres were recorded. Their average response threshold to a noxious cold stimulus was 18.3 ± 1.2 °C but was distributed over a wide range of stimulus temperatures (4-29 °C, an example of a CMC fibre responding to cold is shown in Fig.4.3.10A). Interestingly, the mean response threshold to cold did not differ significantly ($p>0.2$, unpaired t-test) from that of AMC fibres (15.7 ± 2.7, n=10). Encoding of cold intensity by CMC units was studied by plotting the mean discharge rate as a function of stimulus temperature (Fig.4.3.11). This revealed that CMC fibres had a similar response pattern to cold as AMC fibres. On average, CMC fibres displayed an increase in firing frequency during the dynamic phase of the cold stimulus, reaching a peak discharge rate of 1.5 impulses per second, at approximately 7 °C. There was no significant difference ($p>0.7$, unpaired t-test) in the mean response to cold between CMC (26 ± 2 spikes, n=32) and AMC fibres (24 ± 2 spikes, n=10).

29 CMC fibres were tested with menthol. 27 were also tested with mustard oil and capsaicin (see Table.4.3.5). 27 out of 29 (93 %) fibres were excited by menthol: 16 (55 %) responded to the lower concentration of menthol, while 25 (86 %) were excited by the higher concentration of menthol. There was no difference in the proportion of menthol sensitivity among CMC (27/29 fibres) and AMC fibres (5/6 fibres) ($p>0.4$, χ^2 test). An example of a CMC fibre responding to a mechanical stimulus, cold stimulus and menthol is illustrated in Fig.4.3.10. Plotting the mean discharge to menthol application as a function of time revealed that these fibres were excited in a dose dependant manner (Fig.4.3.12A). There was a significantly greater response to the application of 2.5 mM menthol ($p<0.01$, paired t-test, n=27) compared to 250 µM menthol (Fig.4.3.12B). There was no significant difference ($p>0.3$, unpaired t-test) in the mean response to application of 250 µM menthol between CMC (n=27) and AMC fibres (n=5). However, AMC fibres were significantly more responsive (n=5) during application of 2.5 mM menthol ($p<0.01$, unpaired t-test) compared to CMC fibres (n=27) (Fig.4.3.17).
3 units (11 %) responded to the lower concentration of mustard oil, while 8 units (30 %) responded to the higher concentration. 1 unit (<4 %) was found to be

responsive to capsaicin. This particular unit was also responsive to menthol and gave a weak response to mustard oil.

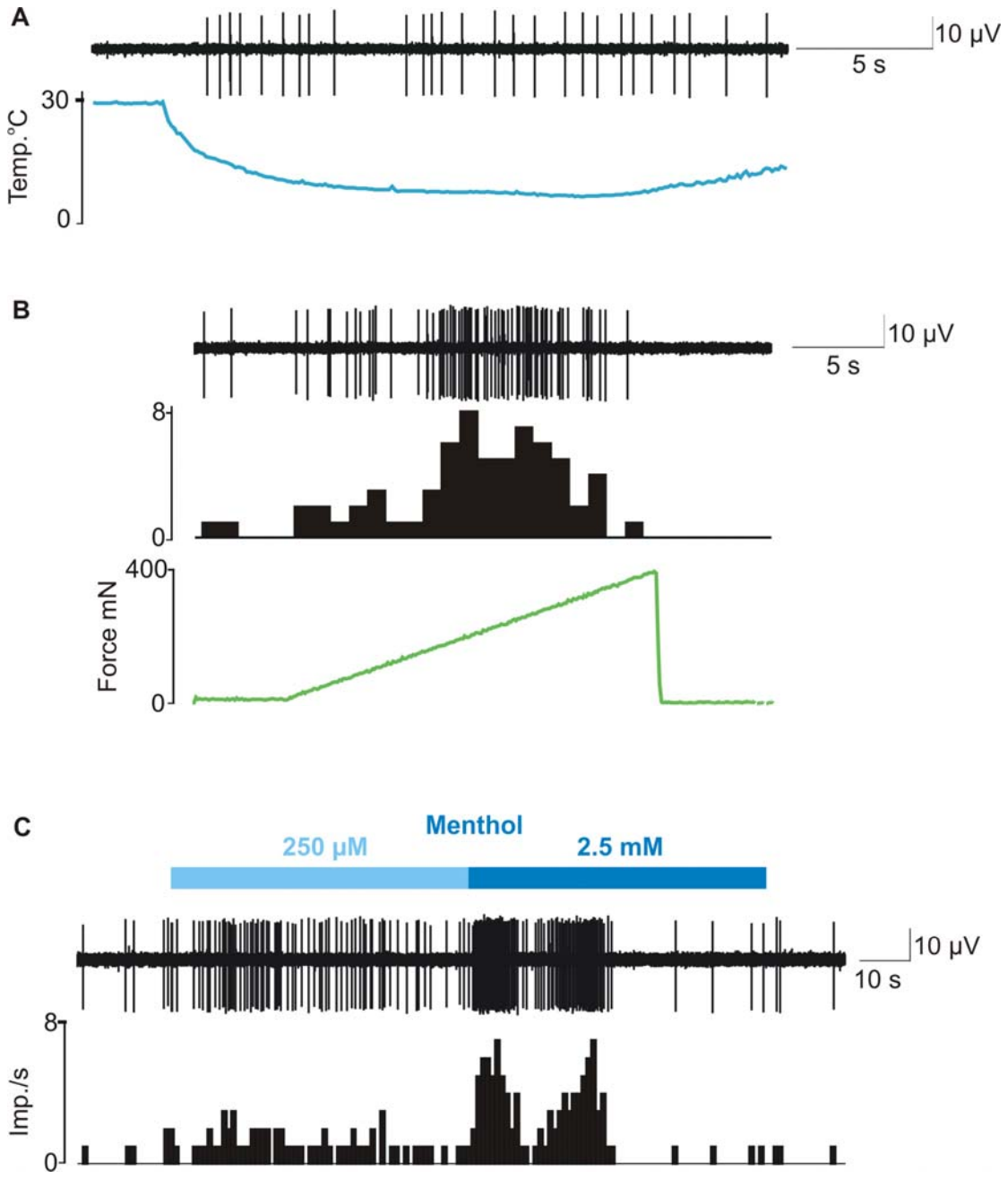

Fig.4.3.10. Example of a C fibre nociceptor (CMC fibre) responding to cold and menthol application

(A) Example of a CMC fibre responding to a cold stimulus. (B) The fibre responded to a 20 s mechanical ramp of 0-400 mN. (C) The fibre later went on to respond in a dose dependent manner to both the low and high concentrations of menthol.

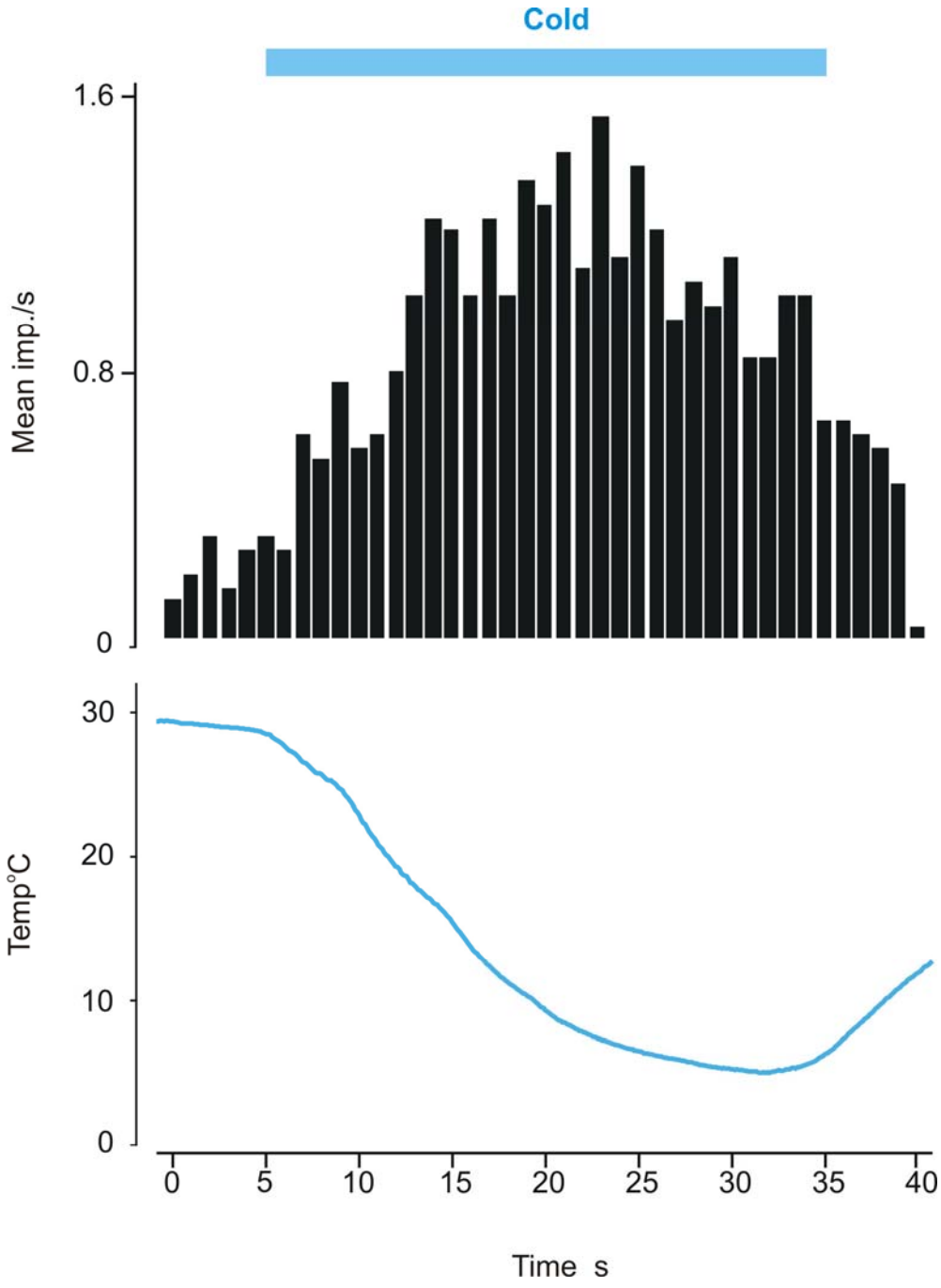

Fig.4.3.11. Average response of a population of CMC fibres to a 30 s cold stimulus (n=27)

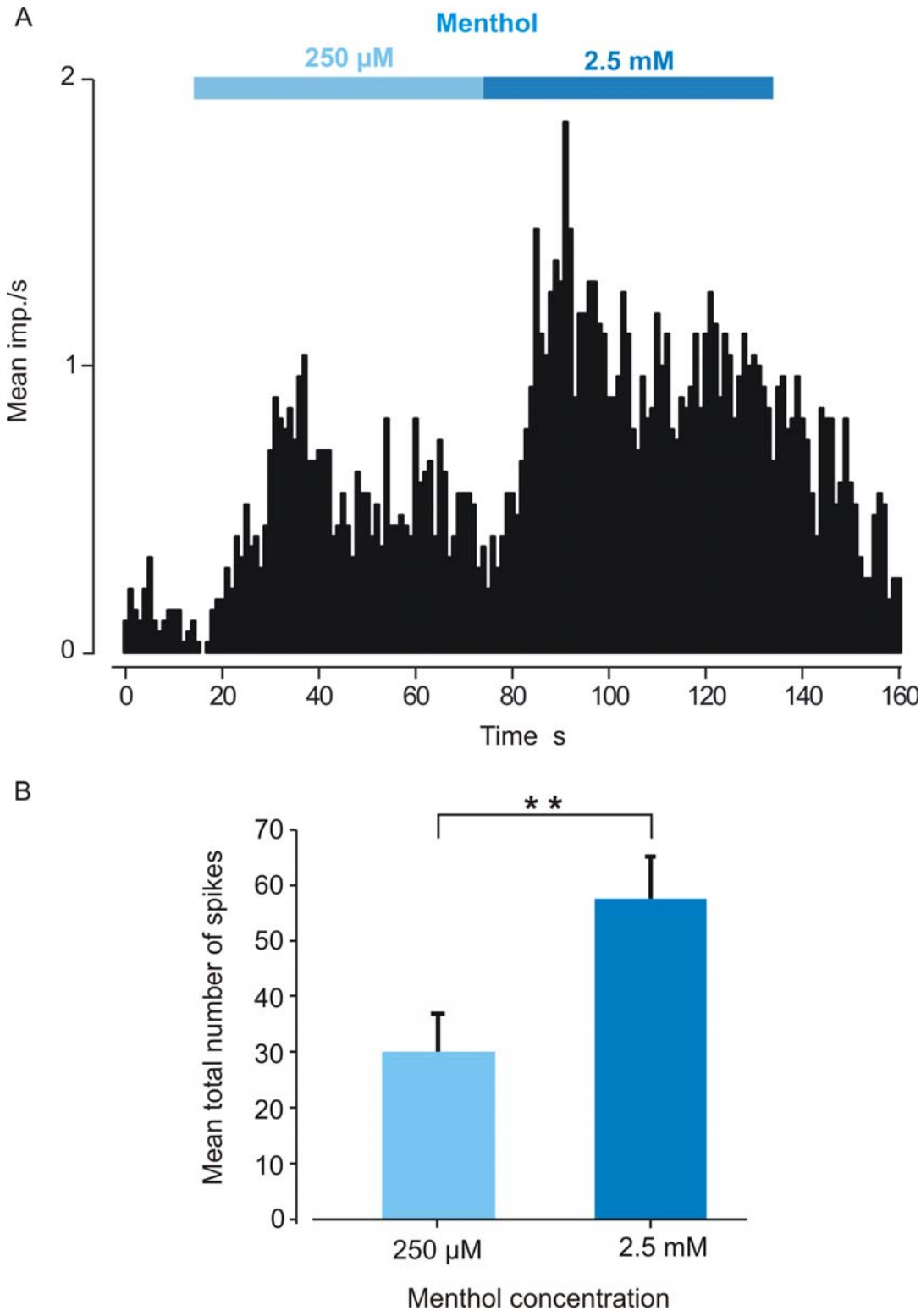

Fig.4.3.12. Average response of CMC fibres to menthol application
(A) Average response of CMC fibres to 250 µM and 2.5 mM menthol (n=27). (B) There was a significantly greater response to the application of 2.5 mM menthol ($p<0.01$, paired t-test, n=27) compared to application of 250 µM menthol.

Mechano- heat- and cold- sensitive C-fibres (CMHC)

8 CMHC fibres were investigated. Their average response threshold to a noxious heat stimulus was 38.6 ± 1.5 °C. Their average response threshold to a noxious cold stimulus was 12.6 ± 2.9 °C, ranging from 2-28 °C.

8 units were tested with menthol and capsaicin. Mustard oil was applied to the receptive field of 7 units. In total 3 units (37 %) responded to menthol. 2 units (25 %) responded to the lower concentration of menthol, while 3 units (37 %) responded to the higher concentration. Of the 7 fibres tested with mustard oil, 2 units (29 %) responded to both the lower and higher concentrations. 3 out of 8 units (37 %) responded to capsaicin application. Two of these units also responded to mustard oil and menthol application.

Cold sensitive mechanically insensitive C fibres (CC)

21 mechano-insensitive cold fibres were investigated in this study. Their general properties are displayed in Table.4.3.2. They were identified as cold specific units on the basis of their activation by innocuous low temperatures and by their insensitivity to mechanical stimuli. 12 of these units displayed ongoing activity at bath temperatures of 32 °C, firing at low frequencies, but usually not in bursts. This steady state activity was suppressed by sudden warming of their receptive fields. 9 units had no initial spontaneous activity and were recruited by a cold stimulus only. In all the cases the discharge frequency increased or a discharge was initiated by cooling the receptive field. Their average response threshold to a cold stimulus was 26.9 ± 1.3 °C ranging from 11.8-34.0 °C. No “paradoxical” heat responses were observed in this study. One unit responded to re-warming after a cold stimulus.

12 units were tested with TRP channel agonist application (see Table.4.3.4). 8 units were excited by the lower concentration of menthol (250 μM), but the higher concentration appeared to decrease activity in many units. Plotting the mean menthol-induced discharge for these eight fibres showed that there was an inverse dose-response relationship (Fig.4.3.13A). The response to 250 μM menthol application was significantly greater ($p<0.01$, Wilcoxon, matched paired test, n=8) compared to the response to 2.5 mM menthol (Fig.4.3.13B).

Interestingly, although CC fibres displayed a significantly greater response to 250 µM menthol application compared to CMC fibres ($p<0.01$, unpaired t-test), the difference was not significantly different from that of AMC fibres ($p>0.1$, unpaired t-test, see Fig.4.3.17). There was no significant difference in response to 2.5 mM menthol between CC fibres and AMC ($p>0.4$, unpaired t-test) and CMC fibres ($p>0.4$, unpaired t-test).

Of the remaining 4 menthol insensitive CC fibres, the ongoing activity of one unit was reversibly inhibited by the high concentration of menthol without excitation at the lower concentration (Fig.4.3.14). Of the three units that were not affected by menthol, one unit behaved atypically and responded to re-warming as well (Fig.4.3.15). None of the 12 units responded to mustard oil or capsaicin.

The cold sensitivity of the fibres was separately analysed for the menthol-sensitive and menthol-insensitive group (Table.4.3.5). The average response threshold of the menthol sensitive group (n=8) to a cold stimulus was 30.0 ± 0.9 $^{\circ}$C, ranging from 27-33.4 $^{\circ}$C. The average change in temperature from a base line temperature required to elicit a response to cold was 1.7 ± 0.5 $^{\circ}$C. On the other hand, the average response threshold of the menthol insensitive group (n=4) to a cold stimulus was 18.9 ± 2.4 $^{\circ}$C, ranging from 11.8-22.8 $^{\circ}$C which was highly significantly different from menthol sensitive fibres (n=8) ($p< 0.001$, unpaired t-test). The average change in temperature from a base line temperature required to elicit a response to cold was 11.8 ± 3.4 $^{\circ}$C, much higher then the change required for the menthol sensitive group. In fact the threshold of the menthol-insensitive cold receptors did not differ from that of CMC- ($p >0.9$, unpaired t-test, n=32) or AMC-units ($p>0.6$, unpaired t-test, n=10). Encoding of cold intensity by both groups of cold units was studied by plotting the mean discharge rate as a function of stimulus temperature (Fig.4.3.16). This revealed that both groups had a different firing pattern and thresholds: the menthol-sensitive group showed an immediate peak discharge when temperature was lowered followed by a lower but sustained discharge throughout the rest of the stimulus. By contrast the peak discharge of the menthol-insensitive group required stronger intensities and was followed by a lower level of activity. Although there was a trend for menthol-sensitive fibres discharging more during a cold stimulus the mean response during the cold

ramp between the menthol insensitive group (149 ± 82, n=4) and the menthol sensitive group (268 ± 150, n=8) was not significantly different ($p>0.1$, unpaired t-test).

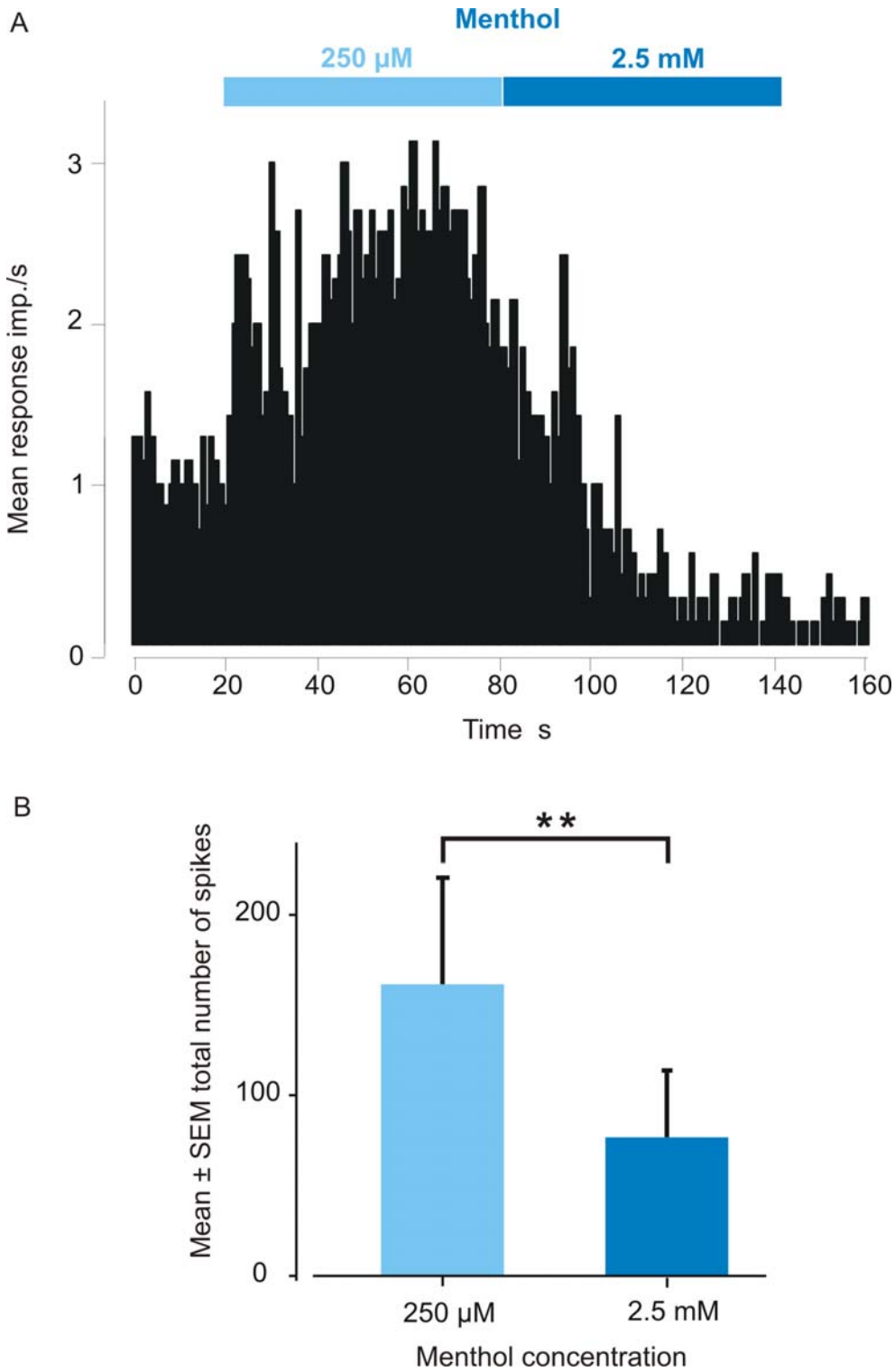

Fig.4.3.13 Average response of cold thermoreceptors to menthol application

(A) Average response of mechanically insensitive cold units (CC) to 250 µM and 2.5 mM menthol (n=8). (B) CC fibres were significantly more responsive during application of 250 µM menthol ($p<0.01$, Wilcoxon, matched paired test, n=8) compared to 2.5 mM menthol.

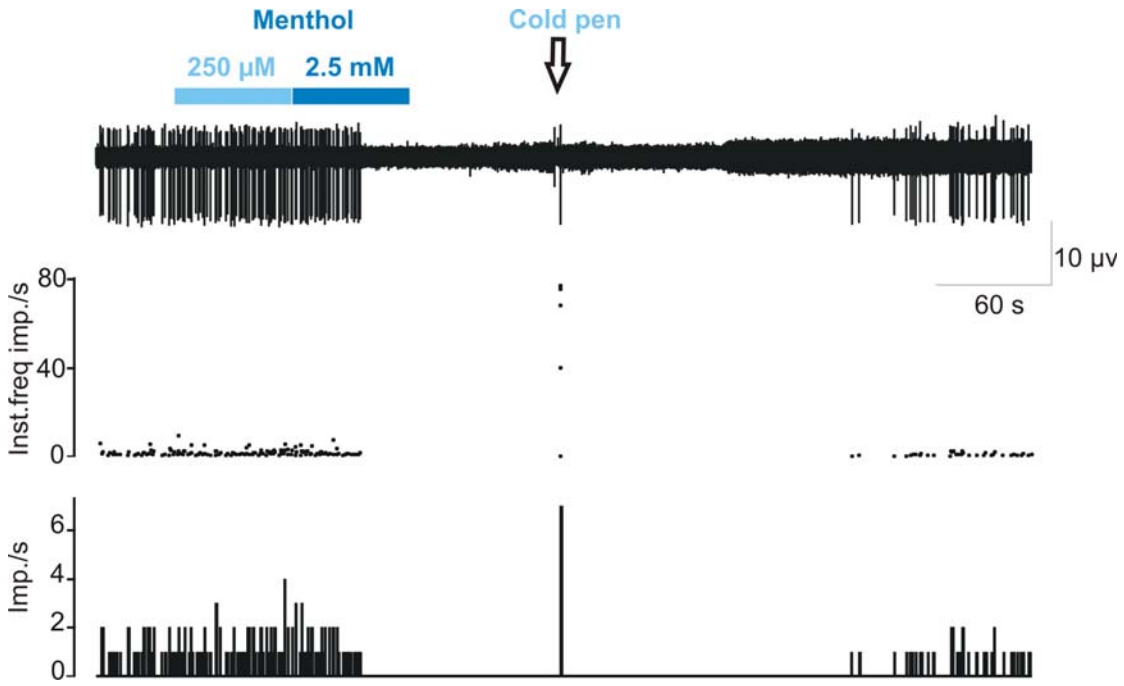

Fig.4.3.14. Example of a cold thermoreceptor during application of menthol

The application of the higher concentration of menthol appeared to abolish all spontaneous activity. The fibre then responded to a cold stimulus in the washout phase. Spontaneous activity resumed approximately after 4 minutes.

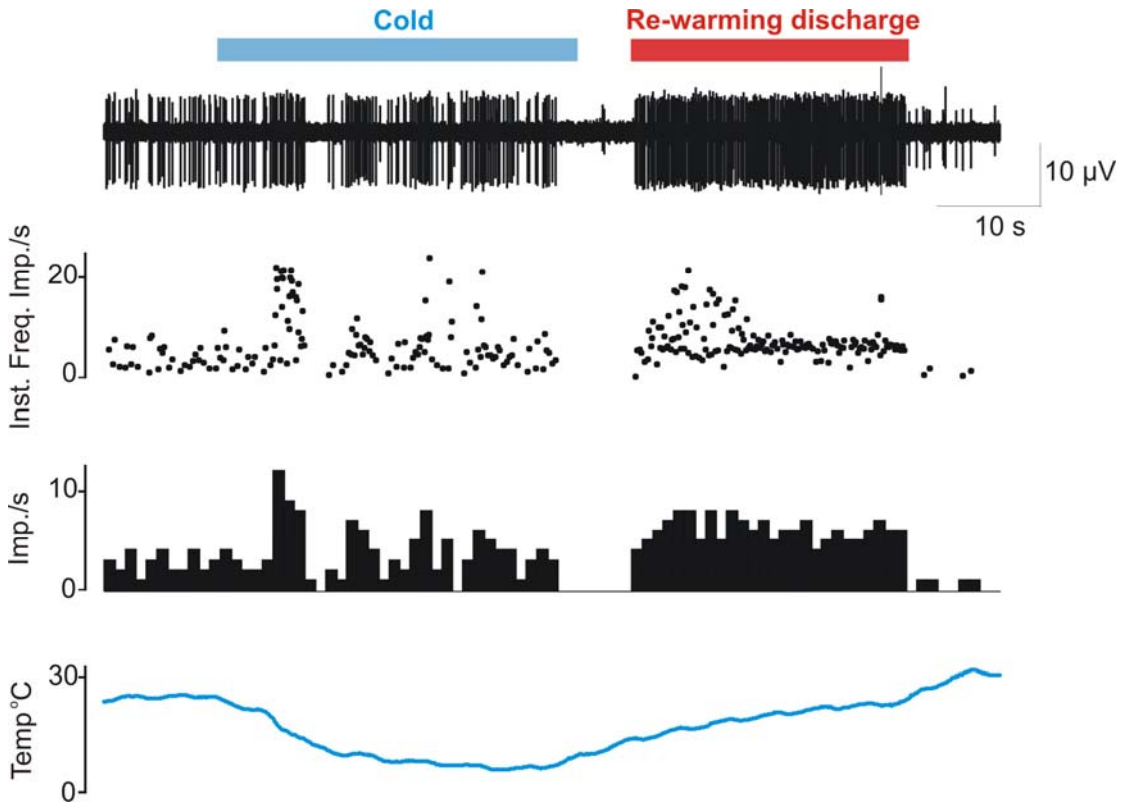

Fig.4.3.15. Example of an atypical cold thermoreceptor which displayed a re-warming discharge during the passive re-warming phase after a cold stimulus

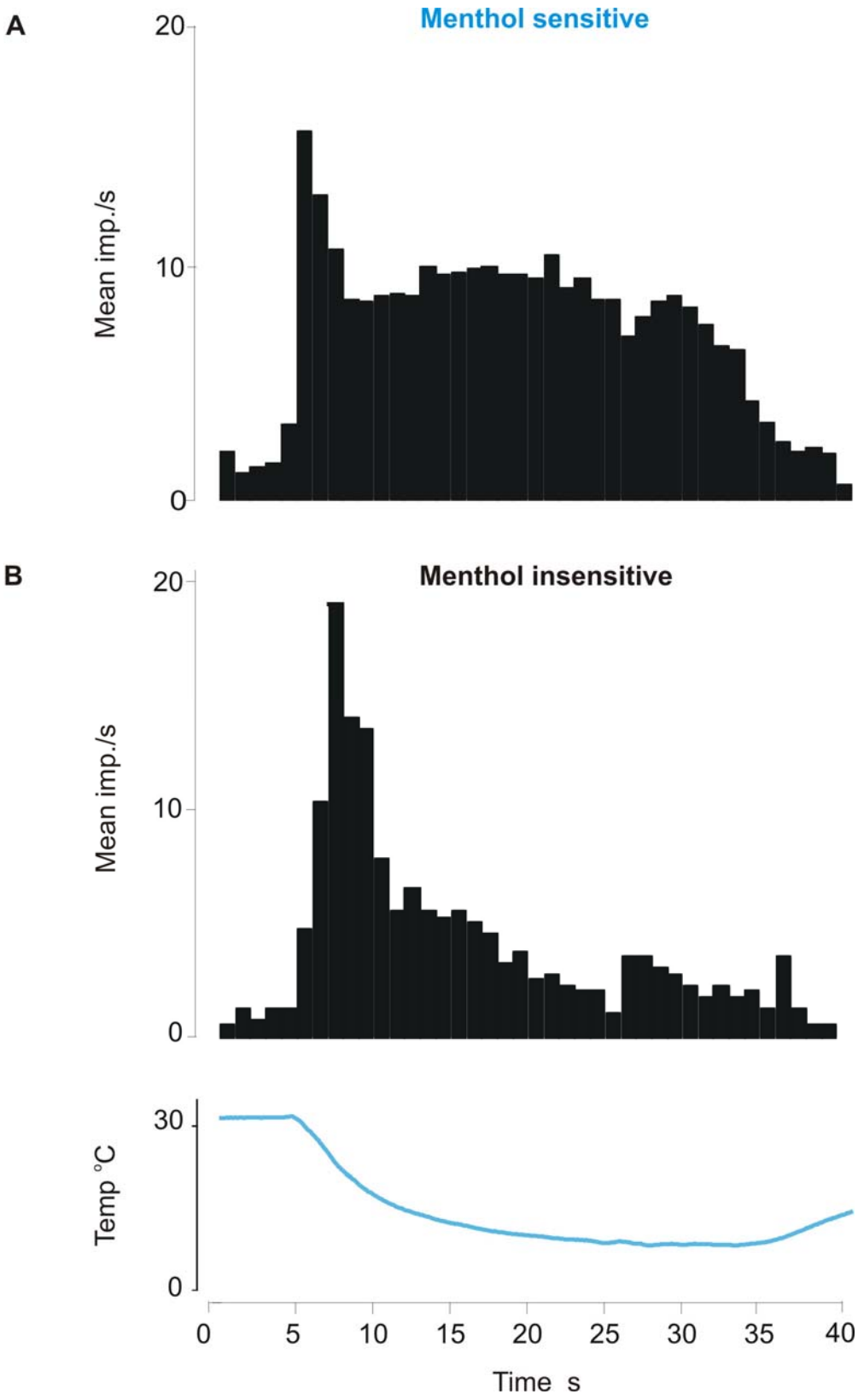

Fig.4.3.16. Two different populations of cold thermoreceptors
(A) The mean response to a cold stimulus of cold thermoreceptors which responded to menthol (n=8) and (B) which were menthol insensitive (n=4) is shown.

TABLE.4.3.5 Conduction velocity, cold threshold and Δ

Type	n	Conduction velocity, m/s	Cold threshold, °C	Δ, °C
Menthol +ve, CC	8	0.47 ± 0.05	30.0 ± 0.9	1.7 ± 0.5
Menthol –ve, CC	4	0.55 ± 0.03	18.9 ± 2.4	11.8 ± 3.4

Values are means ± SE. Menthol +ve CC, menthol responsive mechano-insensitive cold fibres; Menthol –ve CC, menthol insensitive mechano-insensitive cold fibres; Δ, relative change in temperature required to elicit a cold response from base line temperatures.

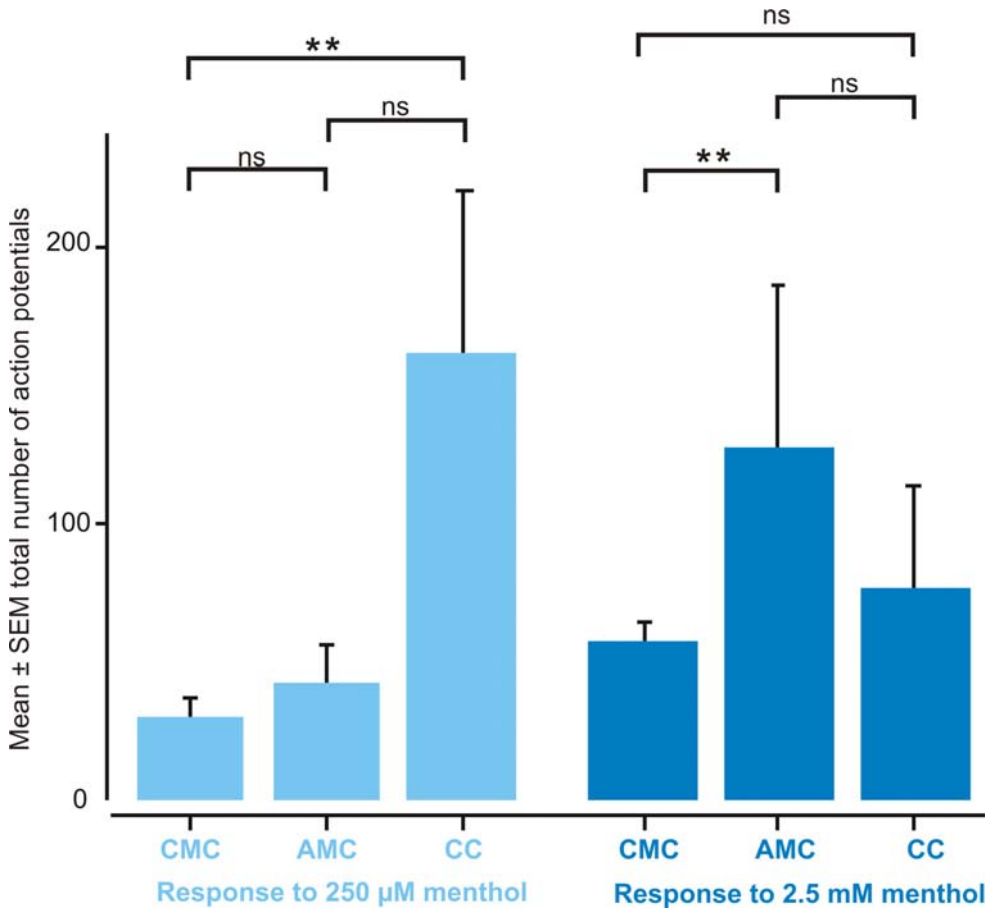

Fig.4.3.17 Comparison of mean menthol responses between AMC, CMC and CC fibres

On average, AMC fibres were significantly more responsive (n=5) to 2.5 mM menthol ($p<0.01$, unpaired t-test) compared to CMC fibres (n=27).
CC fibres displayed a significantly greater response to 250 µM menthol application compared to CMC fibres ($p<0.01$, unpaired t-test). Ns,not significant.

4.4. Discussion

Electrophysiological in vivo and in vitro studies of rodent receptive properties of myelinated or unmyelinated cutaneous afferent fibres have given an insight into the response properties of different sub-populations of fibres (Cain et al., 2001;Koltzenburg et al., 1997;Kress et al., 1992;Leem et al., 1993;Lynn and Carpenter, 1982).

Responses to mechanical and heat stimuli have been well documented and more recently a few studies have also investigated responses to cold stimuli (Cain et al., 2001;Koltzenburg et al., 1997;Simone and Kajander, 1997;Simone and Kajander, 1996).

In addition to these advances the recent molecular cloning of the cold activated channels TRPM8 and TRPA1 have given a further insight into the molecular and cellular mechanisms of cold transduction.

However, no study to date has systematically investigated the sensitivity of sub-population of primary afferent to agonists of all thermosensitive TRP channels that are expressed by sensory neurons. This is the first detailed investigation which has attempted to correlate the thermosensitivity of cutaneous primary afferents with TRP channel agonist sensitivity in vitro, in rat hairy skin.

4.4.1. The majority of low threshold mechano-sensitive A fibres do not respond to thermal stimuli or TRP channel agonists

Thermal responses were absent among the majority of large myelinated Aβ afferents, with the exception of one rapidly adapting (RA) and three slowly adapting (SA) fibres which responded during a cold stimulus. This finding is consistent with previous reports, which have also found some cold sensitivity among SA mechanoreceptors, but no evidence of encoding cold temperatures (Hensel, 1974;Konietzny, 1984;Leem et al., 1993;Simone and Kajander, 1997;Spray, 1986).

Even though some of these fibres respond to thermal stimuli, they have been regarded as irrelevant to the sensation of cold (Hensel, 1974;Konietzny, 1984;Spray, 1986).

Thermal responses were also absent among thin myelinated DH receptors.

Sensitivity to TRP channel agonists were absent among RA, SA and DH receptors, suggesting that these sub-populations do not express TRPM8, TRPA1 and TRPV1. This finding is in agreement with molecular studies which have investigated the expression patterns of TRP channels in relation to the size of the cell body (Peier et al., 2002a;Story et al., 2003). In a recent study, Kobayashi et al (2005) reported that mRNA for TRPV1, TRPA1 and TRPM8 in rat DRG were expressed very rarely on large neurons, suggesting that these TRP channels were not expressed on large myelinated neurons. Here, we have directly shown that this is the case.

The low prevalence of cold sensitivity and the lack of heat and chemo-sensitivity among these subpopulations of low-threshold mechanoreceptors are in support for the view that they contribute little to cold-induced sensations, at least in non-pathological conditions.

However, the presence of cold sensitivity among afferents not responding to TRP channel agonists, indicate the presence of as yet un-discovered cold sensitive TRP channels or TRP-independent cold transduction mechanisms. TRP-independent mechanisms of cold sensitivity shall be discussed in Chapter 6 and 7.

4.4.2. Thermo-insensitive high threshold mechanically sensitive A and C fibre nociceptors in the rat are insensitive to TRP channel agonists

Both thermally insensitive high threshold mechano-sensitive A (AM) and C fibres (CM) are considered to have nociceptive functions. This is due to the fact that both populations have higher mechanical thresholds than low-threshold mechanically sensitive fibres and encode the stimulus intensity. Insensitivity to TRP channel agonists among these groups of fibres, suggest that they do not express these TRP channels. This finding is in agreement with a previous study in which no capsaicin sensitivity was found among 18 AM and 3 CM units in the rat skin (Szolcsanyi et al., 1988).

However, our findings contrast with those of Schmidt et al (1995), in which C fibres were recorded from the human peroneal nerve. They found that topical application of 100 % (10 M) mustard oil for 3-5 minutes activated 5 out of 14

(36 %) of CM units. Whereas in principle species differences could account for this discrepancy there are other explanations:
Our study used much lower concentrations of mustard oil (250 μM and 2.5 mM). It is possible that these concentrations were sub-threshold and therefore failed to activate any CM units. It is also possible that application for 1 minute was not long enough a duration to activate any units. The concentration used in the human study is, however, extremely high and in this concentration range is no longer a “neurogenic” stimulus (i.e. the inflammatory action is not dependent on nerve fibres in this concentration). In fact application of high concentrations of mustard oil in humans has been shown to lead to tissue damage (Magerl et al., 1996) and therefore the C fibre activation could be indirect.
However, in vivo fewer C-fibres can be classified as CMC compared to in vitro recordings (Kress et al., 1992).This is probably due the much less effective cold gradients that can be applied in vivo compared to in vitro. At least some of the rat fibres classified as CM in vivo appear to have cold sensitivity in vitro.

4.4.3. Low threshold-mechano C fibres (CLTM) are TRP agonist insensitive in the rat

CLTM fibres are extremely sensitive to mechanical stimuli. As found in this study some of these fibres are also thermo-sensitive. However, the lack of TRP channel agonist sensitivity in these fibres, suggest that their thermal responses are not mediated by any of these channels.
We only used agonists specific for TRPM8, TRPA1 and TRPV1, so therefore cannot discard the possibility of involvement from other TRP channels.
Since TRPM8 and TRPA1 are so far the only TRP channels thought to be involved in the transduction of cold, lack of sensitivity for agonists specific for these channels suggest that cold responses in CLTM fibres are mediated by other mechanisms. Possible mechanisms include the role of other ion channels and pumps which have been described previously (Askwith et al., 2001;Maingret et al., 2000;Spray, 1986;Viana et al., 2002) and shall be discussed further in Chapter 6.

4.4.4. Heat responsive A and C fibre nociceptors are capsaicin sensitive. Some are also mustard oil sensitive

In the present study 100 % of AMH, 65 % of CMH and 43 % of CMCH were activated by 2 μM capsaicin application. This is in agreement with previous studies which have investigated the effect of capsaicin on A or C fibre nociceptors in rat in vivo and in monkey (Ringkamp et al., 2001;Szolcsanyi et al., 1988). This finding is also consistent with molecular studies which report TRPV1 expression within sensory ganglia in a subset of medium and small sized neurons (Caterina et al., 1997;Kobayashi et al., 2005).
However, it is important to note that these sub-populations of neurons (AMH, CMH and CMCH) are not the only capsaicin sensitive populations.
In this study, the majority of units were located using a mechanical search technique and this is known to introduce a sampling bias. Using an unbiased electrical search technique, Schmidt et al (1995) have shown that in an unbiased sample of C fibres from the human peroneal nerve, 6 % were purely heat responsive (CH) and 24 % were neither responsive to heat nor mechanical stimuli (CM_iH_i).
In the rat hairy skin, up to 26 % of fibres recorded in vivo and 15 % studied in vitro were unresponsive to both mechanical and thermal stimuli (Kress et al., 1992). However, a proportion of these fibres were likely to be postganglionic sympathetic efferents. In sympathectomised animals the percentage of mechanically insensitive afferents was in the order of 10%. Furthermore CH units made up less than 3% of C-fibres whose receptive field had been identified with an unbiased electrical search stimulus.
Capsaicin responses in A fibre nociceptors lacking either heat sensitivity or mechanical sensitivity have been shown in the monkey (Ringkamp et al., 2001), and in fact the largest capsaicin responses were observed in afferents that were insensitive to both heat and mechanical stimuli; these afferents could be classified as chemosensitive specific.
Therefore we can say that in the rat AMH, CMH and CMCH are subpopulations of mechanically sensitive nociceptors which are capsaicin sensitive and other mechanically and heat insensitive afferents probably exist that are also sensitivity to capsaicin.

In the present study, 33 % of AMH, 29 % of CMH and 33 % of CMCH were also activated by mustard oil application. Application of 100 % (10 M) and 10% (1M) mustard oil has been shown to excite 8 out of 9 (89 %) and 2 out of 5 (40 %) of CMH fibres in vivo from the rat saphenous nerve, respectively (Reeh et al., 1986) and up to 58 % of CMH fibres from the human peroneal nerve (Schmidt et al., 1995). Calcium imaging of cultured neurons show that a large proportion of mustard oil sensitive neurons are also sensitive to capsaicin (Bautista et al., 2005;Story et al., 2003).

In the present study, most of the mustard oil responses appeared with a delay of up to 1 minute from time of application. This may correspond to the mustard oil responses from cultured DRG neurons observed by Munns et al (2007). Calcium imaging of DRG neurons showed that some neurons displayed a gradual increase and decrease in calcium levels in response to mustard oil. Responses varied in terms of onset and magnitude (Munns et al., 2007).

Once again it should be noted that these sub-populations (AMH, CMH and CMCH) are not the only mustard oil responsive neurons. One such subpopulation includes the CMC nociceptors, which will be discussed below. Mechanically insensitive C fibres, CM_i are another such population (Schmidt et al., 1995). Application of 10 % cinnamaldehyde (agonist of TRPA1) on the forearm of human subjects has been shown to evoke mild ongoing pain and a small axon reflex flare vasodilation (Namer et al., 2007;Namer et al., 2005). This neurogenic inflammation is thought to be mediated by CM_i afferents. Both studies therefore indicate that TRPA1 is also expressed on CM_i afferents. Since TRPA1 is thought to have a role in noxious cold mediation, it would be interesting to know whether CM_i units respond to cold. This remains unknown at the present.

From the delayed responses evoked by mustard oil in the present study and the fact that mustard oil has been shown to directly activate mechanically insensitive CM_i afferents, it is possible that the role of TRPA1 is important in states of tissue injury involving inflammation.

Although many mustard oil responsive AMH and CMH fibres are initially cold insensitive, they may acquire cold sensitivity in states of tissue injury. Interestingly, in vitro single unit recordings revealed a higher proportion of CMCH fibres in rats with a spinal nerve ligation (SNL) injury compared to naïve

rats (Ji et al., 2007) although overall there was no significant difference in the proportion of cold sensitive C fibres between SNL and naïve rats.

Obata et al (2005) and Katsura et al (2006) reported that expression of TRPA1 levels, but not TRPM8 were increased in a rat model of nerve injury and inflammation induced by complete Freund's adjuvant (CFA) (Katsura et al., 2006;Obata et al., 2005). Intrathecal administration of TRPA1 anti-sense oligodeoxynucleotide was able to suppress inflammation and nerve injury induced cold hyperalgesia in rats, suggesting that the development of cold hyperalgesia is dependent on TRPA1 in DRG neurons. However, these results are in contrast to those of Caspani et al (2007) who found a decrease in the number of DRG neurons expressing TRPA1 after chronic constriction injury of the sciatic nerve in mice and no change in the number of cold responsive neurons (Caspani et al., 2007). Discrepancies in the expression of TRPA1 after nerve injury may be dependent on the injury model used.

Bradykinin, an inflammatory mediator, was shown to activate 78 % of cinnamaldehyde responsive DRG neurons, (Bandell et al., 2004). Calcium imaging of trigeminal neurons in culture revealed that 97 % of bradykinin sensitive neurons were also sensitive to mustard oil (Bautista et al., 2006b). Results from both studies suggest that TRPA1 expressing neurons also express bradykinin receptors. The bradykinin receptor (B2), similar to TRPA1, has been shown to be expressed in a subpopulation of capsaicin sensitive neurons, raising the possibility that TRPV1 acts with TRPA1 to activate nociceptors in response to bradykinin. Bautista et al (2006) have shown that this is the case. They found that in mice lacking TRPA1, bradykinin induced thermal hypersensitivity was absent, suggesting that TRPA1 is a downstream target for bradykinin. They propose that bradykinin induced activation of the PLC pathway in turn leads to activation of protein kinase C which results in the release of intracellular calcium stores. This sensitises and opens TRPV1, resulting in further calcium influx. The intracellular influx of calcium then leads to opening of TRPA1 (Bautista et al., 2006). In this way, TRPA1 is proposed to have an important nociceptive function, whereby it depolarises nociceptors in response to inflammatory mediators.

4.4.5. Cold sensitive nociceptors are menthol sensitive. Some of these neurons are also sensitive to mustard oil

A cold stimulus from 32 to 4 °C excited 22 % of Aδ nociceptors, which is similar to the finding by Simone et al (1997) who reported that 30 % of Aδ nociceptors responded to cold when stimuli of 0 °C were used. However, when stimuli below 0 °C are used, a much higher proportion of Aδ nociceptors are excited (Cain et al., 2001;Simone and Kajander, 1997;Simone and Kajander, 1996). Differences in the proportion of Aδ nociceptors excited by cold, appears to be dependent on the cold stimulus used. A cold stimulus below 0 °C is likely to freeze the skin and may result in non-specific activation of fibres.

In the present study 83 % of AMC and 93 % of CMC were excited by menthol application. Figures.4.3.5 and 4.3.12 demonstrate that both subpopulations of fibres responded in a dose-dependent manner, exhibiting higher rates of firing to the higher dose of menthol, thereby encoding the stimulus.
This is the first study which has directly shown that in the rat, cold sensitive nociceptors conducting in both the A and C fibre range are excited by menthol. Evidence for the contribution of TRPM8 in nociception has been mainly implicated from psychophysical experiments carried out on humans involving menthol application (Namer et al., 2005;Wasner et al., 2004). In both of these studies, topical application of 40 % (4 M) menthol induced a low level of ongoing pain sensation in 8 out of 10 and 7 out of 10 subjects, respectively. Wasner et al (2004) went on to demonstrate that during a differential A fibre block, topical menthol induced a burning pain sensation. Interestingly, reported sensations of coldness were significantly decreased. These findings suggested that menthol activated C fibre nociceptors mediated the burning pain. Our findings are consistent with these findings and in addition we have directly shown that it is the subpopulation of cold responsive A and C fibre nociceptors which respond in a dose dependent manner to menthol.
Further evidence in support of TRPM8's role in nociception has come from Dhaka et al (2007) and Colburn et al (2007). Injection of icilin (a synthetic super cooling agent), an activator of TRPM8, into the hind paw of wild type mice, results in rapid hind-paw withdrawal when mice are placed on a 1 °C cold-plate.

Dhaka and colleagues demonstrate that this behaviour is completely absent in mice lacking TRPM8, therefore suggesting that TRPM8 activation can result in a nociceptive like response (Dhaka et al., 2007). Colburn et al (2007) showed that acetone application to the hind paw of TRPM8 wild type mice resulted in nociceptive behaviour which included brief lifting, licking or shaking of the paw. In contrast, mice lacking TRPM8 displayed no such response, suggesting a role of TRPM8 in the transmission of cold pain. Following chronic constriction injury (CCI) to the sciatic nerve, sensitivity to acetone application was observed in the TRPM8 wild type mice, but not in mice lacking TRPM8, suggesting that TRPM8 is required for the hypersensitivity induced by nerve injury (Colburn et al., 2007).
Menthol application (40 %) does not induce an axon-flare reaction in humans suggesting that mechanically insensitive afferents do not express TRPM8 (Namer et al., 2005;Wasner et al., 2004).
Our findings are also in agreement with molecular studies which state that TRPM8 expression is restricted to neurons of small to medium diameter (Kobayashi et al., 2005;Peier et al., 2002a) and not expressed in heavily myelinated neurons marked by phosphorylated neurofilament heavy chain (Peier et al., 2002a).

60 % of AMC and 28 % of CMC units were activated by mustard oil. Very low levels of co-expression between TRPM8 and TRPA1 mRNA in rat DRG have been reported (Kobayashi et al., 2005). Munns et al (2007) found that within cold sensitive cultured mice DRG neurons, only 5 % responded to both menthol and mustard oil (Munns et al., 2007) suggesting that a small minority of neurons express both TRPM8 and TRPA1. Therefore our fibres which were responsive to both menthol and mustard oil may represent the same small proportion of units which are present in dissociated DRG neurons of the rodent.

The observation that many nociceptive A or C-fibres are not excited by TRP channel agonists but by subfreezing temperature stimuli, indicates that TRP channel are not involved in the transduction of these low temperatures. Dhaka et al (2007) demonstrated that TRPM8 is not involved in sensing very low noxious cold temperatures. When wild-type and TRPM8 deficient mice were

placed on a 1 °C cold plate, both genotypes showed similar nociceptive behaviours with similar withdrawal latencies (Dhaka et al., 2007). This is in agreement with Colburn et al (2007) who found that mice lacking TRPM8 responded vigorously when placed on a 0 °C cold plate, implying the existence of another receptor for the detection of very low temperatures (Colburn et al., 2007). Although heterologously expressed TRPA1 channels have been shown to be activated by noxious cold temperatures (Story et al., 2003), results from two independent studies on TRPA1 deficient mice have led to different conclusions on the role of TRPA1 in sensing noxious cold. Kwan et al (2006) placed wild-type and mice lacking TRPA1 on 0 °C cold plates and demonstrated that mice lacking TRPA1 responded with significantly less paw withdrawals than wild-type mice. In contrast Bautista et al (2006) found that when wild-type and mice lacking TRPA1 were allowed to chose between surfaces adjusted to room temperature versus 15 °C or 10 °C, all animals spent >98 % of the time on the room-temperature side, irrespective of genotype. Therefore the role of TRPA1 in cold sensing remains unclear. Interestingly, a third of cold sensitive neurons in the mouse DRG were found to be insensitive to both menthol and mustard oil, implying a TRPM8 and TRPA1 independent mechanism for the transduction of cold (Munns et al., 2007). The generation of a TRPM8/TRPA1 double knockout mouse will be useful in identifying TRP independent mechanisms of cold sensitivity.

4.4.6. CMCH fibre nociceptors may represent a class of menthol and capsaicin sensitive neurons

CMCH fibres represent a class of nociceptor that are responsive to both noxious cold and heat stimuli, a small number of which were studied in the present study. It is thought that up to a third of CMH fibres in rat hairy skin (Koltzenburg, 2004) and up to 40 % in humans (Campero et al., 1996) recorded in vivo are responsive to noxious cold, i.e. they are CMHC fibres. The proportion in the current sample is smaller and previous studies using unbiased electrical search stimuli have shown that only approximately 10 % of the CMH fibres also cold sensitive (Kress et al., 1992).

43 % of CMCH units were excited by menthol, 33 % responded to mustard oil and 43 % responded to capsaicin application, two of which were also menthol and mustard oil sensitive.

The TRP channel agonist responses in these afferents illustrate that they are capable of responding to menthol, mustard oil and capsaicin, suggesting that they may express all three TRP channels.

In the present study, of the 3 CMCH units that responded to capsaicin, 2 also responded to menthol. This class of nociceptor may represent a subpopulation that expresses both TRPM8 and TRPV1.

However, there have been conflicting molecular findings associated with co-expression of TRPM8 and TRPV1, some of which have reported no or little co-expression (Kobayashi et al., 2005;Peier et al., 2002a;Story et al., 2003) and some studies which have observed co-expression (Okazawa et al., 2004;Viana et al., 2002). Calcium imaging studies have shown that TRPM8-expressing neurons are 5-10 % of the population at large and that 30-50 % of these neurons are capsaicin sensitive. This means that approximately 1-5 % of all neurons express both TRP channels. The total number of TRPV1-positive neurons is an order of magnitude higher. This corresponds well to the 1:10 ratio of cold sensitivity found in TRPV1-expressing heat sensitive CMH fibres. Interestingly a recent study found that 24 % of mice DRG neurons which expressed a genetically encoded axonal tracer of TRPM8 also expressed TRPV1 (Takashima et al., 2007) suggesting co-expression of TRPM8 and TRPV1 in a subset of neurons.

Interestingly it has been shown that that in culture conditions without nerve growth factor (NGF) none of the menthol sensitive cold neurons were activated by capsaicin (Story et al., 2003). However, with NGF, 50 % of menthol sensitive neurons showed a response to capsaicin. This difference in co-expression of TRPM8 and TRPV1 may therefore be dependent on culture conditions. These results suggest that in states of inflammation, high levels of NGF may lead to co-expression of TRMP8 and TRPV1 on fibres which usually just express TRPM8 and this may contribute to thermal hypersensitivity during injury (Anand, 2003).

4.4.7. Cold thermoreceptors are only activated by low concentrations of menthol

21 specific cold fibres were recorded in the present study. The receptive fields of these fibres were identified using a cold search stimulus. This revealed that not all cold specific afferents displayed ongoing activity at the bath temperature of 32 °C. Many previous investigators have not used a cold stimulus as their search strategy, recording only from fibres displaying on-going activity at the ambient temperature (in vivo recordings or organ bath in vitro). If different populations of cold thermoreceptors exist (which shall be discussed below), this may introduce a bias into which sub-population of cold fibre is recorded from. Konietzny (1984) reported the existence of different populations of specific cold receptors in humans which had peak firing frequencies at different static temperatures. Campero et al (2001) found that in their sample of human cold units all displayed a resting discharge at a room temperature of 21 °C.
In this study, fibres that did display ongoing activity displayed no burst discharges and all had conduction velocities in the C fibre range (<1 m/s). These findings are in agreement with findings from human cold fibres (Campero et al., 2001;Konietzny, 1984). However studies in monkey have found that cold fibres conduct in the Aδ range (Darian-Smith et al., 1973;Dubner et al., 1975;Long, 1977). Human cold sensations are thought to be mediated mainly by Aδ fibres, as shown by studies using differential nerve blocks (Torebjork and Hallin, 1973). If this was the case, impulses from C cold fibres would not elicit sensations of cold. However, it has been suggested that cold fibres may not be uniformly myelinated throughout their entire length, so that distally they conduct in the C fibre range, and more proximally they conduct in the Aδ range (Campero et al., 2001).
Low concentrations of menthol have been shown to increase the rate of cold fibre activity (Jiang et al., 2002;Schäfer et al., 1986). Hensel and Zotterman (1951) found that low concentrations of menthol sensitized cold receptors, so that they would respond to higher temperatures (Hensel, 1981).
In the present study, application of 250 μM menthol increased the rate of firing in 80 % of such cold units. In 2 units, no change in fibre activity was observed. We found that application of 2.5 mM decreased firing rate in all cold fibres (n=8)

which had previously been activated by the lower concentration. This has not been observed before. None of the units responded to mustard oil or capsaicin. This suggests that these units express TRPM8 but do not express TRPA1 or TRPV1. The fact that high concentrations of menthol decreases discharge is reminiscent of the view that menthol may desensitize TRPM8 expressing cold fibres, similar to the desensitizing effect of high capsaicin concentration on CMH units.

Dhaka et al (2007) demonstrated that placing mice with formalin induced inflammation on 17 °C cold plates reduced the behavioural nociceptive response. Mice lacking TRPM8 did not display this cold induced analgesia (Dhaka et al., 2007), suggesting that TRPM8 mediates the effects of cold analgesia. The cooling effect of menthol may be mediated by the activation of menthol sensitive cold fibres recorded in this study.

When cold units were grouped in terms of their menthol sensitivity, the menthol insensitive group (n=4) had a higher (colder) cold threshold of 18.9 ± 2.4 °C while the threshold was much lower (warmer) for the menthol sensitive group (n=8). Both groups also showed different firing pattern during a cooling ramp (Fig. 4.3.16). From these observations we propose that their may be two sub-populations of cold fibres: (i) one which has a higher cold threshold and are menthol insensitive and (ii) another group that has a lower cold threshold and are menthol sensitive.

The hypothesis for the existence of more than one sub-population of cold fibre is not a new concept. As briefly mentioned already, Konietzny (1984) reported two different populations of cold thermoreceptors in humans: one which displayed peak frequencies at static temperatures between 20 and 27 °C and those which showed increasing activity down to 10 °C.

LaMotte and Thalhammer (1982) similarly reported the existence of mechanically insensitive, “high-threshold” cutaneous cold receptors in the monkey. These afferents displayed no-ongoing activity and were only activated at temperatures below 27 °C and were not excited by mechanical stimuli. It is possible that the menthol insensitive thermoreceptors with thresholds below 23 °C which were recorded in this study correspond to this class of high threshold cold sensitive thermoreceptors.

Studies on cultured neurons have also shown that there are cold sensitive neurons that are insensitive to menthol and have lower activation thresholds (Nealen et al., 2003).

4.4.8. Conclusions

In conclusion, cold responses were not found in rapidly adapting A fibres that innervate hair follicles.
Some cold sensitivity was found among slowly adapting A fibres and low threshold-mechano C fibres (CLTM). However, cold responses in these fibres do not appear to be mediated by either TRPM8 or TRPA1, since they were insensitive to their respective agonists, menthol and mustard oil. Interestingly heat sensitive CLTM fibres were also not responsive to capsaicin.
We have shown for the first time that menthol in the micromolar and millimolar range is directly able to activate Aδ and C fibre nociceptors which also respond to cold, in a dose dependent manner, suggesting a nociceptive role for TRPM8. On the other hand, menthol is only able to activate innocuous cool C fibres in the micromolar range and higher concentrations appear to suppress their activity, suggesting that TRPM8 also has a role in signalling innocuous cool sensations. A summary diagram representing the TRP channel expression on the peripheral nerve endings of the different fibre types is shown in Fig. 4.4.1 below.
We propose that the “cooling” affect of menthol is therefore mediated by the activation of cold specific fibres and the “burning” quality of menthol is mediated by the activation of Aδ and C cold nociceptors that respond to the higher concentrations. At these concentrations the activity of cold specific thermoreceptors is suppressed and therefore the inhibitory action of cold inputs in the CNS (Craig and Bushnell, 1994) appears to be diminished.

The role of TRPA1 in transducing noxious cold still remains unclear. The results of the present study have shown that TRPA1 expression appears to be on nociceptors which respond to heat (AMH and CMH fibres), cold (AMC and CMC fibres) and those which respond to both noxious heat and cold (CMCH fibres). Since both cold and heat activated fibres express TRPA1 it suggests

that it is not specifically activated by noxious cold. Furthermore, in nearly all the cases, fibres responded to mustard oil in a delayed manner, suggestive either of an indirect action of mustard oil or another mechanism of TRPA1 activation. In this study a high proportion of all cold sensitive nociceptors (Aδ and C) and thermoreceptors were activated by menthol application, but not during application of mustard oil. Therefore it suggests that TRPM8 and not TRPA1 is important for the transduction of cold.

The next results chapter (chapter 5) investigates the role of the TRPV2 receptor, which has been been shown to be activated by strong, noxious heat in heterologous expression systems. However, the role of this ion channel in the native system currently remains unknown. For the first time, the receptive properties of afferents innervating the hairy and glabrous skin from mice lacking the TRPV2 receptor will be investigated.

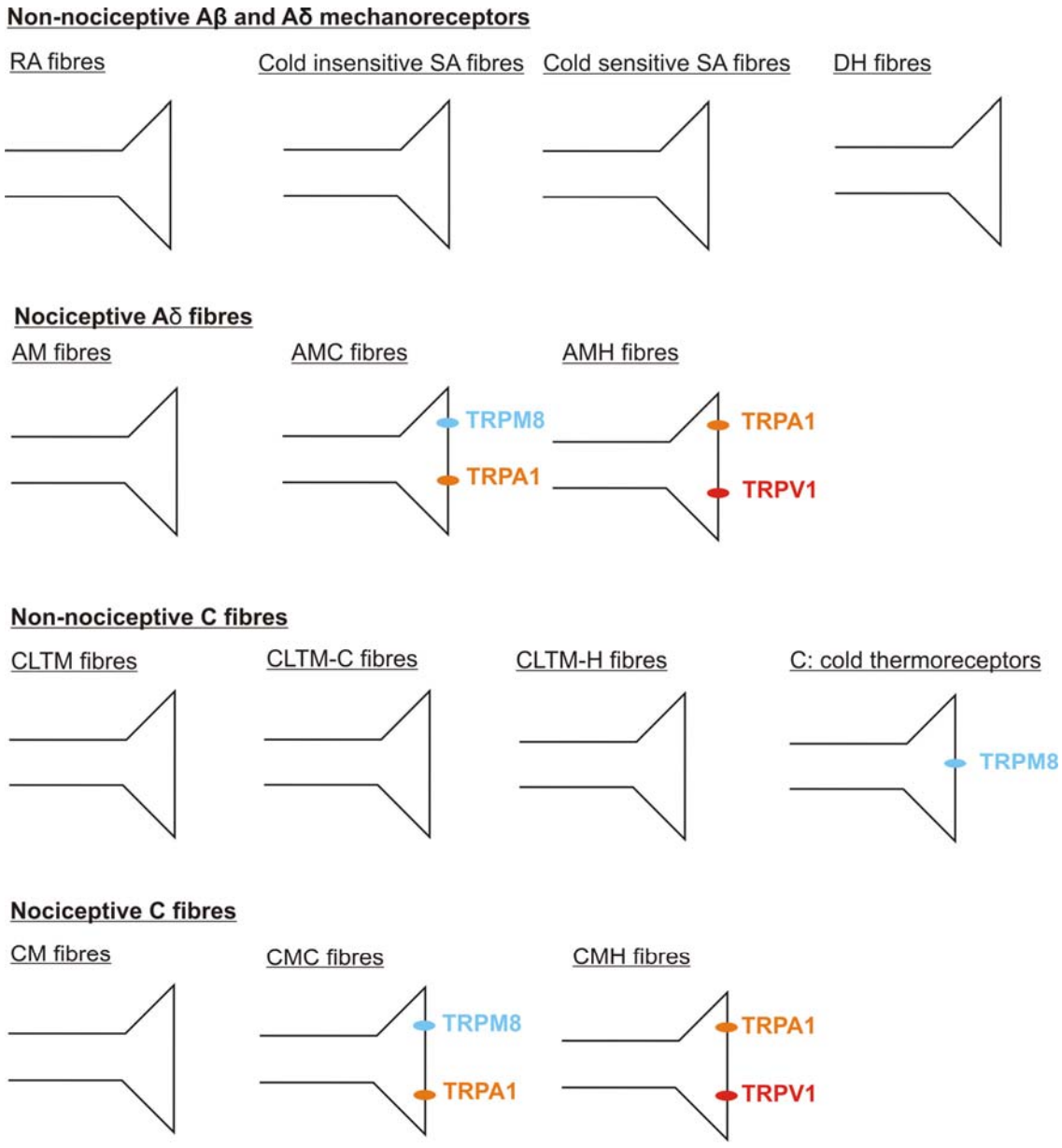

Fig. 4.4.1 Schematic representation of TRPM8, TRPA1 and TRPV1 expression on terminals of afferent nerve fibres

Aβ mechanoreceptors and Aδ DH fibres were unresponsive to TRP channel agonists and therefore are proposed not to express any of the TRP channels. Similarly, thermally insensitive Aδ and C fibre nociceptors (AM and CM fibres) lacked sensitivity to TRP channel agonists and are therefore proposed not to express any of these TRP channels also.

In contrast there was a high correlation of menthol sensitivity among cold sensitivity A and C fibres nociceptors (AMC and CMC fibres) as well as thermoreceptors (CC fibres) and these fibres are proposed to express TRPM8. Capsaicin sensitivity was high among heat sensitive A and C fibres nociceptors and are therefore proposed to express TRPV1. Mustard oil sesntivity was found among some cold and heat sensitive A and C fibre nociceptors.

Thermally sensitive CLTM fibres were not responsive to TRP channel agonists and therefore are proposed not to express TRPM8, TRPA1 or TRPV1.

Chapter 5

Thermal and mechanical sensitivity of afferents lacking the TRPV2 receptor in mice

5.1. Introduction

Psychophysical experiments in humans have shown that a single heat stimulus applied to the hairy skin (between 39-51.5 °C) evokes two distinct sensations. An initial, well localised pain (first pain) sensation described as ‘sharp’ or ‘pricki ng’ is followed by second pain described as ‘burning’ or ‘throbbing’ one second later (Campbell and LaMotte, 1983;Price et al., 1977). Conduction times to first and second pain are found to be correlated with conduction in Aδ and C fibres, respectively (Price et al., 1977).

Recordings from C and Aδ fibre primary afferents that respond to noxious heat stimulation in monkey, cat and human have advanced the knowledge of mechanisms of heat transduction (Bessou and Perl, 1969;Campbell et al., 1979;Gybels et al., 1979;LaMotte and Campbell, 1978;Price et al., 1977). These studies have also revealed that heat sensitive neurons differ in their thermal thresholds, C fibres typically responding over 40 °C, while Aδ fibres require even higher temperatures for activation.

In primates, A-fibre nociceptors can be divided into two groups based on their response pattern to heat stimuli. Type I (AMH type I) afferents respond to intense heat (53 °C, 30 seconds) with a long latency, a late peak discharge and average heat thresholds of >53 °C. In contrast, type II (AMH type II) afferents have lower heat thresholds (~47 °C), display an earlier peak discharge during a heat stimulus (similar to CMH fibres) and have much higher mechanical thresholds (Davis et al., 1993;Treede et al., 1995;Treede et al., 1998). In response to repeated heat stimuli, AMH type I fibres have been shown to become sensitised, whereas AMH type II fibres fatigue (Treede et al., 1995;Treede et al., 1998). Based on these observations it is suggested that the ‘first pain’ sensation to heat is conveyed by AMH type II nociceptors, while AMH

type I fibres are likely to signal pain to long duration heat stimuli and signal first pain sensations to mechanical stimuli (Treede et al., 1998).

Heat responses also depend on skin type. Campbell and LaMotte (1983) found that in humans, a noxious heat stimuli elicited a double pain sensation on the hairy skin of the arm, but not when applied on the glabrous skin of the hand (Campbell and LaMotte, 1983). The absence of 'first' pain suggested a lack of AMH type II fibres in the glabrous skin. Consistent with the above finding, Treede et al (1995) only found AMH type I fibres in monkey glabrous skin. However, a recent study in humans by Iannetti et al (2006) found that laser evoked heating of the hairy and glabrous skin of the hand at the same energy elicited similar psychophysical ratings, providing evidence that first pain to heat does exist in glabrous skin (Iannetti et al., 2006).
Although heat responses in C fibres did not differ between hairy and glabrous skin (Treede et al., 1995), CMH fibres that innervated hairy skin were readily sensitized to heat, whereas those that innervated the glabrous skin did not share this property (Campbell and Meyer, 1983).

Insight into the molecular mechanisms of heat transduction came with the molecular cloning of the heat and capsaicin activated TRPV1 receptor in 1997 (formerly known as vanilloid receptor type 1 (VR1)) (Caterina et al., 1997). TRPV1 is found to be predominantly expressed in small to medium diameter sized DRG neurons and has an average activation threshold of ~43 °C (Caterina et al., 1997) consistent with its role as a heat sensor in A and C fibre nociceptors. Heat responses in CMH and AMH type II fibres have been suggested to be mediated by the TRPV1 receptor. In support of this notion, capsaicin has been shown to excite polymodal CMH nociceptors in the rat skin (Szolcsanyi et al., 1988) and AMH type II nociceptors in the monkey (Ringkamp et al., 2001).

In mice lacking TRPV1, neurons lose their sensitivity to capsaicin, heat evoked currents in acutely dissociated neurons in culture are significantly reduced and a lower proportion of heat sensitive C fibres are found in the hairy skin (Caterina et al., 2000a). Interestingly however, high threshold responses above

55 °C remain intact in cultured neurons and C fibres retain a substantial sensitivity to heat (Caterina et al., 2000a), suggesting the existence of another heat transducer not associated with capsaicin sensitivity.
In 1999, the identification of TRPV2 (formerly known as vanilloid receptor like protein 1 (VRL-1)), a capsaicin insensitive, heat activated ion channel with an activation threshold of ~52 °C, made it an ideal candidate for this role (Caterina et al., 1999). Structurally related to TRPV1, TRPV2 was found to be predominantly expressed on medium to large diameter sized neurons and approximately 80 % of TRPV2 immunoreactive cells stained with anti-neurofilament antibody RT97, a marker for myelinated neurons (Caterina et al., 1999). The histological data together with the heat response profile in heterologous systems suggested that TRPV2 could be the heat transducer in AMH type I afferents. Consistent with this hypothesis, Nagy and Rang (1999) reported of two classes of heat sensitive neurons in the rat DRG. They found that small to medium sized neurons had an average heat threshold of 45 °C and were capsaicin sensitive whereas a second class of heat sensitive neurons that were larger in size, had a higher heat threshold of 51° C and were insensitive to capsaicin (Nagy and Rang, 1999).

However, the role of TRPV2 as a noxious heat sensor came into question when Woodbury et al (2004) using an ex-vivo preparation, showed that in mice lacking TRPV1, only 1 out of 12 heat responsive C fibres was immuno-positive for TRPV2. They showed that heat responses indistinguishable from those seen in wild-type mice could be recorded in the absence of TRPV1 or TRPV2 (Woodbury et al., 2004). They suggested that the difference to previous results (Caterina et al., 2000a) might be explained by the differences in skin type in the experimental model used. They also suggested that dissociated cell or sensory neuron culture lacked normal and central peripheral processes and therefore represented models of axotomized rather than normal neurons. On the other hand, the ex-vivo preparation in which the skin, DRG and spinal cord are intact represent complete intact afferents. They hypothesized that a TRPV1/TRPV2 heat transducing mechanism may be important during states of tissue injury in isolated cells and that a TRPV1/TRPV2 independent mechanism exists which functions in intact afferents.

The role of TRPV2 in the development of inflammation induced hyperalgesia was investigated by Shimosato et al (2005). An increase in TRPV2 expression in NF200 expressing presumably myelinated neurons was identified, which peaked two days after inflammation induced with complete Freund's adjuvant (CFA) (Shimosato et al., 2005). They further went on to show that hyperalgesia to heat was abolished at 50 °C, but not at 56 °C in inflamed rats pre-treated with resiniferatoxin (RTX, a capsaicin analog which binds to TRPV1). At 56 °C, RTX was able to reduce the response to heat in non-inflamed rats. This suggested that in normal conditions activation of both TRPV1 and TRPV2 are involved in heat transduction at 56 °C, but during inflammation TRPV2 up-regulation and not TRPV1 is important in mediating hypersensitivity to noxious high temperature stimuli.

Unlike TRPV1 which is exclusively expressed in the trigeminal and dorsal root ganglia (Caterina et al., 1997) expression of TRPV2 is not limited just to the sensory system (Caterina et al., 1999). TRPV2 messenger RNA was found to also be expressed in the lungs, spleen, intestine and brain, indicating that it may be activated by stimuli other than noxious heat (Caterina et al., 1999). Interestingly, Muraki et al (2003) demonstrated that TRPV2 was expressed in mouse vascular smooth muscle cells in the heart and functioned as a calcium entry channel which was activated by cell swelling. They also demonstrated that when TRPV2 was heyerologously expressed into Chinese hamster ovary (CHO) cells, the channel could be activated by membrane stretch (Muraki et al., 2003). This suggested that activation of TRPV2 could be involved in mechanosensitive membrane depolarization.

The above studies suggest that TRPV2 maybe involved in the detection of noxious heat (above 52 °C) and play an important role in the development of heat hyperalgesia in states of inflammation. The expression pattern of TRPV2 suggests that it may also be involved in the detection of other stimuli apart from noxious heat. While the generation of TRPV1 null mice has contributed to a clearer picture of the patho-physiological roles of TRPV1 (Caterina et al., 2000a;Davis et al., 2000), the involvement of TRPV2 in noxious heat sensing is incompletely understood.

Aim of the present study

This is the first study which has investigated the receptive properties of single cutaneous primary afferent neurons in TRPV2 null mice. The present study aims to investigate the role of TRPV2 in noxious heat and mechanical sensing in both the hairy skin which innervates the hind limb and glabrous skin which innervates the foot pad of the mouse.

5.2 Methods

5.2.1. Animals

TRPV2 receptor wild type (+/+) and knockout (-/-) 129SV+C57/Blk6 hybrid transgenic adult male and female mice were kindly provided by Dr Micheal Caterina, Johns Hopkins School of Medicine, Baltimore. These mice were used for in vitro electrophysiologcal analysis.
TRPV2 heterozygous (+/-) 129SV and C57/Blk6 adult male and female mice were also provided by Dr Micheal Caterina. Breeding was set up between TRPV2 heterozygous (+/-) male 129SV and female C57/Blk6 mice or between TRPV2 heterozygous (+/-) male C56/Blk6 mice and female 129SV mice to generate further hybrid TRPV2 wild type (+/+) and knockout (-/-) offspring. Animals were bred in the animal house of the Institute of Neurology, University College London, UK. Mice were then genotyped and TRPV2 wild type and knockout mice were then used for electrophysiological analysis.
Hybrid (129SV+C57/Blk6) mice with normal TRPV2 receptor genes will be referred to as TRPV2 wild type (TRPV2 WT) or TRPV2 (+/+) mice from here onwards. Similarly, hybrid mice lacking the TRPV2 receptor will be referred to as TRPV2 knock out (TRPV2 KO) or TRPV2 (-/-) from here onwards.

5.2.3. Genotyping

To confirm the genotype, tissue from the ear of each offspring was taken before electrophysiological analysis. The ear punctures were digested in lysis buffer and proteinase K to extract the DNA from each tissue sample. PCR was performed using 2 µl of the reverse primer, 5'-AAC-AGC-AAA-TCC-TGG-AAC-CTC-AC per tissue sample and 1 µl per tissue sample of the following forward primers: 5'-CAG-CTA-TCC-TGA-GCA-GAA-GAG-AGC and 5'-AGC-ATC-TAC-CCC-AAC-CTT-CAG-AC. The PCR products were then separated using electrophoresis on a 1.5 % agarose gel. An example is shown below.

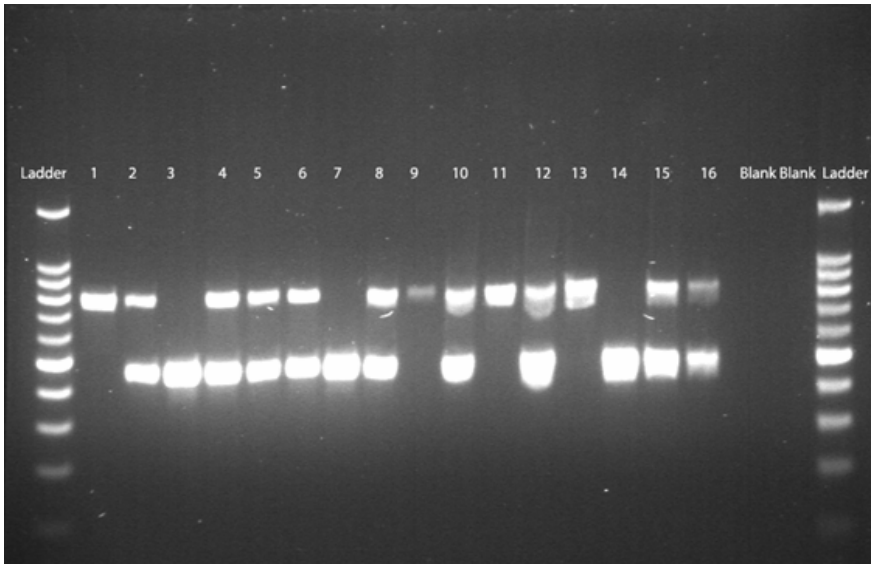

Fig. 5.2.1 Agarose gel electrophoresis of DNA produced from PCR experiments*.*

The predicted PCR product size of the TRPV2 WT is 470 base pairs (lanes 3, 7 and 14) and 750 base pairs for the TRPV2 KO (lanes 1, 9, 11 and 13) DNA ladders are shown on the left and right.

5.2.3. Skin-nerve in vitro preparation and recording technique

The tibial nerve with the skin of the foot pad and part of the leg attached was dissected from adult mice as described in chapter 2. The skin was mounted corium inside up in an organ bath and superfused with warm oxygenated SIF. Receptive fields of individual primary afferent neurons were then identified with a mechanical search stimulus and characterised using electrical, mechanical and thermal stimuli as described in chapter 2.

5.3. Results

Single unit recordings were made from a total of 172 afferent fibres, 100 from TRPV2 wild type (WT) mice and 72 from TRPV2 knock out (KO) mice. Recordings were made from a total of 39 animals, 24 WT mice and 15 KO mice.

5.3.1. Hairy Skin

Overall properties of fibres recorded from the hairy skin are presented below in Table.5.3.1. Recordings were made from a total of 16 animals, 8 TRPV2 WT and 8 TRPV2 KO. A total of 77 units were recorded, 38 from WT and 39 from KO animals.

TABLE.5.3.1. Conduction velocity and von Frey hair thresholds of myelinated Aβ, thin myelinated Aδ and unmyelinated C fibres in TRPV2 wild type and knockout animals, recorded from hairy skin

Genotype	Fibre type	Fibre type	n	Conduction Velocity, m/s	von Frey threshold
TRPV2 WT	Aβ	RA	1	22.2	1
		SA	3	15.0 ± 3.1	0.25 (0.25, 0.25)
	Aδ	DH	2	9.2	0.25
		AM	18	3.8 ± 0.4	8 (5.7, 11.3)
		AMCH	1	1.2	8
	C	CM	4	0.68 ± 0.2	19.2 (10, 192)
		CMH	3	0.69 ± 0.2	8 (5.7, 8)
		CMC	4	0.67 ± 0.2	21.7 (9.7, 80)
		CMIH	2	0.6	-
TRPV2 KO	Aβ	RA	7	14.6 ± 1.4	0.5 (0.3, 1)
		SA	5	13.9 ± 1.2	1 (0.35, 1)
	Aδ	DH	3	6.4 ± 1.5	0.25 (0.25, 0.3)
		AM	12	5.5 ± 0.9	8 (5.7, 20)
		AMC	1	3.3	2
	C	CM	6	0.7 ± 0.1	11.2 (8, 11.3)
		CMH	3	0.5 ± 0.1	32 (22.4, 32)
		CMC	2	0.9	5.7

All values are means ± SE; except for von Frey hair thresholds which are median and first (Q_1) and third (Q_3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMC, high threshold mechano-cold A fibre; AMH, high threshold mechano-heat A fibre; DH, D-hair receptors; CM, C mechano-sensitive nociceptor; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CMIH, C mechanically insensitive, heat sensitive nociceptor

Aβ mechanoreceptors

The mean conduction velocity of Aβ fibres from WT mice was 14.3 ± 1.7 m/s (n=4). The mean conduction velocity of Aβ fibres from KO mice was 14.3 ± 0.1 m/s (n=12). There was no significant difference between these values (p>0.9, unpaired t-test). On average there was no significant difference in the von Frey hair threshold between Aβ fibres from WT (0.25 mN (0.25, 0.63)) and KO animals (0.75 mN (0.3, 1)) animals (p>0.1, Mann-Whitney U test).

Rapidly adapting (RA) mechanoreceptors

1 RA unit was recorded from WT mice, whereas 7 were recorded from the KO. None responded to noxious cold or heat stimulation.
Firing properties to constant force stimulation were studied, by plotting the mean discharge rate as a function of force from 5-150 mN (Fig.5.3.1). The time for analysis began at the start of the rise of the force plateau and lasted for 11 seconds as described previously (Koltzenburg 1997). This time window was chosen to include the neural activity at the onset of the force stimulus and to capture the discharge at the end of the force plateau when stimulus intensity returned to 1 mN. The total mean number of impulses generated during constant force stimulation for each of the forces applied is shown in Fig.5.3.2. This revealed that RA units recorded from KO mice appeared to be mechanically more sensitive in comparison to the RA unit recorded from the WT. However, since only 1 unit was recorded from the WT, no proper comparison was possible.

Slowly adapting (SA) mechanoreceptors

3 SA units were recorded from WT mice and 5 were recorded from the KO. None of the units responded to noxious heat. In the KO animal, 1 out of the 5 (20 %) SA fibres responded to cooling, with a response threshold of 27.8 °C. There appeared to be no difference in the response pattern to constant force stimuli between SA fibres from WT and KO animals (Fig.5.3.3). This was supported by the finding that there was no significant difference (p>0.8 one way

ANOVA) in the mean total number of impulses generated during each of the forces applied (see Fig.5.3.4). Interestingly, SA fibres from mice lacking TRPV2 were more responsive during the onset and offset of the mechanical force stimulus compared to fibres from WT mice (Fig.5.3.3). However, the difference in von Frey hair thresholds of SA fibres from WT and KO animals ($p=0.07$, Mann-Whitney U test, see Table.5.3.1) was not significant.

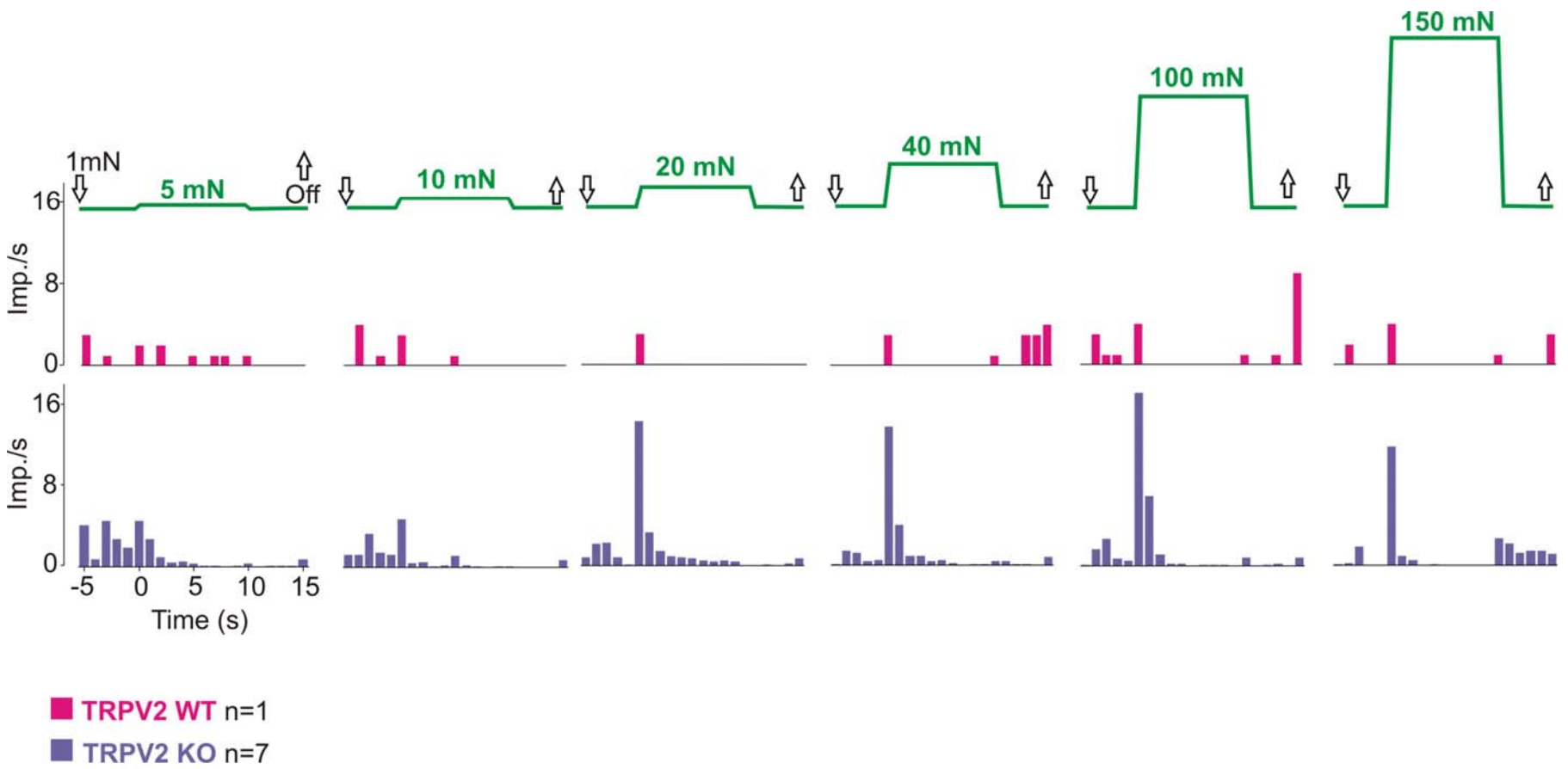

Fig.5.3.1. Mean response of rapidly adapting (RA) fibres to constant force stimulation in hairy skin
RA fibres recorded from mice lacking TRPV2 (n=7) were more responsive to constant force stimulation compared to the RA fibre (n=1) recorded from the TRPV2 wild type (WT). However, a statistical comparison was not possible since only one unit from the TRPV2 WT animal was recorded.

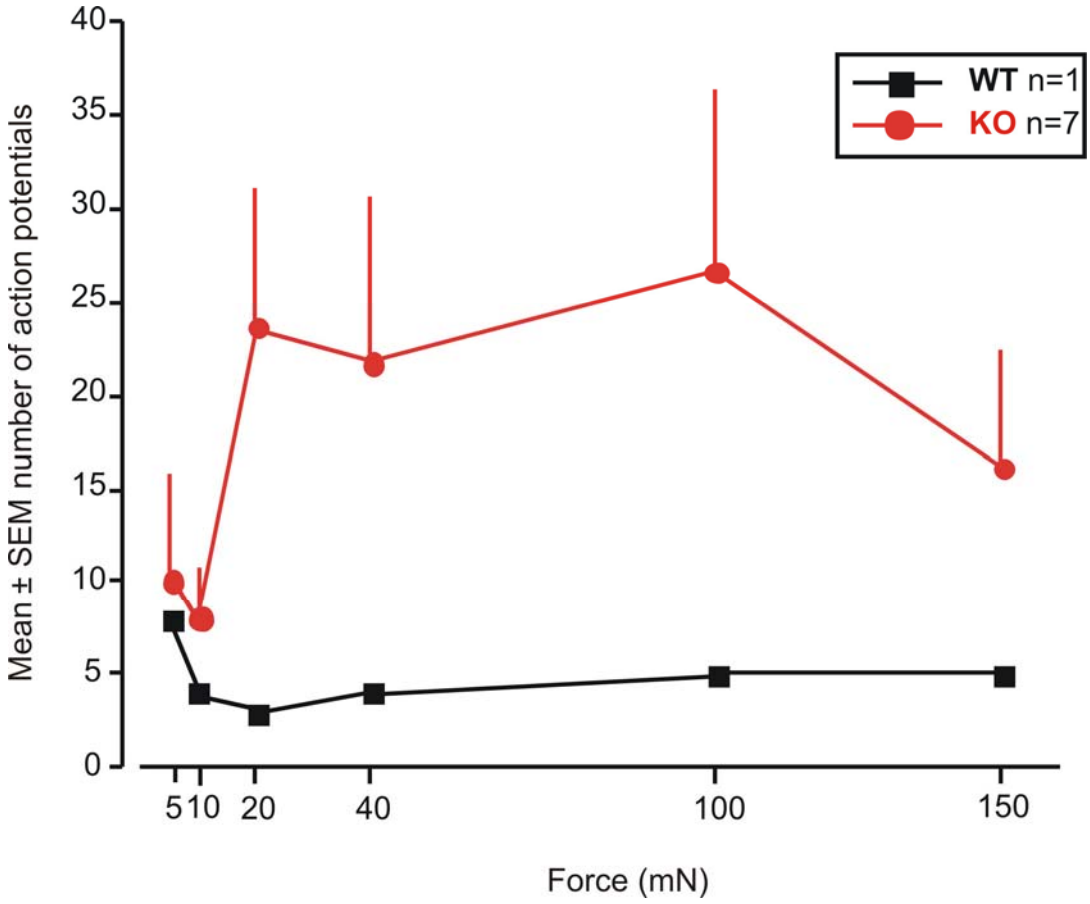

Fig.5.3.2. Mean response of rapidly adapting (RA) fibres to constant force stimuli in hairy skin
On average, RA fibres from KO mice generated more impulses during the constant force stimulation compared to fibres from WT animals. However, a statistical comparison was unable to be made since only one unit from the WT animal was recorded.

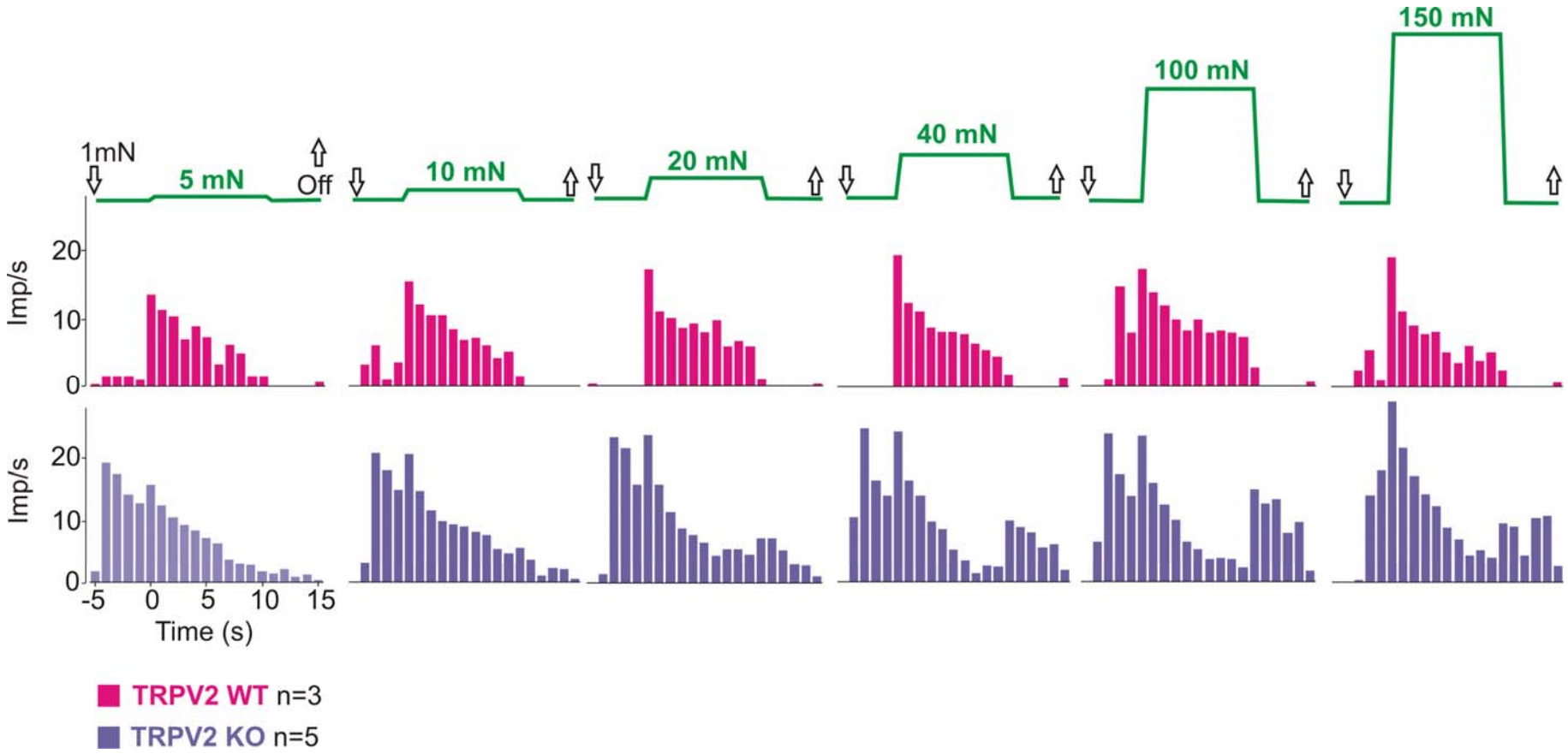

Fig.5.3.3. Mean response of slowly adapting (SA) fibres to constant force stimulation in hairy skin
There was no major difference in firing pattern or rate of discharge during the constant force stimulation between SA fibres lacking TRPV2 (n=5) and those from WT (n=3) animals. However, on average, SA fibres from the KO animal were much more responsive during the 1 mN baseline stimulus compared to those from the WT animal.

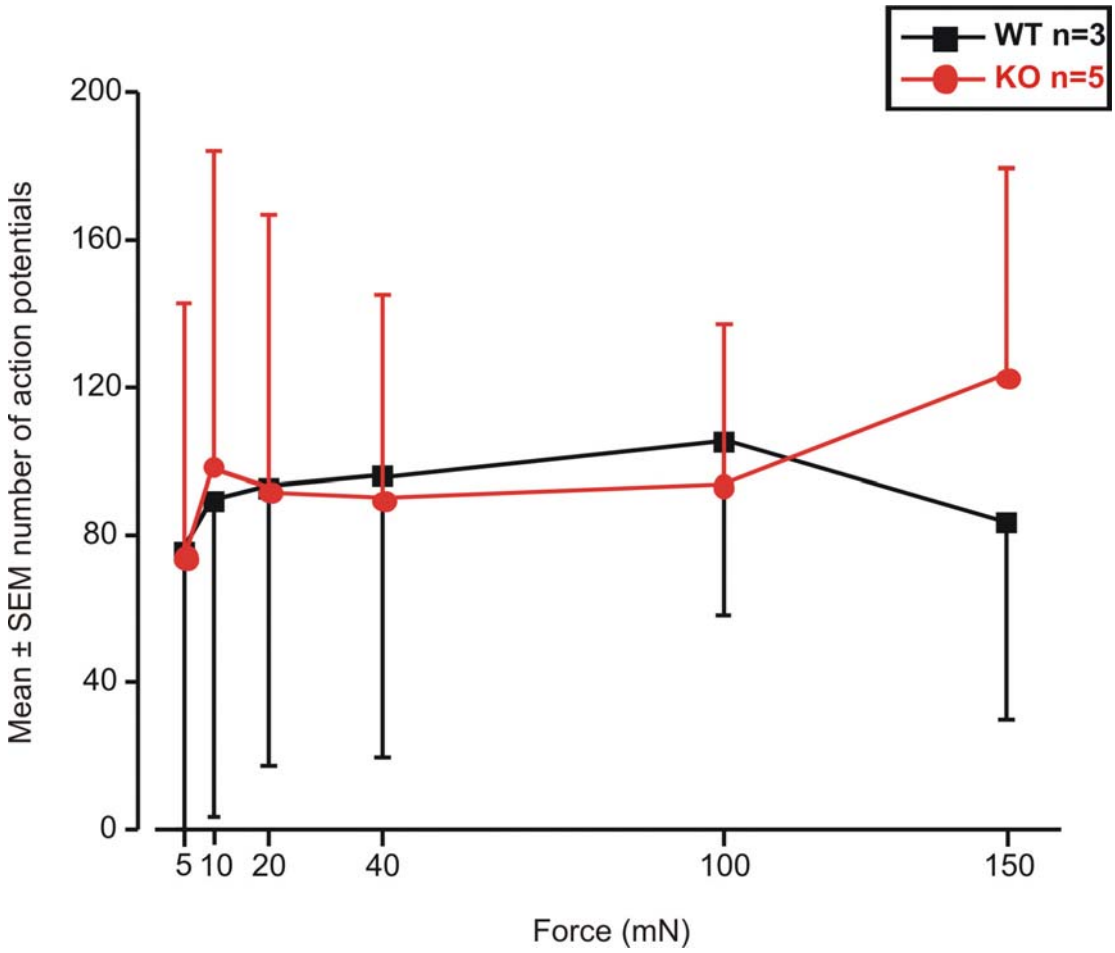

Fig.5.3.4. Mean response of slowly adapting (SA) fibres to constant force stimuli in hairy skin

There was no significant difference in the response to constant force stimuli between SA fibres from WT and KO animals ($p>0.8$, one way ANOVA).

Aδ fibres

D-hair (DH) fibres

2 DH units were recorded from the WT and 3 were recorded from the KO animals. None responded to noxious heat or cold stimulation.
An example of a DH from a WT and one from a KO animal responding to a constant force stimulus of 100 mN are illustrated in Fig.5.3.5A and B respectively. Plotting their mean discharge rate as a function of force (Fig.5.3.6) revealed that fibres from TRPV2 WT mice were clearly more responsive during constant force stimuli of 5-20 mN compared to DH fibres from the KO animal. They were also more responsive during the onset of the mechanical stimulation. A clear increase in the mean total number of impulses during the constant force stimuli of 5-20 mN is illustrated in Fig.5.3.7.

High threshold mechano-A (AM) nociceptors

19 AM fibres were recorded from WT and 13 were recorded from the KO mice. In particular, a higher proportion of AM fibres were recorded, since we were interested in investigated whether heat sensitivity among these fibres differed from TRPV2 WT and KO mice. The mean conduction velocity of AM fibres from WT mice was 3.7 ± 0.4 m/s and 5.3 ± 0.9 m/s from those lacking TRPV2. Although there was a trend of Aδ nociceptors from KO animals to conduct faster than those from WT, this did not reach statistical significance ($p=0.06$, unpaired t-test).
1 out of 19 (5 %) AM fibres from the TRPV2 WT animal responded to both noxious cold and heat stimulation (AMHC, see Fig.5.3.8A). The response threshold was 43 °C (see Table.5.3.3) and 15.3 °C to noxious heat and cold, respectively. To ensure that we were not underestimating the proportion of heat sensitive Aδ nociceptors, a second noxious heat stimulus peaking 50 °C (see Fig.5.3.8B for an example) on the corium side of the skin was applied on 9 out of the 18 AM units. However, this did not evoke a heat response.

None of the AM fibres from the KO animal responded to noxious heat. 1 out of 13 (8 %) AM fibres from the KO animal responded to cold (AMC) with a response threshold of 25 °C. 8 out of 13 AM units were further tested with a noxious heat stimulus of 50 °C, but none became responsive.
There was no significant difference in von Frey hair thresholds between AM fibres from WT (8 mN (5.7, 11.3), n=18) and KO animals (8 mN (5.7, 8), n=13) ($p>0.8$, Mann-Whitney U test). Plotting the mean response to a mechanical stimulus ramp (0-200 mN) revealed that there was no major difference in the firing pattern between AM fibres from WT and mice lacking TRPV2 (Fig.5.3.9A). Although on average the peak discharge rate was higher in KO animals (8.6 impulses/s in WT, 13.2 impulses/s in the KO), statistically there was no difference in the total mean number of spikes generated during the mechanical ramp between the two genotypes ($p>0.4$, unpaired t-test, Fig.5.3.9B).

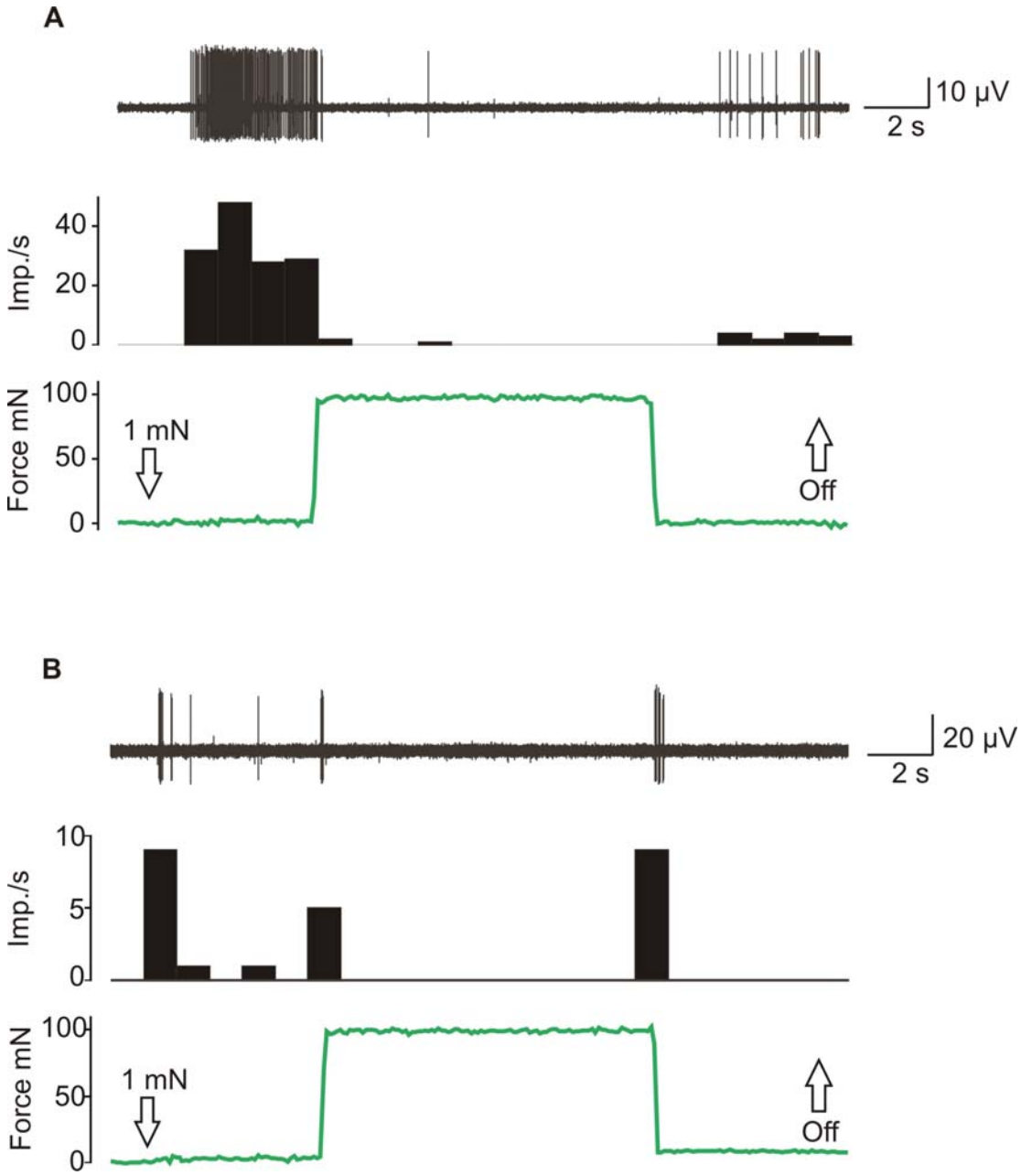

Fig.5.3.5. Example of a D-hair fibre recorded from a TRPV2 wild type (WT) and knockout (KO) animal responding to 100 mN constant force stimulus

DH fibres from both WT (A) and KO (B) animals responded typically during the onset, beginning and at the end of the mechanical stimulus.

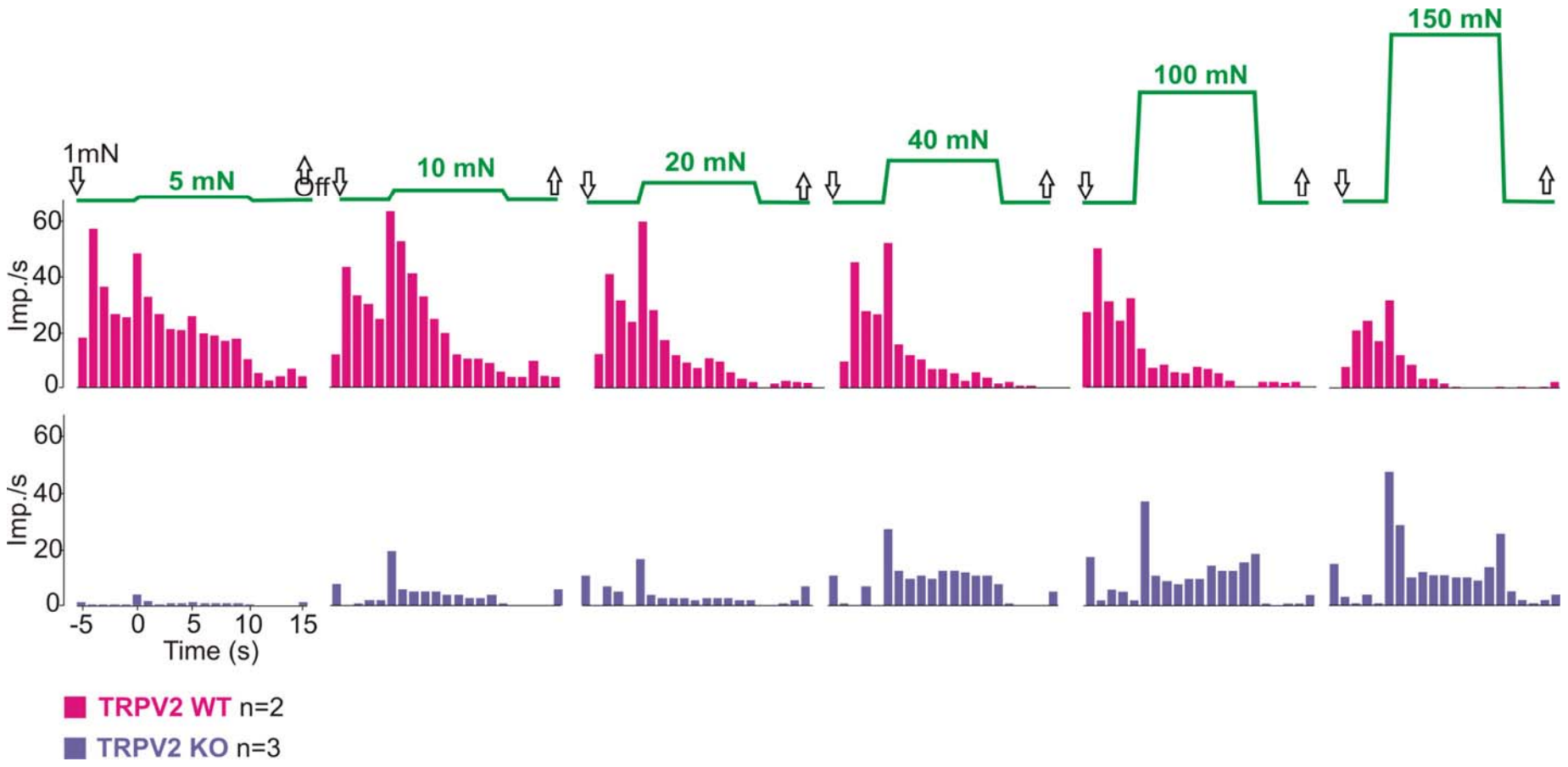

Fig.5.3.6. Average response of D-hair (DH) fibres to constant force stimulation in hairy skin
DH fibres recorded from TRPV2 WT animals (n=2) displayed higher firing frequencies, especially during the onset of the mechanical stimulus compared to DH fibres from mice lacking TRPV2 (n=3).

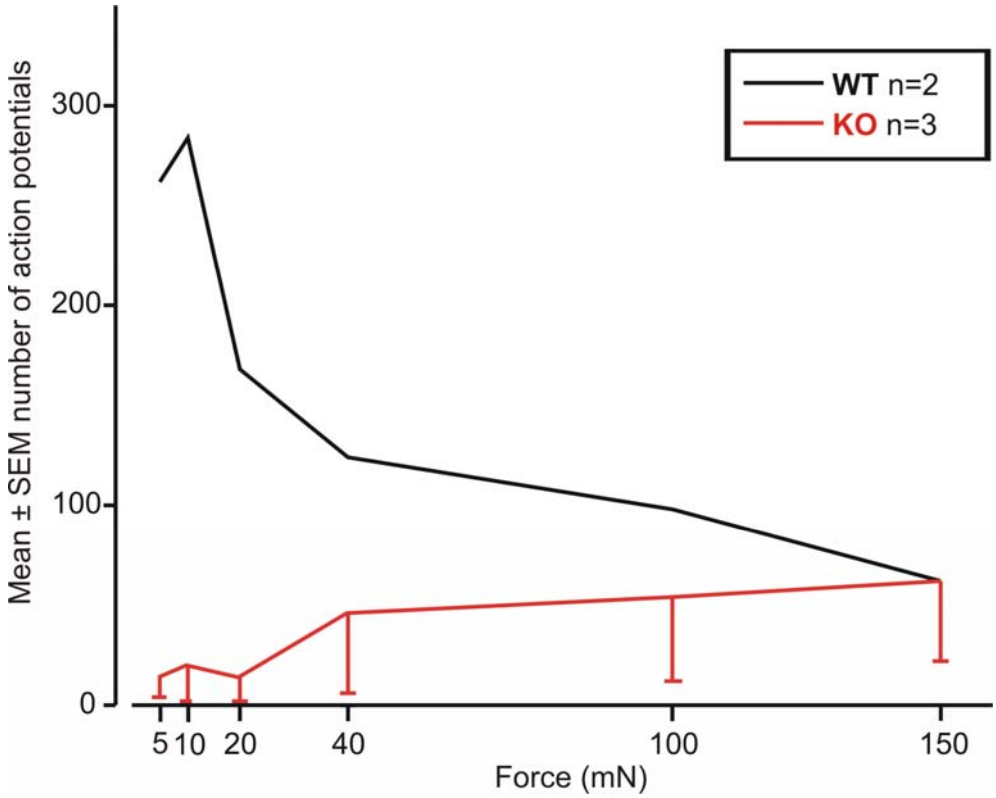

Fig.5.3.7. Mean response of D-hair (DH) fibres to constant force stimuli in hairy skin

The average response (± SD) of DH fibres from WT animals (n=2) was clearly greater than the average response (± SEM) from the KO animals (n=3) between mechanical forces of 5 to 40 mN.

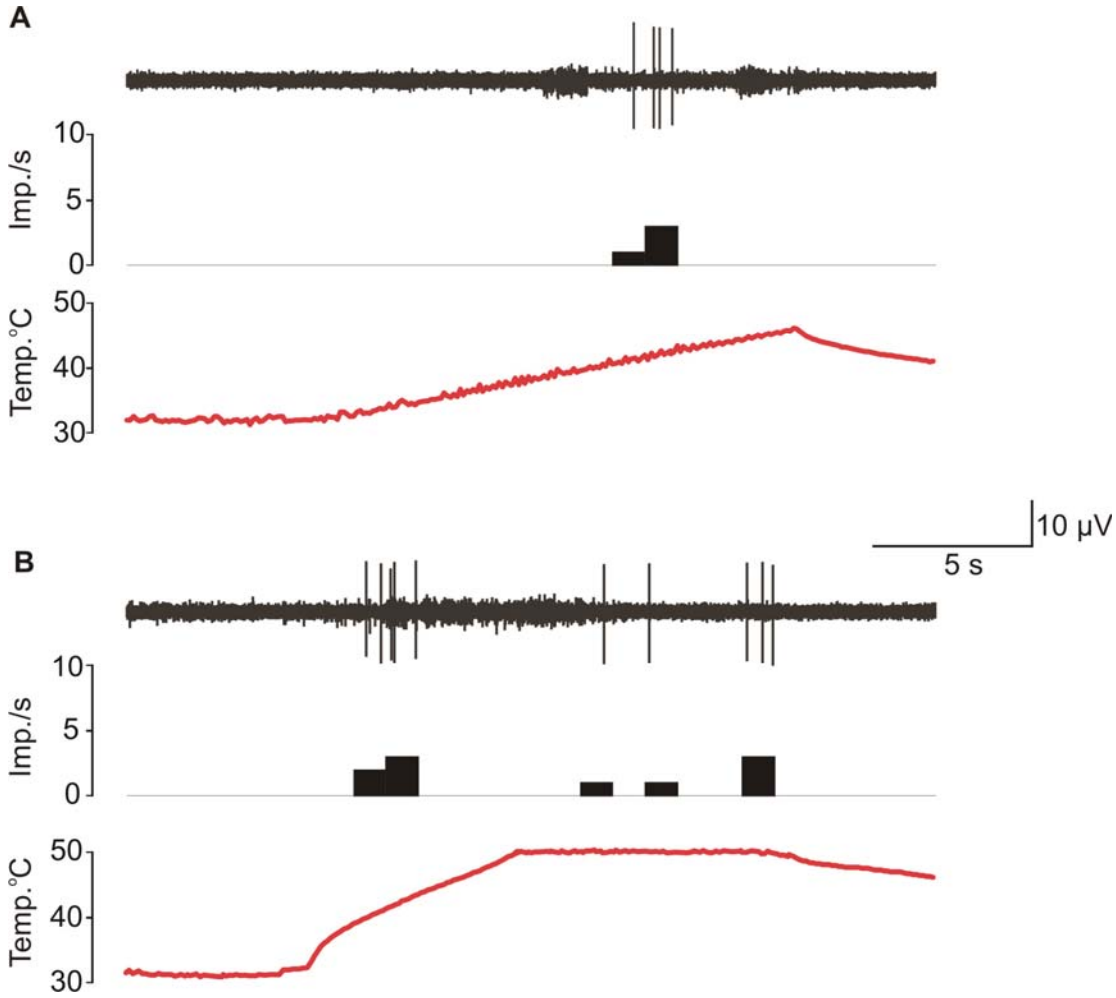

Fig.5.3.8. Aδ nociceptor response to noxious heat (AMH) in hairy skin
(A) The AM fibre responded to the first heat stimulus (47 °C).
(B) A second heat stimulus, reaching 50 °C was then applied. The fibre responded during both the dynamic (ramp) and static phase (hold) of the heat stimulus.

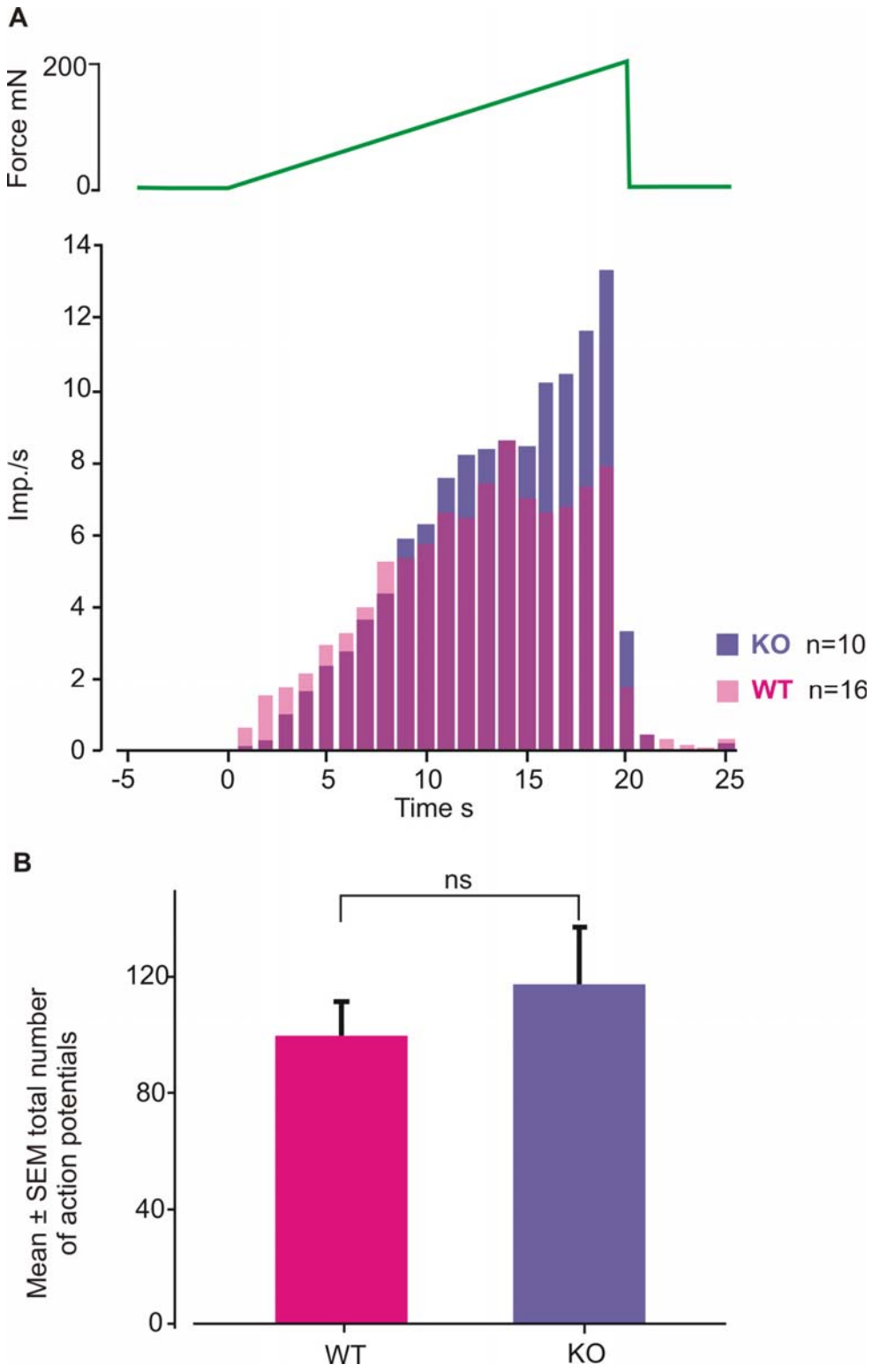

Fig.5.3.9 Mean response of high threshold mechano-sensitive nociceptors (AM) to a mechanical ramp stimulus (0-200 mN) in hairy skin

(A) There was no difference in the firing pattern between fibres from WT and KO mice. AM fibres lacking TRPV2 however displayed a higher peak discharge rate of 13.2 imp./s compared to 8.6 imp./s in WT animals. (B) There was no significant difference in the mean total number of discharges generated during the mechanical ramp between AM fibres of WT and KO mice ($p>0.4$, unpaired t-test).

C fibres

11 high threshold mechanically sensitive C (CM) fibres were recorded from both the WT and KO mice. The mean conduction velocity of CM fibres from WT mice was 0.7 ± 0.09 m/s and 0.7 ± 0.07 m/s for KO animals. There was no significant difference in the conduction velocity between WT and KO animals ($p>0.8$, unpaired t-test).

High threshold mechano-heat sensitive C fibres (CMH)

3 out of 11 (27 %) CM fibres responded to noxious heat from both the WT and KO mice (see Table 5.3.3). Examples of heat responses are illustrated in Fig.5.3.10A and B.
The mean response threshold to heat in WT mice was 34.7 ± 1.7 °C. The mean response in KO mice was 37.8 ± 3.9 °C, which was not significantly different from that of the WT ($p>0.8$, Mann-Whitney U test). Although the mean total number of discharges during a heat stimulus in the WT was not significantly different from that in KO ($p>0.5$ Mann-Whitney U test, Fig.5.3.11B), plotting the average response as a function of temperature revealed a difference in firing pattern. On average, CMH fibres from WT mice displayed an increase in firing as the temperature increased up to 47 °C, reaching a peak discharge of 5.8 impulses per second. In contrast, CMH fibres from mice lacking TRPV2 displayed a higher peak discharge rate of 8.6 but at a lower temperature than that of fibres from the WT mice (Fig.5.3.11A).

High threshold mechano-cold sensitive C fibres (CMC)

4 out of 11 (36 %) CM fibres responded to noxious cold (CMC) in WT mice, compared to 2 out of 11 (18 %) from the KO. The average response threshold to cold was 16.4 ± 1.7 °C in the WT and 16.3 °C (15.2-17.4 °C) in the KO. Overall, there appeared to be no major difference in firing pattern during noxious cold between the two genotypes (Fig.5.3.12A). On average CMC fibres from WT mice generated a slightly greater response to a cold stimulus than CMC fibres from the KO mice (Fig.5.3.12B).

Mechanical sensitivity in C fibre nociceptors

The average response to a mechanical ramp (0-200 mN) was compared between CM fibres from WT (n=10) and KO (n=8) animals. On average as the stimulus force increased the firing discharge also increased in fibres from both genotypes. However, fibres from mice lacking TRPV2 clearly displayed a greater response to the suprathreshold stimulus compared to fibres recorded from WT mice (Fig.5.3.13A). CM fibres from the KO were significantly more responsive during the mechanical ramp compared to CM fibres from WT mice ($p<0.05$, unpaired t-test, Fig.5.3.13B). However, there was no significant difference ($p>0.6$, Mann-Whitney U test) in von Frey hair thresholds between CM fibres from both genotypes.

Mechanically insensitive, heat sensitive C fibres (CMIH)

While carrying out the 50 °C heat stimulus, 2 mechanically insensitive, heat sensitive C (CMIH) fibres were recorded. Both were found in TRPV2 WT animals. The receptive fields of both fibres were located via electrical stimuli. Upon mechanical probing with a glass rod, both fibres remained unresponsive. Interestingly they did not respond to the first noxious heat stimulus which reached 47 °C and only responded to the 50 °C ramp and hold heat stimulus. An example of one such unit is shown in Fig.5.3.14.

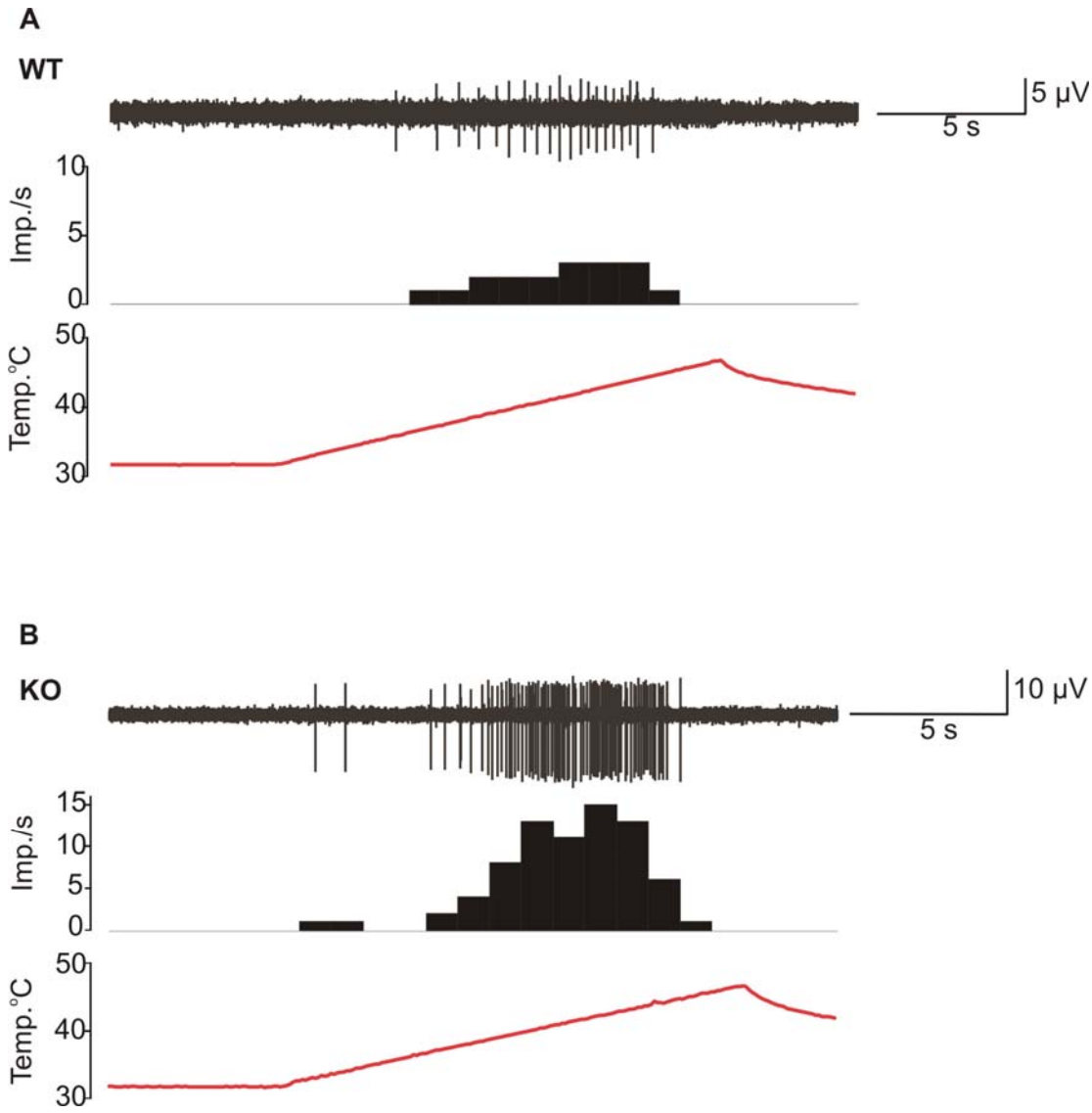

Fig.5.3.10. Example heat responses of CMH fibres recorded from TRPV2 wild type (WT) and knockout (KO) mice in the hairy skin
Example of a CMH fibre recorded from a TRPV2 WT (A) and KO mouse (B) is shown above. There was no difference in the proportion of heat sensitive C fibres between WT and KO animals.

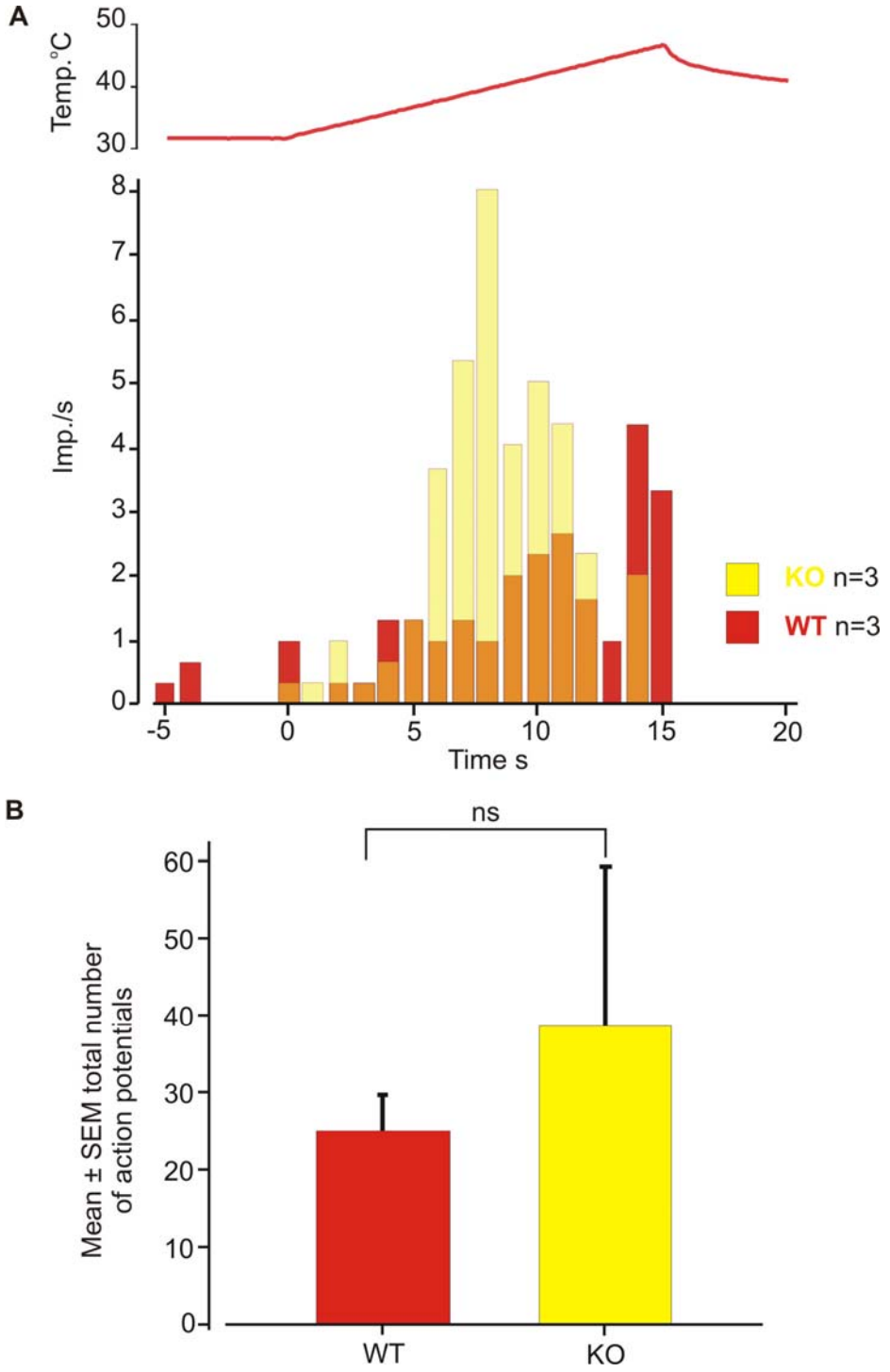

Fig.5.3.11. Average response of CMH fibres responding to a noxious heat stimulus in hairy skin

(A) CMH fibres from WT mice displayed an increasing discharge as the temperature increased, displaying a peak response of 5.8 impulses per s at the end of the heat stimulus. In contrast, CMH fibres from the KO animal displayed a slightly different firing pattern. They were more responsive to heat, with a peak discharge rate of 8.6 impulses per second earlier into the heat stimulus. (B) Although CMH fibres recorded from KO animals displayed a higher mean total number of discharges during the heat stimulus compared to fibres from the WT, this difference was not statistically significant (p>0.5, Mann-Whitney U test).

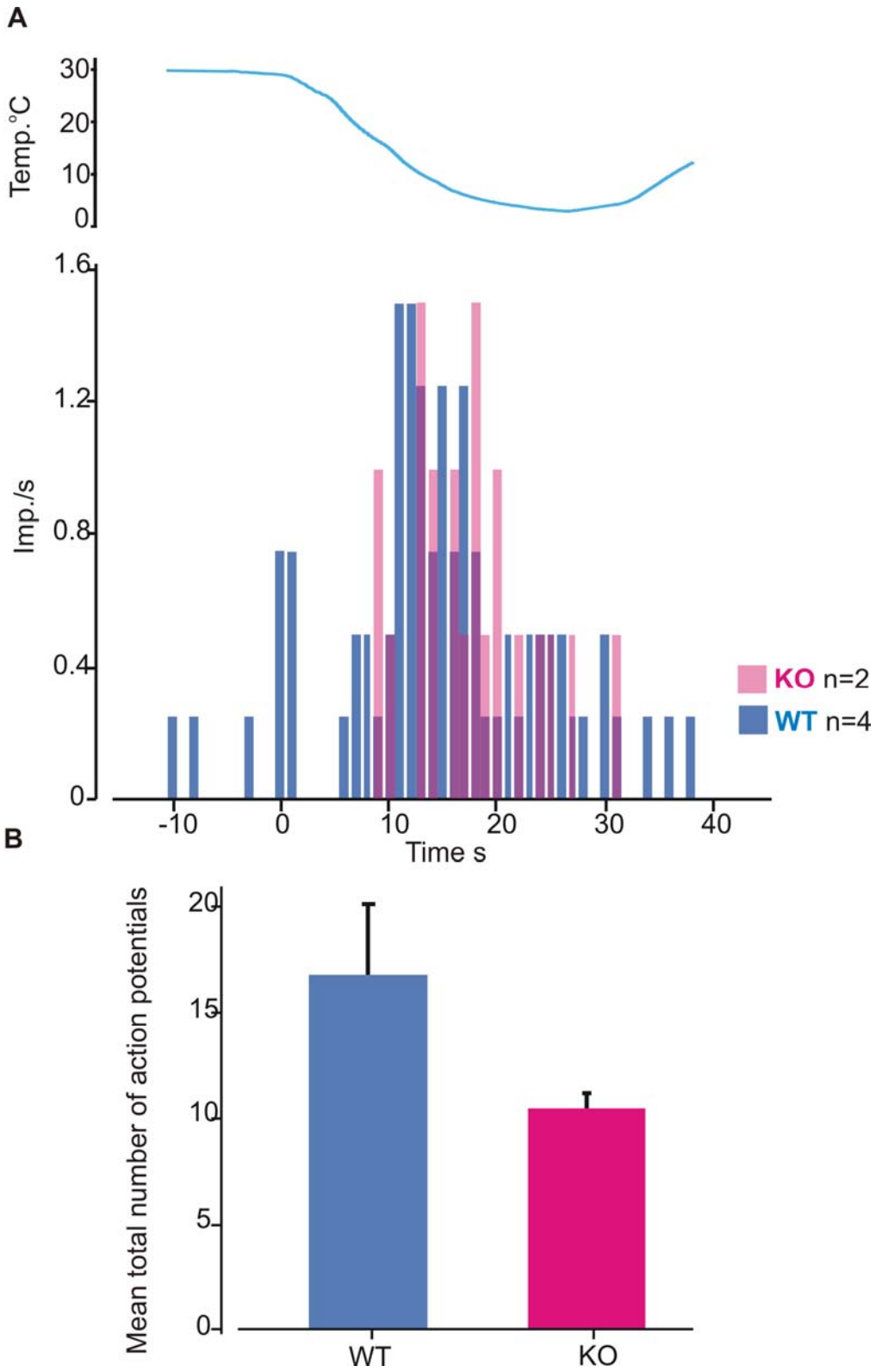

Fig.5.3.12. Average cold response of CMC fibres in the hairy skin
(A) There appeared to be no major difference in the firing pattern to cold between CMC fibres from wild type (WT) and knockout (KO) animals. (B) On average, CMC fibres from WT mice displayed a slightly greater response to cold compared to fibres from the KO mice. However, since only two CMC units were recorded from KO animals, a statistical comparison was not possible.

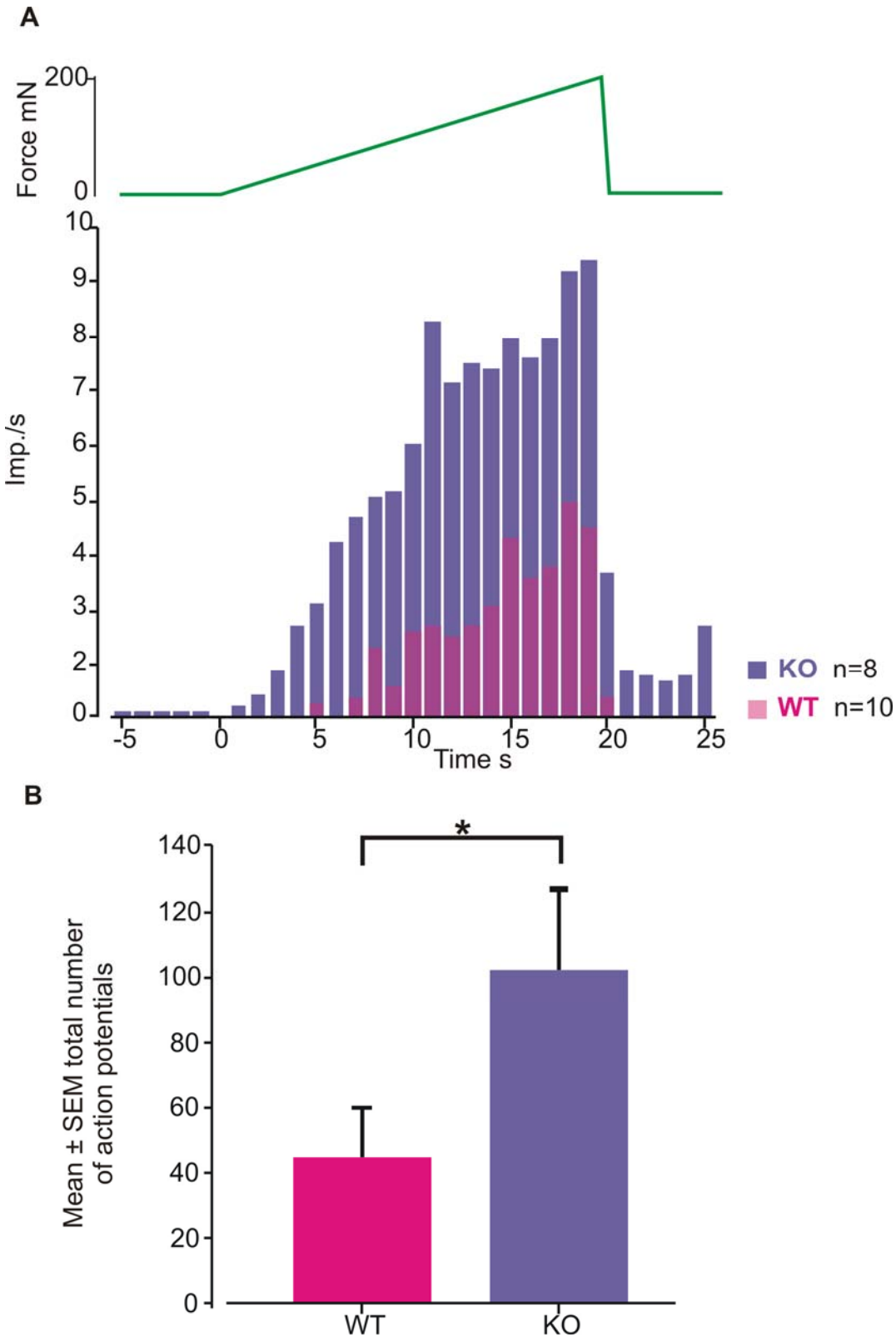

Fig.5.3.13. Average response of mechanically sensitive C fibre nociceptors (CM fibres) to mechanical stimulation in the hairy skin
(*A) CM fibres from KO mice clearly displayed a greater response during the mechanical ramp compared to CM fibres from the WT. (B) There was a significant increase (p<0.05, unpaired t-test) in the mean total number of spikes generated by CM fibres from KO mice compared to those from WT mice.*

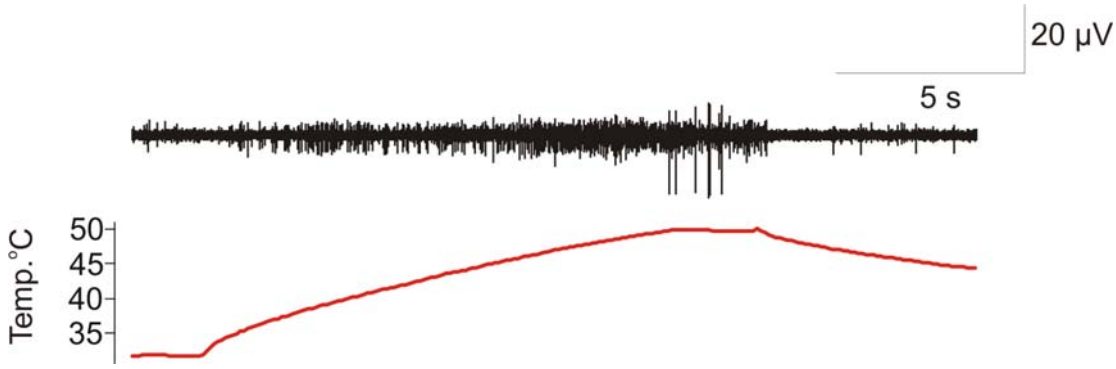

Fig.5.3.14. Example of a mechanically insensitive, heat sensitive C (CM_IH) fibre recorded from hairy skin

2 CM_IH units were recorded from the hairy skin of TRPV2 WT mice. They were both found during the application of a 50 °C heat stimulus.
Their receptive fields were then located using electrical stimuli, as they did not respond to mechanical probing with a glass rod. Once the receptive field was accurately located, a 47 °C heat stimulus was applied, to which they never responded. Interestingly, they only discharged during a 50 °C heat stimulus.
An example of one of the units is shown above (the CM_IH fibre is the larger unit responding towards the end of the heat stimulus. Other smaller units are firing in the background).

5.3.2. Glabrous skin

Overall properties of fibres recorded from glabrous skin are presented below in Table.5.3.2. Recordings were made from a total of 23 animals, 16 TRPV2 WT and 7 TRPV2 KO. A total of 95 units were recorded, 62 from WT and 33 from KO animals.

TABLE.5.3.2. Conduction velocity and von Frey Hair thresholds of myelinated Aβ, thin myelinated Aδ and unmyelinated C fibres in TRPV2 wild type and knockout animals, recorded from glabrous skin

Genotype	Fibre type	Fibre type	n	Conduction Velocity, m/s	von Frey threshold
TRPV2 WT	Aβ	RA	6	17.8 ± 2.6	1.4 (1, 1.4)
		SA	4	14.2 ± 3.2	1 (1, 2.5)
	Aδ	DH	0	-	-
		AM	28	6.8 ± 0.87	8 (5.7, 16)
		AMH	0	-	-
		AMC	1	3.1	32
	C	CM	12	0.57 ± 0.04	19.3 (9.7, 32)
		CMH	6	0.43 ± 0.04	16 (11.3, 16)
		CMC	4	0.71 ± 0.1	8 (8, 26)
		CMCH	1	0.42	16
TRPV2 KO	Aβ	RA	8	18.3 ± 2.3	0.5(0.43, 1)
		SA	5	17.7 ± 0.9	0.71 (0.35, 1)
	Aδ	DH	0	-	-
		AM	9	7.3 ± 1.7	8 (8, 16)
		AMH	1	3.4	22.6
	C	CM	5	0.57 ± 0.05	8 (5.7, 32)
		CMH	4	0.53 ± 0.03	16 (12, 30.7)
		CMCH	1	0.49	16

All values are means ± SE; except for von Frey hair thresholds which are median and first (Q_1) and third (Q_3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMC, high threshold mechano-cold A fibre; AMH, high threshold mechano-heat A fibre; DH, D-hair receptors; CM, C mechano-sensitive nociceptor; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor

Aβ mechanoreceptors

The mean conduction velocity of Aβ fibres from WT mice was 16.2 ± 1.2 m/s (n=9). The mean conduction velocity of Aβ fibres from KO mice was 18.1 ± 1.4 m/s (n=13). There was no significant difference between the values ($p>0.4$, unpaired t-test). On average Aβ fibres from KO mice had significantly lower von Frey hair thresholds (0.5 mN (0.35, 1.0), n=13) than those from WT mice (1.2 (1.0, 1.4), n=9) ($p<0.01$, Mann Whitney-U test).

Rapidly adapting (RA) mechanoreceptors

6 RA units were recorded from WT mice, whereas 8 were recorded from the KO. None responded to noxious cold or heat stimulation.
Plotting their mean response to constant force stimulation ranging from 5-150 mN revealed that RA units from WT mice were mechanically more sensitive, especially during the onset and offset of the stimulus (Fig.5.3.15). RA fibres from WT mice were significantly more responsive during constant mechanical forces of 5 and 40 mN ($p<0.001$, one-way ANOVA, Fig.5.3.16). However, overall von Frey hair thresholds of RA fibres from KO mice were significantly lower than that from WT animals ($p<0.05$, Mann Whitney U test, see Table.5.3.2).

Slowly adapting (SA) mechanoreceptors

4 SA units were recorded from WT mice, whereas 5 were recorded from the KO. None responded to noxious cold or heat stimulation.
There appeared to be no apparent difference in the firing pattern or firing frequency to constant force stimuli in SA fibres between both genotypes (Fig.5.3.17). Although on average there was a trend for fibres from KO mice to be more responsive during constant forces between 40-150 mN, the difference was not statistically significant (see Fig.5.3.18). There was no significant difference in the von Frey hair thresholds of SA fibres from KO and WT mice ($p=0.09$, Mann-Whitney U test).

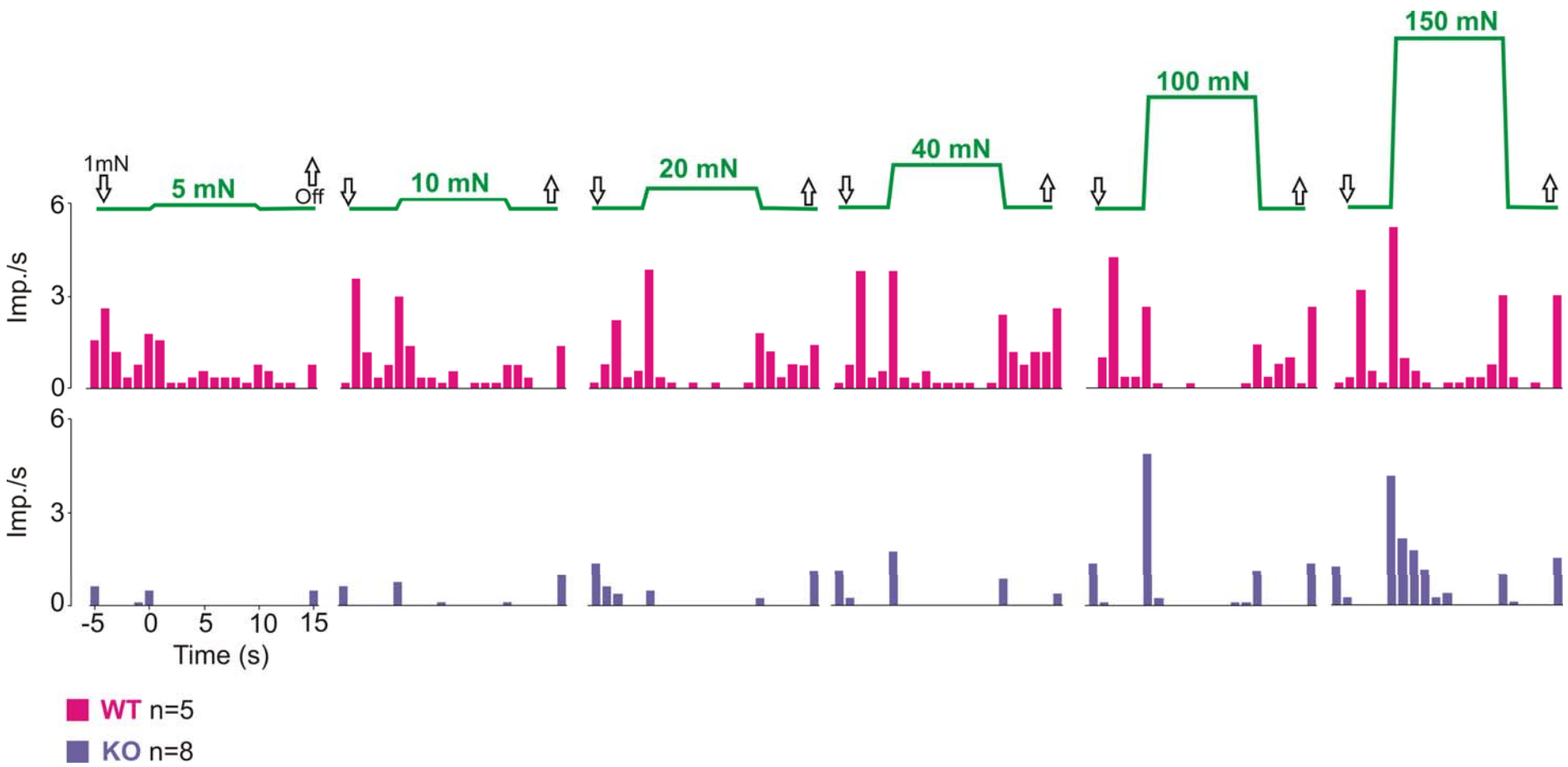

Fig.5.3.15. Mean response of rapidly adapting (RA) fibres to constant force stimuli in glabrous skin
RA units from WT animals were much more sensitive during mechanical stimulation of their receptive fields compared to RA fibres from KO mice. They were especially more responsive during the onset and offset of the stimulus.

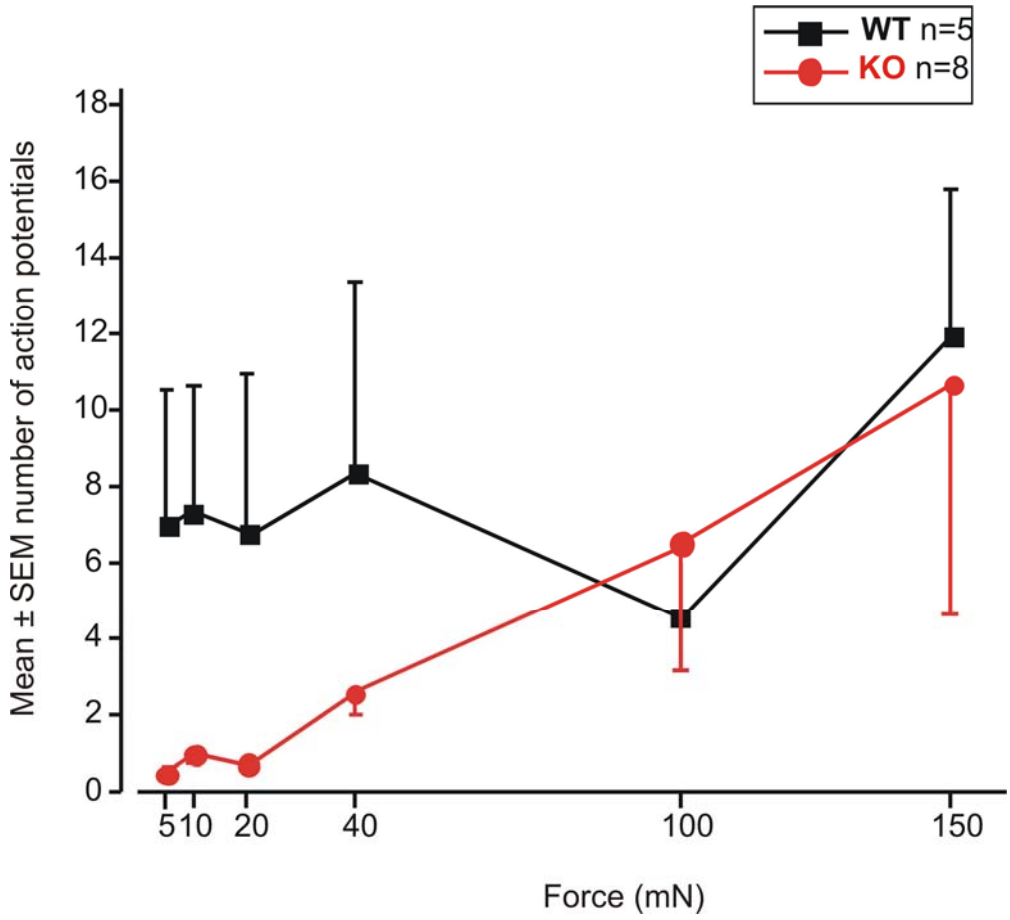

Fig.5.3.16. Mean response of rapidly adapting (RA) fibres to constant force stimuli in glabrous skin
RA fibres from WT mice generated significantly more impulses ($p<0.001$, one way ANOVA) between constant forces of 5 and 40 mN compared to fibres from KO mice.

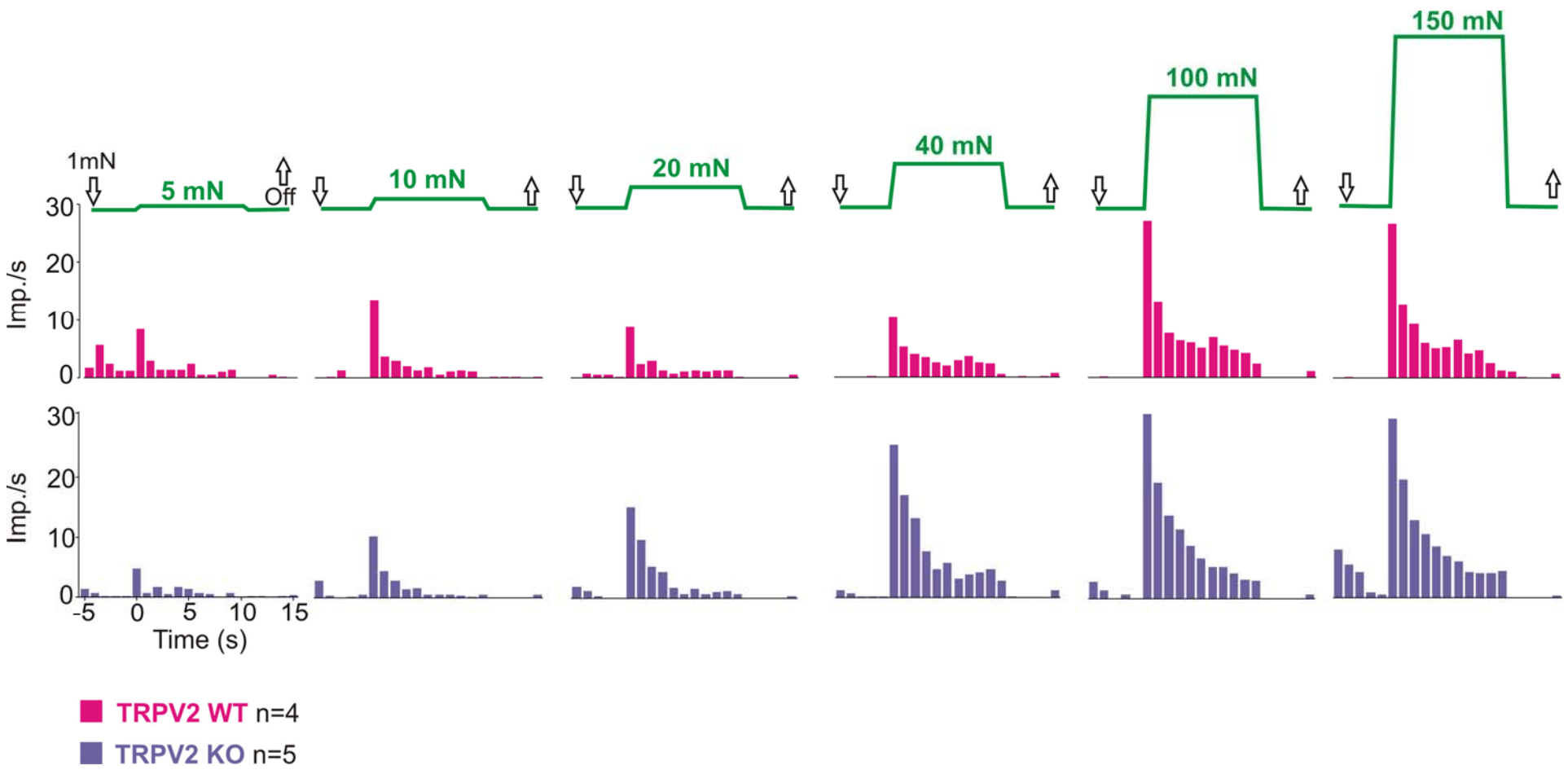

Fig.5.3.17. Mean response of slowly adapting (SA) fibres to constant force stimulation in glabrous skin
The average response of SA fibres from WT and KO animals revealed that there was no noticeable difference in the firing pattern or response to constant force stimuli.

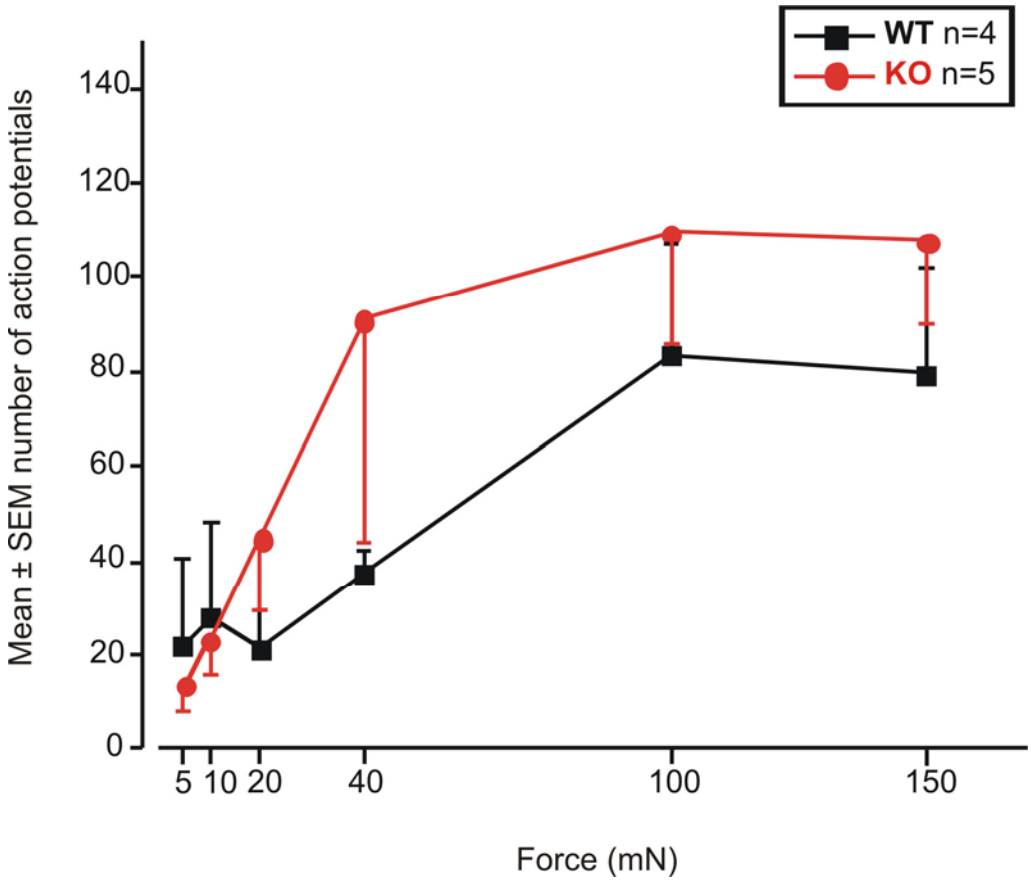

Fig.5.3.18. Mean response of slowly adapting (SA) fibres to constant force stimuli in glabrous skin

There appeared to be a trend of SA fibres from the KO to be more responsive to constant force stimuli of 20 mN-150 mN. However, statistically there was no significant difference in the response between fibres from WT and KO mice ($p>0.2$, one way ANOVA).

Aδ fibres

High threshold mechano-A (AM) nociceptors

29 AM fibres were recorded from WT and 10 from KO mice. In particular, a higher proportion of AM fibres were recorded, since we were interested in investigated whether heat sensitivity among these fibres differed from TRPV2 WT and KO mice. The mean conduction velocity of AM fibres from the WT mice was 6.7 ± 0.9 m/s and 6.9 ± 1.5 m/s from KO mice. There was no significant difference in the conduction velocity between both genotypes ($p>0.9$, unpaired t-test).

None of the AM fibres from WT mice responded to noxious heat. 12 out of 29 AM fibres were further tested with a 50 °C heat stimulus, but none responded. 1 AM fibre (3 %) responded to a cold stimulus (AMC) with a response threshold of 20 °C. 1 out of 10 (10 %) AM fibres from the KO responded to heat (AMH), with a response threshold of 41.7 °C (Fig.5.3.19, see Table.5.3.3). 8 out of 10 AM fibres were further tested with a heat stimulus of 50 °C, but none became heat responsive. None responded to cold.
Plotting the mean response to a mechanical ramp (0-200 mN) revealed that there was no major difference in the firing pattern between AM fibres from WT and KO animals (Fig.5.3.20A). Although on average there was a trend of AM fibres from WT mice to display a greater response than fibres from the KO during the mechanical ramp, this did not reach statistical significance ($p>0.8$, unpaired t-test, Fig.5.3.20B). There was no significant difference in von Frey hair thresholds between WT (8 mN (5.7, 16), n=29) and KO (8 mN, (8, 22.6), n=10) fibres ($p>0.8$, Mann Whitney U test).

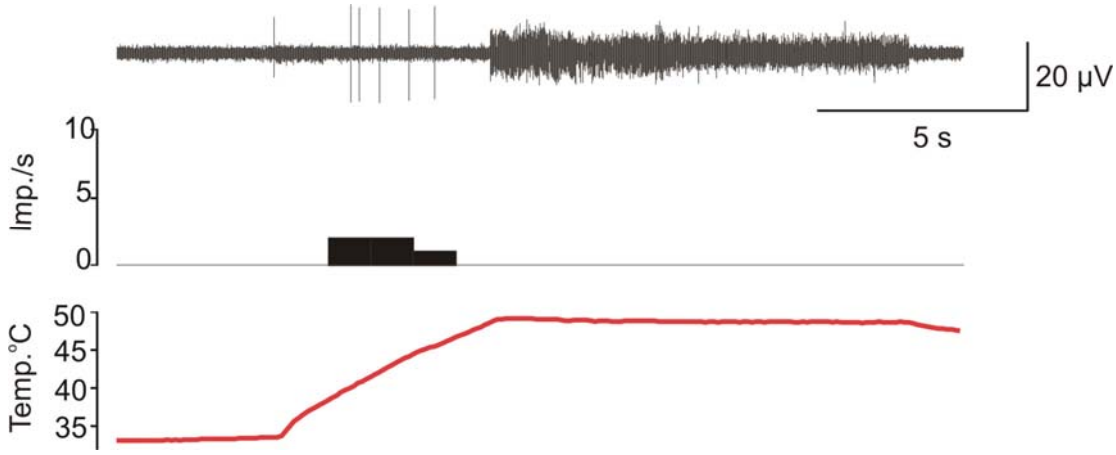

Fig.5.3.19. Aδ nociceptor response to noxious heat (AMH) in glabrous skin

The AM fibre responded to both the first heat stimulus (not shown 47 °C) and to a second heat stimulus of 50 °C.

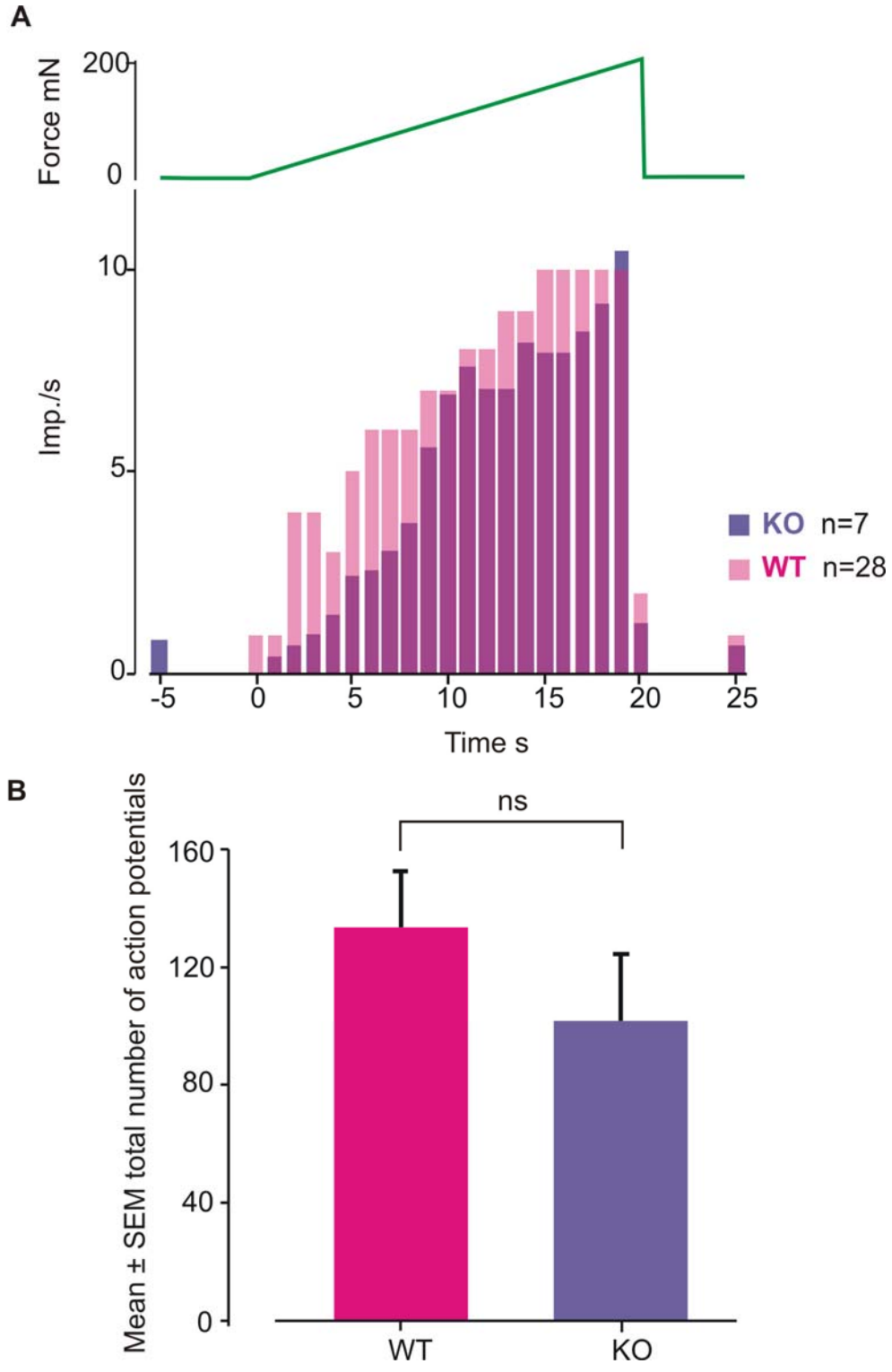

Fig.5.3.20. Mean response of high threshold mechanically sensitive nociceptors (AM) to a mechanical ramp stimulus (0-200 mN) in glabrous skin

(A) AM fibres from both WT and KO mice displayed an increase in firing as the force of the mechanical stimulus increased. AM fibres from WT mice discharged at a slightly higher rate compared to fibres from KO mice. (B) There was no significant difference in the mean total response during the mechanical ramp between AM fibres from WT and KO mice (p>0.8, unpaired t-test).

C fibres

23 high threshold mechanically sensitive C (CM) fibres were recorded from WT and 10 were recorded from KO mice. The mean conduction velocity of CM fibres from WT mice was 0.55 ± 0.03 m/s and 0.55 ± 0.02 m/s from KO mice. There was no significant difference in C fibre conduction velocity between WT and KO animals ($p>0.9$, unpaired t-test).

High threshold mechano-heat sensitive C fibres (CMH)

7 out of 23 (30 %) CM fibres responded to heat (CMH) in WT mice, with a mean response threshold of 35.2 ± 1.1 °C (see Table.5.3.3). 5 out of 10 (50 %) CM fibres responded to noxious heat from KO mice, with a mean threshold response of 36.0 ± 0.8 °C, which was not significantly different from that of the WT ($p>0.2$, Mann-Whitney U test). There was no difference in the proportion of heat sensitive C fibres between WT and KO animals ($p>0.3$, χ^2 test). An example of a CMH fibre recorded from WT and KO animals are illustrated in Fig.5.3.21A and B respectively. Plotting the mean response to a heat stimulus revealed that in contrast to CMH fibres from mice lacking TRPV2, fibres from the WT did not encode the stimulus (Fig.5.3.22). CMH fibres from the KO displayed a higher peak discharge rate of 10.9 impulses per second compared to 5.9 impulses per second in WT mice. Although the mean total number of discharges during the heat stimulus from KO animals was higher than that from WT animals, the difference did not reach statistical significance ($p>0.7$, Mann-Whitney U test, see Fig.5.3.22B).

High threshold mechano-cold sensitive C fibres (CMC)

5 out of 23 (22 %) of CM fibres responded to noxious cold (CMC) in the WT, compared to 1 out of 10 (10 %) in the KO animal. The average response threshold to cold in the WT was 14.0 ± 2.1 °C and 11.7 °C in the KO animal. CMC fibres from the WT appeared to be more responsive to cold compared to the fibre recorded from the KO animal (Fig.5.3.23), but since only 1 unit was recorded from the KO, no comparison was possible.

Mechanical sensitivity in C fibre nociceptors

The average response to a mechanical ramp (0-200 mN) was compared between AM fibres from WT (n=18) and KO (n=9) animals. On average as the stimulus force increased, the discharge rate also increased from fibres of both genotypes. On average fibres from WT mice displayed a slightly greater response compared to those from the KO (Fig.5.3.24A). Although, CM fibres from the WT generated a higher mean total number of spikes during the mechanical ramp, the difference was statistically insignificant ($p>0.2$, unpaired t-test, Fig.5.3.24B). There was no significant difference ($p>0.9$, Mann-Whitney U test) in von Frey hair thresholds between CM fibres recorded from WT (16 mN (8, 32), n=23) and KO (16mN (8, 32), n=10) mice.

TABLE.5.3.3. Heat thresholds and responses of thin myelinated Aδ and unmyelinated C fibres in TRPV2 wild type and knockout animals, recorded from hairy and glabrous skin

Genotype	Preparation	Fibre type	n	Heat threshold °C	Mean discharges during heat ramp
TRPV2 WT	Saphenous	AMH	1	43	4
		CMH	3	34.7 ± 1.7	25 ± 4
TRPV2 KO	Saphenous	AMH	0	-	-
		CMH	3	37.8 ± 3.9	38.7 ± 20
TRPV2 WT	Tibial	AMH	0	-	-
		CMH	7	35.2 ± 1.1	52.8 ± 20
TRPV2 KO	Tibial	AMH	1	41.7	8
		CMH	5	36.0 ± 0.8	87.2 ± 18

All values are means ± SE; AMH, high threshold mechano-heat A fibre; CMH, C mechano-heat sensitive nociceptors

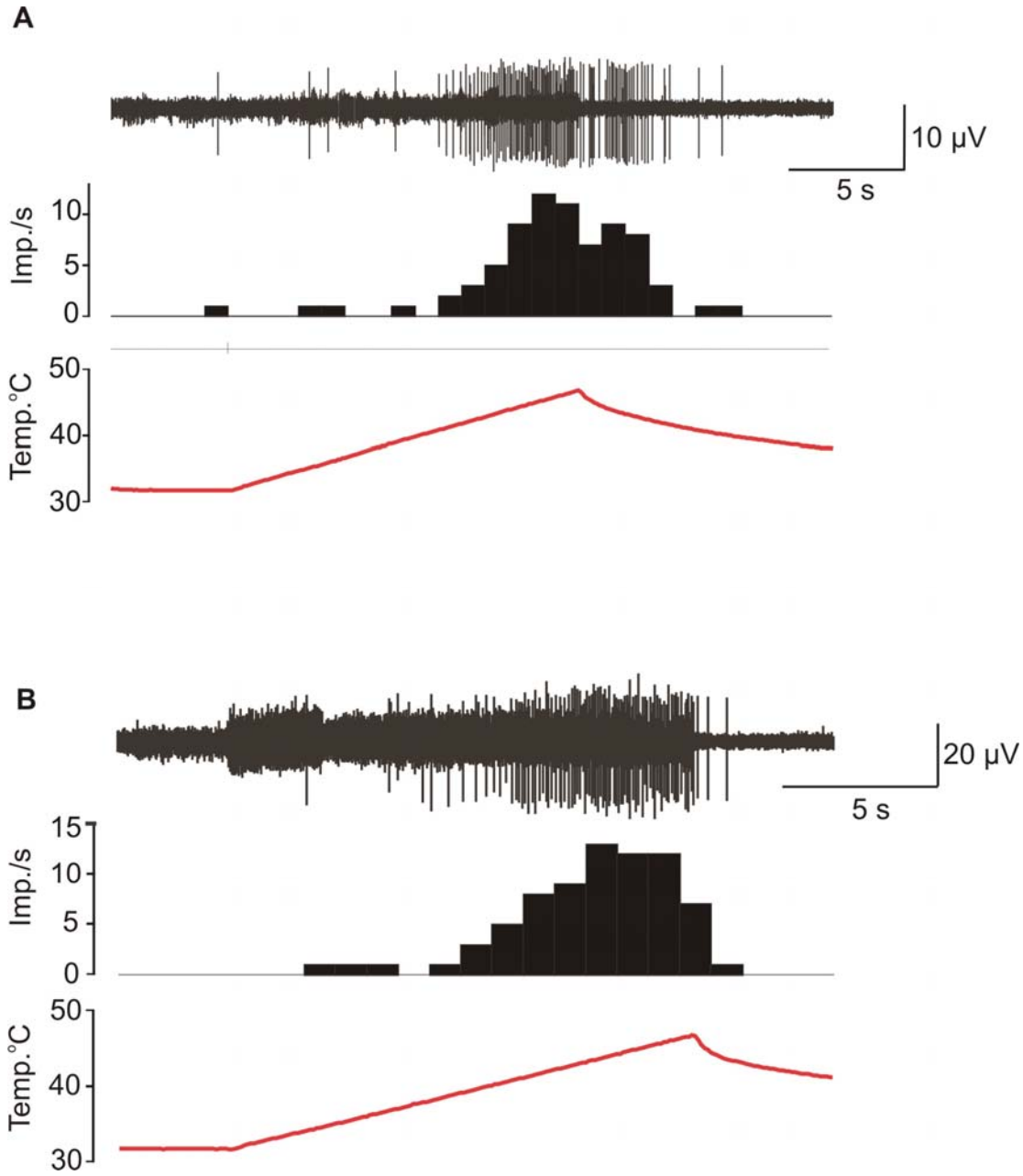

Fig.5.3.21. Example heat responses of CMH fibres recorded from TRPV2 wild type (WT) and knockout (KO) mice in the glabrous skin
An example of a CMH fibre recorded from a TRPV2 WT (A) and KO mouse (B) is shown above. There was no significant difference ($p>0.3$, χ^2 test) in the proportion of heat sensitive C fibres between WT and KO animals.

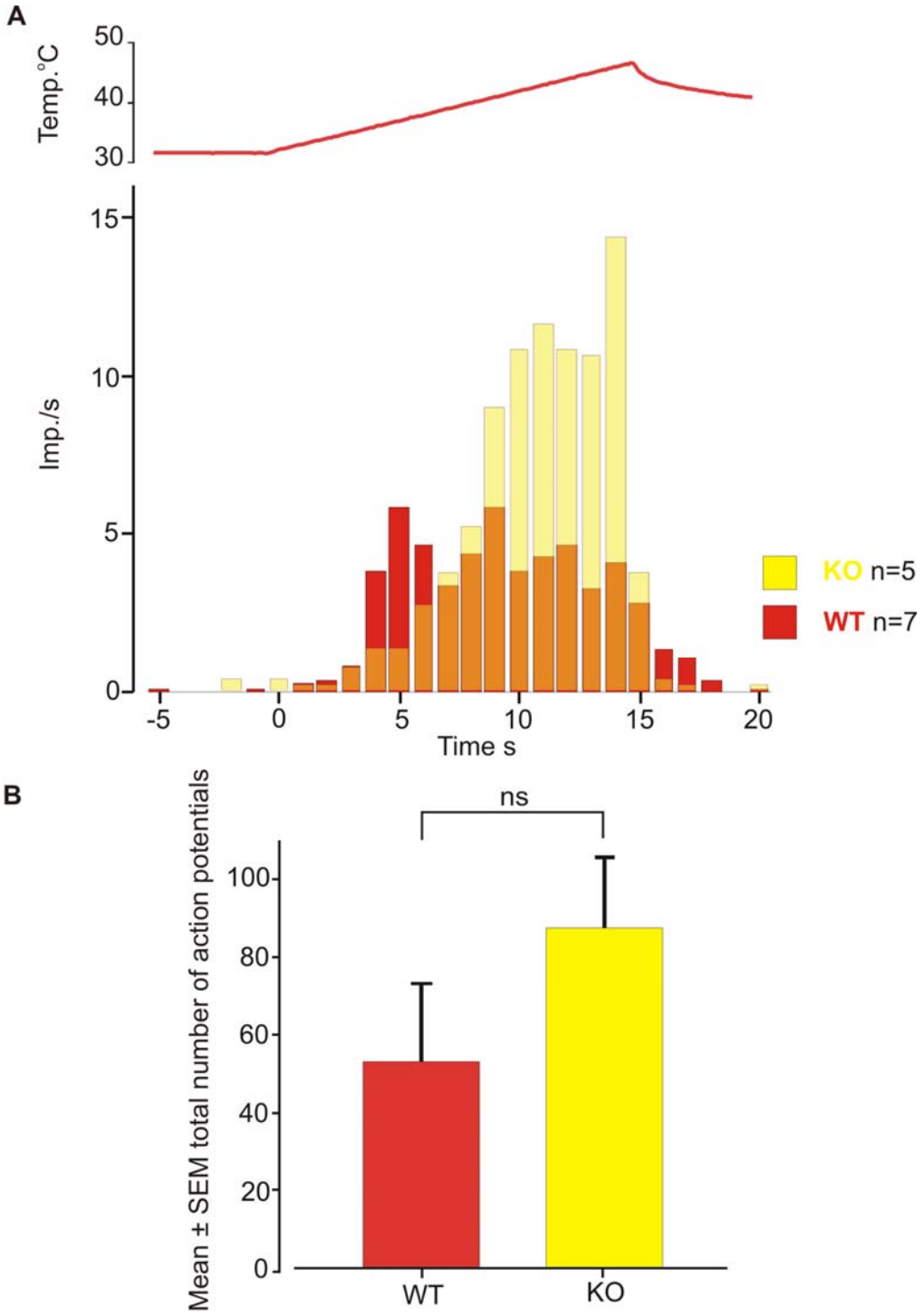

Fig.5.3.22. Average response of C mechano-heat sensitive (CMH) fibres to a noxious heat stimulus in glabrous skin

(A) CMH fibres from KO mice displayed an increase in firing frequency as the temperature increased, and therefore encoded the heat stimulus. This was in contrast to CMH fibres from the WT, which did not appear to encode the stimulus intensity. CMH fibres lacking TRPV2 also displayed a greater peak discharge rate of 10.9 imp./s compared to only 5.9 imp./s in fibres from WT mice. (B) Although CMH fibres recorded from KO animals displayed a higher mean total number of discharges during the heat stimulus compared to those from WT, this difference was statistically insignificant ($p>0.7$, Mann-Whitney U test).

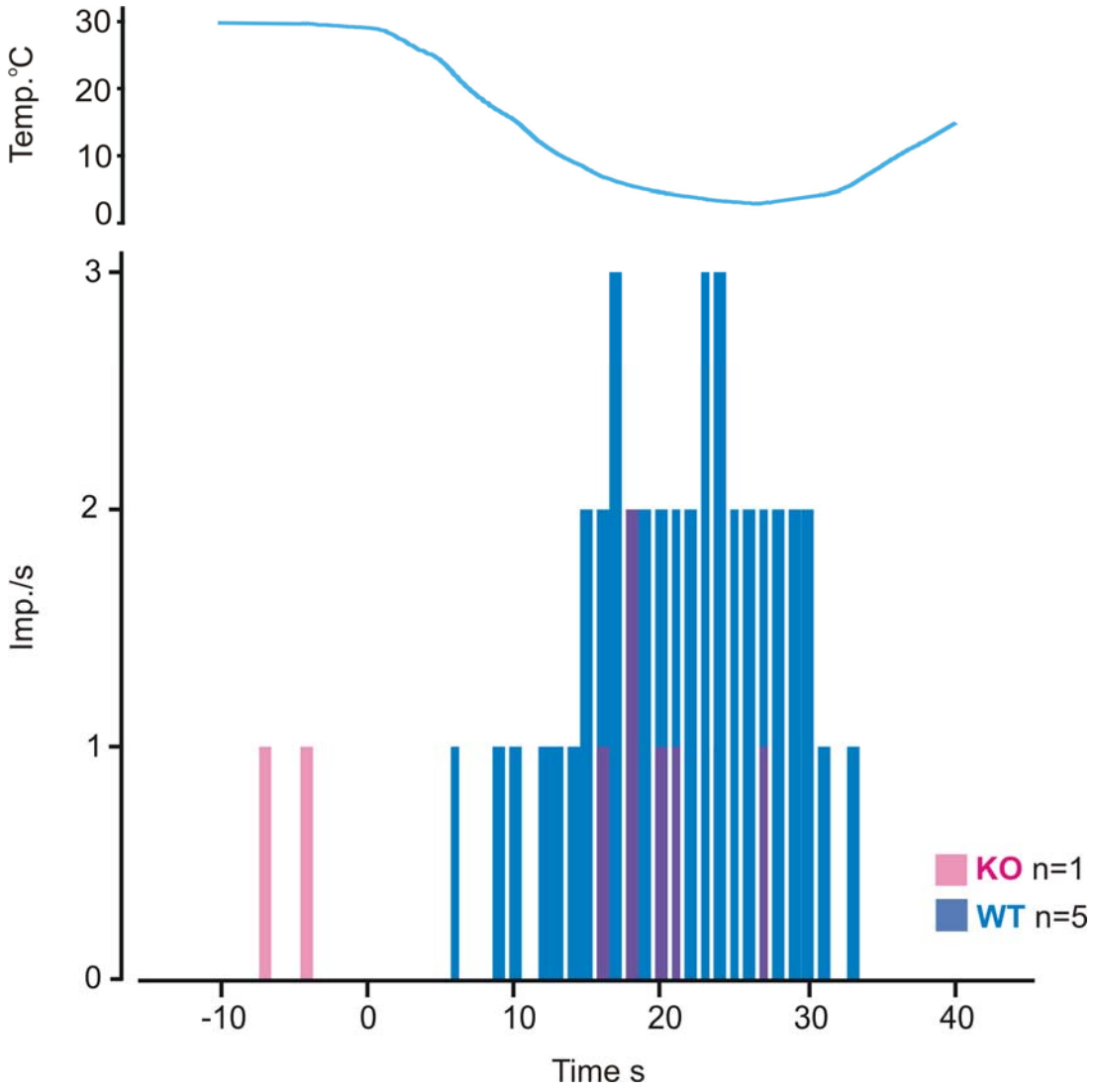

Fig.5.3.23. Average cold response of mechano-cold sensitive C (CMC) fibres in the glabrous skin

On average, CMC fibres from WT animals (n=5) displayed a higher response to cold compared to the CMC fibre recorded from the KO animal (n=1). However, since only one CMC unit was recorded from the KO, a statistical comparison could not be made.

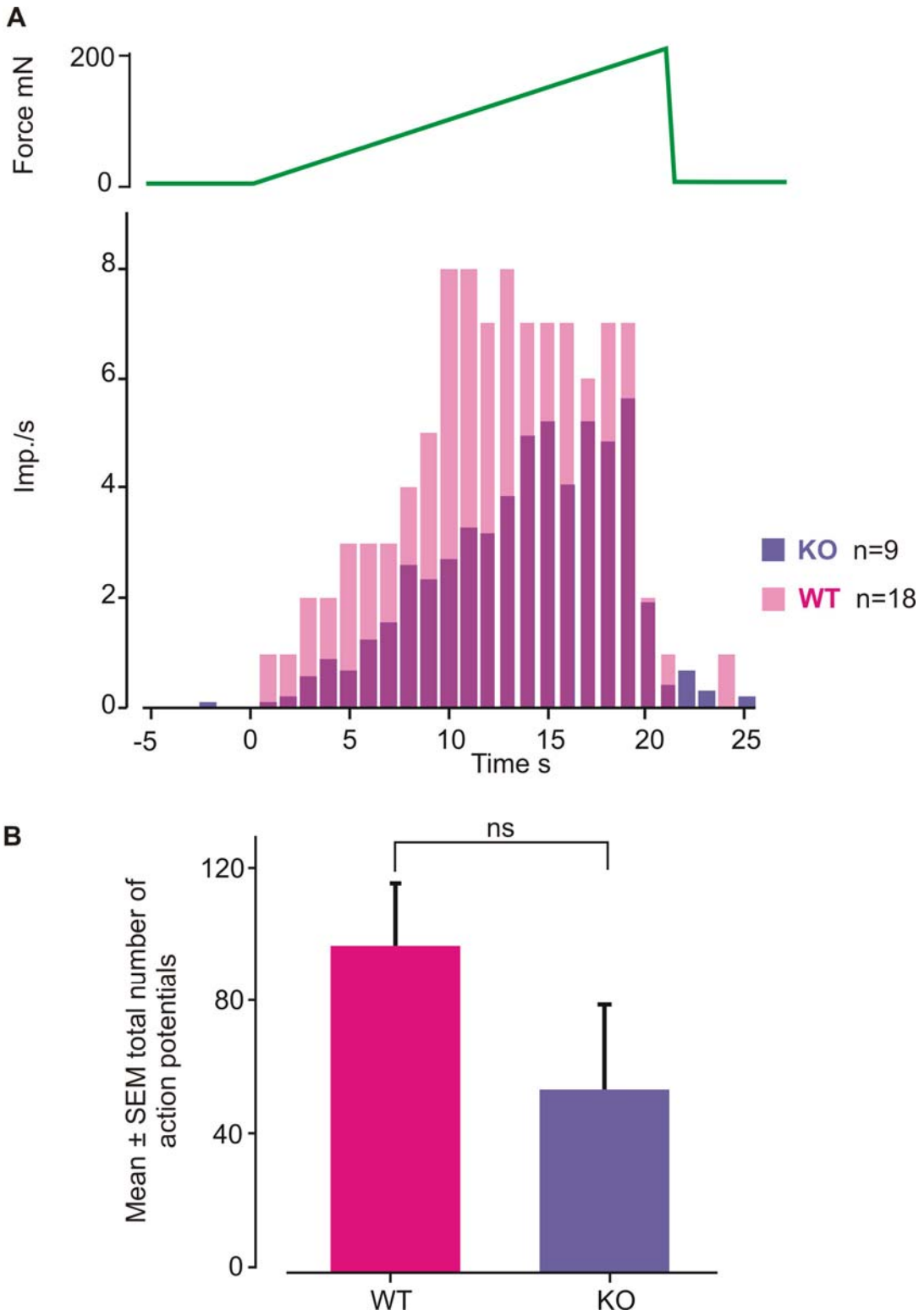

Fig.5.3.24. Average response of C mechano-sensitive nociceptors (CM) to mechanical stimulation in the glabrous skin

(A) CM fibres from the WT displayed a greater response during the mechanical ramp compared to CM fibres from the KO. (B) There was no significant difference in the total mean response during the mechanical ramp between fibres from WT and KO animals (p>0.2, unpaired t-test).

5.4 Discussion

This is the first time that the receptive properties of cutaneous afferents innervating the hairy and glabrous skin of mice lacking the TRPV2 receptor have been studied. We found that overall there were only small differences in receptive properties of afferents lacking the TRPV2 receptor in both hairy and glabrous skin.

The same proportion of mechanically heat sensitive A and C fibre nociceptors were found in mice of both genotypes. Heat thresholds of C fibres recorded from mice lacking TRPV2 did not differ significantly from those recorded from wild-type mice. However, CMH fibres from TRPV2 null mice did differ in their firing pattern during a noxious heat stimulus. No significant differences in cold sensitivity were observed.

In general we found that fibres lacking TRPV2 retained their mechanical sensitivity, but subtle differences in firing pattern to controlled mechanical stimuli were observed in both RA and DH fibres.

The role of TRPV2 as a heat transducer on mechanically heat sensitive A-fibre nociceptors (AMH) remains undefined

TRPV2 is hypothesised to be the heat transducer in AMH type I fibres and therefore we were interested in investigating whether the proportion of heat sensitive AM fibres differed between the two genotypes.

We found that only 1/19 (5 %) of Aδ nociceptors responded to noxious heating (AMH) in the hairy skin of a TRPV2 wild-type mouse, compared to none (0/13) in the TRPV2 null mouse. In glabrous skin none (0/29) of the AM fibres responded to noxious heat in the TRPV2 wild-type mice, whereas 1/10 (10 %) responded in the TRPV2 null mouse. The heat threshold of the AMH fibre recorded from the hairy skin was 43 °C, and 41.7 °C for the AMH fibre recorded from the glabrous skin. Due to the low occurrence of AMH fibres in both hairy and glabrous skin, we cannot say for certain whether TRPV2 is essential for the transduction of heat in these fibres.

To ensure that we were not underestimating the proportion of heat sensitive A-fibres because of the use of a low intensity stimulus, a second heat stimulus reaching 50 °C on the corium side of the skin (reaching ~55 °C on the

epidermal side) was applied for 30 s. However, this failed to excite any previously heat insensitive units.

However, the low proportion of heat sensitive A-fibre nociceptors found in this study is consistent with results from previous studies that carried out single unit recordings in the rodent (and consistent with the low proportion of heat sensitive AM fibres recorded from mouse hairy and glabrous skin in chapter 3). Lynn and Shakhanbeh (1988) investigated the responses of Aδ nociceptors in rat hairy and glabrous skin to repeated heat stimulation in vivo. Fourteen AM fibres were tested with two consecutive heat stimuli of 55 °C, of which none responded (Lynn and Shakhanbeh, 1988). When a 60 °C heat stimulus was applied, 1/15 AM fibres in the hairy and 1/14 in the glabrous skin fired weakly (between 1-4 spikes). 27 AM units were tested with another 1-4 heat trials (60 °C, 15 s) and from these another 4 units responded to the third trial. Therefore rat AM fibres were almost unresponsive to skin heating. Similarly, only 1/6 AM fibres responded to a second noxious heat stimulus in the rat hairy skin (Lynn and Carpenter, 1982). In mouse glabrous skin in vivo, 3/25 (12 %) AM fibres responded to noxious heat with an average heat threshold of 42 ± 3 °C (Cain et al., 2001). Other studies found a slightly higher proportion of AMH fibres, 5/19 (26 %, average heat threshold 42.5 ± 1.4 °C) and 8/41 (20 %, heat threshold between 47-52 °C) in mouse (Koltzenburg et al., 1997) and rat hairy skin (Koltzenburg et al., 1997;Leem et al., 1993)Leem et al., 1993) respectively. Much higher proportions of heat sensitive A-fibre nociceptors have been recorded from the hind limb of cat (~50 %) and monkey (Beck et al., 1974;Campbell et al., 1979), suggesting that there may be species differences. The differences in heat induced flexion reflexes suggests that there might also be strain differences. In the hybrid mice used in the present study, virtually no heat sensitive Aδ fibres exist.

It is also important to note that A-fibre nociceptors make up between 12-20 % of total cutaneous sensory neurons (Lewin and Moshourab, 2004;Lynn and Shakhanbeh, 1988) and if approximately 20 % of these are heat sensitive (AMH) this only accounts to 2.4-4 % of the total sensory neurons in a nerve. This may explain why very few AMH fibres were found in this study.

Since we only recorded from mechanically sensitive afferents we cannot rule out the possibility that TRPV2 may have an important role in transducing noxious heat in mechanically insensitive A-fibre nociceptors or those which have very high mechanical thresholds. Interestingly, during the study 2 mechanically insensitive C fibres which responded to the 50 °C heat stimulus in the hairy skin of TRPV2 wild-type mice were found, but such A-fibres were never found. Moreover, the majority of mechanically insensitive A fibres have been shown to be responsive to capsaicin (Ringkamp et al., 2001) suggesting that heat responses in these fibres might be mediated by TRPV1. Nevertheless, it is possible that TRPV2 may also be expressed with TRPV1 on these fibres. This may seem unlikely, since TRPV1 and TRPV2 have been shown to be expressed on distinct populations of sensory neurons in the mouse (Caterina et al., 1999) and in the rat DRG (Ahluwalia et al., 2002). However, TRPV1 was found to be expressed in around 20 % of TRPV2 immuno-positive rat DRG neurons, representing co-expression in 1.7 % of all neurons (Greffrath et al., 2003). In the rat DRG, 38 % of TRPV1 positive neurons were found to express TRPV2 (Rutter et al., 2005). Shimosato et al (2005) found that 7 % of TRPV1 expressing neurons expressed TRPV2 in rat DRG (Shimosato et al., 2005). Other studies have also reported TRPV2 co-localisation with TRPV1 in some medium sized cells in the rat DRG (Liapi and Wood, 2005;Rau et al., 2007). Therefore co-expression of TRPV1/2 in a small percentage of neurons may correspond to the AMH fibres which express both TRPV1 and TRPV2.

Using the ex-vivo preparation, in which intracellular recordings were carried out from mice lacking TRPV1, it was shown that only 1 out of 12 heat sensitive C fibres stained positively for TRPV2 (Woodbury et al., 2004). Using the same preparation it was shown that only 1 out of 6 A-fibre heat sensitive nociceptors stained positive for TRPV2 and only a small number of TRPV2 immuno-positive cells responded to heat (3/21) (Lawson et al., 2008). Both these studies indicate that TRPV2 is not well correlated with heat sensitivity and therefore is unlikely to be the heat transducer in rodent A-fibre nociceptors.

In mice lacking TRPV2, mechanically sensitive C-fibre nociceptors retain their heat sensitivity. However, CMH fibres lacking TRPV2 display a different firing pattern during a noxious heat stimulus

There was no difference in the proportion of heat sensitive C fibres found in hairy or glabrous skin between mice of either genotype. There was no significant difference in the mean heat threshold or the average number of action potentials elicited to a standard heat stimulus in CMH fibres lacking TRPV2, from both hairy and glabrous skin. This indicates that TRPV2 is not essential for transducing heat in these fibres or setting their thermal thresholds.

Interestingly however, when we compared the average discharge pattern to heat, we noticed that differences existed. CMH fibres from the hairy skin of TRPV2 wild type mice appeared to encode the heat stimulus better, increasing in discharge frequency as the temperature increased. In contrast, CMH fibres from mice lacking TRPV2 did not encode the stimulus and displayed an early peak discharge half-way into the heat stimulus, which was then followed by a lower level of firing.
The opposite trend was found in the glabrous skin. On average CMH fibres from mice lacking TRPV2 appeared to encode the stimulus much better than those fibres from the TRPV2 wild-type mice. CMH fibres from the wild-type mice displayed an earlier peak discharge which then appeared to plateau for the remaining duration of the stimulus. A common finding in both hairy and glabrous skin was that, CMH fibres from TRPV2 null mice displayed a higher peak discharge rate than fibres from the TRPV2 wild-type mice.

Although TRPV2 has been mainly shown to be expressed on medium sized neurons, presumably Aδ fibres, expression on C fibres has also been reported. Lawson et al (2008) found that 5 out of 24 (~21 %) of mechanically sensitive C fibres stained positive for TRPV2 and this included 2 CMH fibres (Lawson et al., 2008). In the rat DRG it was reported that TRPV2 labelled neurons included some small sized neurons (Ma, 2001) and in the dorsal horn TRPV2 staining was found in laminae I and II, suggesting TRPV2 expression on both C and Aδ fibres. In the rat DRG 5 % of peripherin (a marker for unmyelinated neurons)

expressing neurons, were found to be positive for TRPV2 (Shimosato et al., 2005). These results would support the hypothesis that TRPV2 may be co-expressed with TRPV1 on some C fibres, including CMH fibres.
In CMH fibres which express both TRPV1 and TRPV2, noxious heat may result in initial activation of TRPV1 followed by activation of TRPV2 at higher temperatures. TRPV2 may therefore contribute to the heat response at higher temperatures. This may be a possible explanation as to why CMH fibres from the hairy skin of mice lacking TRPV2 displayed an early peak discharge. The lack of TRPV2 may explain the lower level of discharge seen at higher temperatures. Caterina et al (1999) reported that co-expression of TRPV1 and TRPV2 in HEK cells resulted in a bi-modal response to heat (Caterina et al., 1999) that appeared to represent an initial response from TRPV1 followed by TRPV2. Liapi and Wood (2005) demonstrated the ability of TRPV1 and TRPV2 to interact with each other in the adult rat (Liapi and Wood, 2005). If this is the case, then it suggests the formation of heteromultimers with new physiological properties. CMH fibres expressing both TRPV1 and TRPV2 may therefore have different physiological properties compared to those that express just TRPV1.

Interestingly though, Rau et al (2007) failed to find immunoreactivity for both TRPV1 and TRPV2 from retrogradely labelled cell bodies classified as unmyelinated nociceptors (Rau et al., 2007). Futhermore, they demonstrated that in nociceptors which did express both TRPs, heat response curves were no different to those nociceptors which expressed TRPV1 alone. They also found no evidence of bimodal or distinct high-threshold heat reactivity in nociceptors co-expressing both TRP channels. This suggests that TRPV2 does not play a significant role in mediating heat responses in TRPV1 expressing heat sensitive C-fibres. Our results would agree with this, since there was no difference in the overall response or heat threshold. Nevertheless, differences in firing pattern to heat observed in this study indicate that TRPV2 may play a part in shaping the response to heat and this requires further investigation.

The involvement of TRPV2 in states of inflammation and nerve injury

In heterologous expression system Caterina et al (1999) demonstrated that when bath temperatures were raised from 22 °C to above 53 °C, inward currents were evoked in 70 % of HEK cells and 75 % of oocytes transfected with TRPV2 (Caterina et al., 1999). These findings have been confirmed by more recent studies which have demonstrated that noxious heat is capable of evoking TRPV2 activation when transfected in HEK cells (Leffler et al., 2007;Neeper et al., 2007). Despite this however, the evidence for a physiological role for TRPV2 as a transducer of noxious heat in sensory neurons remains poor (Lawson et al., 2008;Woodbury et al., 2004). Furthermore, Benham et al (2003) were unsuccessful at measuring temperature gated currents in TRPV2 transfected cells (Benham et al., 2003) and noxious heat was unable to activate human TRPV2 when transfected in HEK cells (Neeper et al., 2007). Therefore the involvement of TRPV2 in noxious heat sensing remains controversial.

Interestingly however, it has been demonstrated that when heterologously expressed, TRPV2 can be sensitised to repeated heat stimuli (Caterina et al., 1999;Leffler et al., 2007), suggesting the possibility that TRPV2 may contribute to heat sensitivity following peripheral injury.
Consistent with this hypothesis, an increase in TRPV2 expressing neurons was found after CFA induced inflammation in rats (Shimosato et al., 2005) and behaviourally this upregulation was suggested to be important in mediating heat hyperalgesia at 56 °C in inflamed tissue. A recent study reported of an increase in TRPV2 mRNA in the DRG of rats with chronic constriction-induced sciatic nerve injury (CCI) (Frederick et al., 2007).
In the present study, it was found that in previously heat insensitive A and C fibres, repeated application of heat was unable to induce sensitisation. In fact, CMH fibres desensitised to repeated applications of heat. Leffler et al (2007) demonstrated that in capsaicin-sensitive DRG neurons, the heat-activated currents displayed pronounced tachyphylaxis during three consecutive heat stimuli. It is therefore very likely that that we recorded from CMH fibres expressing TRPV1 and further to support this we have already shown (see

chapter 4) that the majority of CMH fibres recorded from this preparation are capsaicin sensitive. AM fibres did not display sensitisation to heat either and this is consistent with findings from Lynn and Shakhanbeh (1988) who demonstrated that very few AM fibres from hairy skin of the rat responded to noxious heating after repeated heat stimuli and even then the responses were not always reproducible (Lynn and Shakhanbeh, 1988). This is in contrast to findings from primates. Interestingly, Rau et al (2007) state that not all the nociceptors which expressed TRPV2 sensitised to noxious heat and this suggested that the ability of nociceptors to sensitise to heat was dependent on which cells were selected for recording (Rau et al., 2007). This also applies to the present study, since we may have recorded from fibres which do not express TRPV2 and therefore sensitisation to repeated heat stimulation was never observed.

The role of TRPV2 as a noxious heat sensor appears to be analogous to the role of TRPA1 as a noxious cold sensor. Both TRPV2 and TRPA1 have been shown to be activated by noxious heat and cold, respectively, when expressed in heterologous systems. However, convincing evidence for a physiological role in normal sensory neurons is lacking for both these receptors. It now appears that the role of TRPA1 is more significant in states of injury and inflammation and that its interaction with TRPV1 may be important in mediating responses to inflammatory mediators. Similarly, further investigation might reveal whether this is also the case for TRPV2.

TRPV2 may play a role in mechano-transduction

In 2003, Muraki and colleagues demonstrated TRPV2 expression in mice aortic myocytes and also that it functioned as a calcium entry channel that could be activated by cell swelling induced by hypotonic solutions (Muraki et al., 2003). Treatment of mouse aorta with TRPV2 antisense DNA, reduced the amount of TRPV2 protein and suppressed the activation of the channel in response to hypotonic stimulation. They further went on to show that when TRPV2 was transfected into Chinese hamster ovary (CHO) cells, the channel could be activated by membrane stretch (Muraki et al., 2003). Another study suggested that TRPV2 may mediate calcium entry in response to physical stimulation of

mast cells (Stokes et al., 2004). Findings of these studies indicate that TRPV2 is capable of responding to mechanical stimuli and that therefore it may play a role in regulating mechanosensitivity. However, whether TRPV2 contributes to the ability of sensory neurons to respond to mechanical stimulation is currently unknown.

We show for the first time that in mice lacking TRPV2, fibres innervating both the hairy and glabrous skin retain their mechanosensitivity, indicating that TRPV2 is not essential for the transduction of mechanical stimuli. However, we observed several differences in the stimulus response functions to controlled mechanical stimuli in some groups of fibres, indicating that TRPV2 may regulate the receptive properties of some groups of fibres.
In the hairy skin, slowly adapting (SA) fibres from mice lacking TRPV2 were particularly more responsive during the onset and offset of stimulus than fibres recorded from wild type animals. The opposite trend was observed in D-hair fibres; those recorded from the wild type animals were mechanically more sensitive during the application of low forces (between 5-20 mN). No significant differences were observed in A-fibre nociceptors. C-fibre nociceptors from TRPV2 null mice were significantly more responsive during application of a mechanical ramp compared to fibres recorded from wild type animals.
In the glabrous skin, RA fibres from TRPV2 wild type mice were more responsive during the onset and offset of the mechanical stimulus. SA fibres and A and C-fibre nociceptors did not display any significant differences in their response properties to mechanical stimuli. These observations suggest that the absence or presence of TRPV2 may affect the sensitivities of certain fibre populations to mechanical stimuli.

Tamura et al (2005) investigated the neurotrophin dependence of TRPV2 positive neurons in the developing and adult mouse DRG. Immunofluorescence staining revealed that the neurotrophin-3 (NT3) receptor, TrkC was expressed in most TRPV2-positive DRG neurons at E11.5 and E13.5. In the adult DRG, TrkC was expressed in ~68 % of TRPV2 positive neurons and the expression of TrkB was observed in ~9 % of TRPV2 positive neurons (Tamura et al., 2005). This suggests that TRPV2 may be expressed on sensory neurons which are mainly dependent on NT3 and possibly also to BDNF.

Interestingly, using the skin nerve preparation, Airaksinen et al (1996) reported that in adult heterozygous NT3 null mice, 78 % of SA fibres and 50 % of DH fibres were absent (Airaksinen et al., 1996), indicating that NT3 is required for their survival. Over-expression of NT3 in mice increases mechanical sensitivity in SA and AM fibres (McIlwrath et al., 2007).
In mice lacking BDNF, SA fibres and not other types of cutaneous afferents displayed a reduction in their mechanical sensitivity (Carroll et al., 1998). Since mechanical sensitivity in SA, DH and AM is dependent upon levels of NT3 and/or BDNF, it raises the possibility that TRPV2 may also be expressed on some of these fibres.

Further support for TRPV2's role in mechanotransduction has come from a study by Lawson et al (2008). They found that almost half (12/23) of A-fibre nociceptors and 43 % (3/7) RA fibres stained positive for TRPV2. Some staining was also seen in SA fibres (1/6) and 21 % (5/24) of C fibre nociceptors (Lawson et al., 2008). They reported no difference in the firing rates to mechanical stimulation between AM TRPV2 positive and negative fibres. They concluded by stating that mechanical sensitivity and not heat was the strongest functional correlate of TRPV2 immunostaining.

We did not observe a clear trend as to whether lack of TRPV2 correlated with a decrease or increase in mechanosensitivity. This may indicate that different sub-populations of mechanically sensitive fibres express differential amounts of TRPV2. Or else the contribution of TRPV2 in response to mechanical stimulation may vary according to which fibre type it is expressed in. Since we have shown that fibres retain their mechanosensitivity in the absence of TRPV2, it indicates that other receptors or ion channels (discussed below) are present which mediate mechanotransduction in these fibres. TRPV2 may interact with these mechanisms to ultimately 'shape' their response to a mechanical stimulus.
Using the skin nerve preparation, Price et al (2000) found that in mice lacking the brain sodium channel 1 (BNC1, also called Acid sensitive ion channel 2 (ASIC2)), RA mechanoreceptors displayed a reduced discharge frequency in

response to mechanical stimuli (Price et al., 2000) and further went onto show that the BNC1 was expressed in fibres which surrounded hair follicles.
Shin et al (2003) identified a T-type calcium channel, $Ca_v3.2$ specifically expressed in D-hair receptors (Shin et al., 2003). Dubreuil et al (2004) further showed that T-type calcium current functioned by lowering the activation threshold of D-hair afferents, which accounted for the high mechanical sensitivity in these fibres (Dubreuil et al., 2004).

Taking the above findings into consideration, it is possible that mechanical deformation of sensory terminals leads to activation of a channel complex made up of many components, such as the ones mentioned above and also TRPV2. TRPV2 may therefore be involved in generating a normal graded response to mechanical stimuli.

Conclusions

This is the first time that the receptive properties of afferents innervating the hairy and glabrous skin from mice lacking the TRPV2 receptor have been studied in vitro.
We conclude that TRPV2 does not appear to be essential in mediating noxious heat sensitivity in nociceptors innervating the hairy or glabrous skin. Since we failed to observe heat sensitisation in A and C fibre nociceptors in the hairy skin of wild type (C57Blk/6) mice, this does not appear to be an important mechanism in rodents. It is important to note however, that since our recording technique was biased towards recording from mechanically sensitive afferents, we cannot exclude sensitisation of mechanically insensitive afferents (MIA). Using an electrical, instead of a mechanical search stimulus to locate receptive fields would help to find these MIAs and test whether heat sensitivity is present. It is also possible that TRPV2 plays a more significant role in nociceptors which innervate deep tissue sites rather than the skin as shown by Rau et al (2007). This requires further investigation.
Interestingly, mice lacking the TRPV2 receptor display no behavioural deficits in response to noxious heat over 52 °C in vivo (M.J.Caterina, 2008, personal communication) and therefore indicates that TRPV2 is not required for the detection of noxious heat in vivo.

Since TRPV2 has been hypothesised to mediate high threshold heat responses in a subpopulation of capsaicin insensitive neurons one would expect to see a lack of these responses in cultured neurons lacking TRPV2.

Finally we demonstrated that some subpopulations of fibres differed in their response properties to controlled mechanical stimulation. Importantly, mechanosensitivity was retained in all fibre populations indicating that TRPV2 is not essential for the transduction of mechanical stimuli. In particular we showed that D-hair units and RA fibres from glabrous skin of mice lacking TRPV2 were mechanically less sensitive. Recording from a larger sample of RA units would confirm whether this is also the case for RA fibres from hairy skin. It would be interesting to investigate whether TRPV2 is localised in nerve endings around hairs in rodent skin.

The current and previous results chapter (chapter 4) have so far focussed on investigating the role of thermally activated TRP ion channels and their role in thermal sensation. The next results chapter (chapter 6) will investigate the role and contribution of potassium conductances in mediating cold sensitivity in primary afferent neurons in the rat.

Chapter 6

The role of voltage gated potassium channels in mediating cold sensitivity in primary afferent neurons

6.1 Introduction

Potassium (K^+) channels have an important role in regulating neuronal excitability. Their role involves setting the resting membrane potential, keeping fast action potentials short, terminating periods of intense activity and regulating the time between successive action potentials (Hille, 1992).

K^+ channels are made up of four pore forming α subunits that associate with several different types of β-subunits which modify their function (Ashcroft F, 2000). K^+ channels are classified structurally according to the number of transmembrane domains contained within the α subunit. The classic voltage gated K^+ channels (K_V channels), also called delayed rectifiers, have six transmembrane domains (S1-6) and fall into nine sub gene families (K_V1-9). They are typically closed at the resting membrane potential, but open on membrane depolarisation. When open they drive the membrane potential towards the potassium equilibrium, repolarising the cell and thereby decreasing the excitability of the cell (Julius and McCleskey, 2005). K_V channels can form homomeric channels (formed from identical α subunits) and functional heteromeric channels (from different α subunits of the same family).

Several studies have investigated the expression and role of K^+ currents in rodent dorsal root ganglion (DRG) neurons using the traditional K^+ channel blockers 4-aminopyridine (4-AP) and tetraethylammoniam ion (TEA) (Akins and McCleskey, 1993;Everill and Kocsis, 2000;Everill and Kocsis, 1999;Fedulova et al., 1998;Gold et al., 1996). Using the patch clamp technique, Akins and McClesky discovered that cultured sensory neurons from the adult rat expressed both transient and sustained outward K^+ currents. The transient current was found to be blocked by 4-AP, whereas the sustained current was blocked by TEA (Akins and McCleskey, 1993). Later, in 1996, Gold and colleagues provided evidence for the existence of at least six types of voltage gated K^+ currents in sensory neurons from adult rat, three transient ones and three sustained ones (Gold et al., 1996). Of the transient currents, one was

found mainly to be expressed in larger sized neurons and displayed sensitivity to 4-AP. The second transient current was selectively expressed in small diameter, capsaicin responsive neurons and was sensitive to both 4-AP and TEA. The third transient current was also found in capsaicin sensitive cells, but was only sensitive to 4-AP. Two of the sustained currents were blocked by TEA, but not 4-AP, whereas the other sustained current was insensitive to both TEA and 4-AP. Safronov et al (1996) found that potassium currents in small DRG neurons were made up of one fast inactivating current and four delayed rectifying currents (Safronov et al., 1996). Fedulova et al (1998) isolated three potassium currents from rat DRG neurons and found that all cells expressed varying amounts of all three currents (Fedulova et al., 1998). They found that the fast inactivating K^+ current was blocked by 2 mM 4-AP, but not 10 mM TEA. A transient slow inactivating K^+ current was partially reduced by both 4-AP and TEA and a non-inactivating current was reduced by TEA, but not 4-AP. Everill and colleagues identified three potassium currents in large sized DRG neurons; a dominant sustained current (I_K) and two transient currents I_A and I_D (Everill et al 1993, Everill and Kocsis 2000).

In summary, the results of these studies indicate the existence of at least three different K^+ currents in the DRG; two transient currents and one sustained or non-inactivating current. The two transient currents, also referred to as A-type currents, are composed of one rapidly inactivating (I_A) and one slowly inactivating (I_D) current which are sensitive to 4-AP. The sustained current is sensitive to TEA. However, it is clear, as shown by Gold et al (1996) that more than three K^+ currents exist in the DRG and this seems likely considering the ability of α subunits to form heteromultimers.

Interestingly, potassium currents have been shown to be involved in mediating cold sensitivity in DRG neurons (Reid and Flonta, 2001;Viana et al., 2002). Reid and Flonta hypothesised the involvement of an ionic current in cold thermoreception and predicted that cold would either activate an inward current or inhibit an outward one. They found that cooling increased the input resistance of all neurons indicating that it inhibited an outward current which was active at the resting membrane potential. Upon cooling, there was a greater increase in the input resistance of cold sensitive compared to cold

insensitive neurons. The current was identified as a cold sensitive outward potassium current, I_{COLD} and was found to be largely resistant to 4-AP and TEA. During cooling, inhibibition of this current brings cold sensitive neurons to firing threshold. The four transmembrane (4TM) family of potassium channels, also known as the two-pore domain potassium (K_{2P}) channels, are open at all voltages and are thought to underlie leak currents in neurons. TREK-1 which belongs to this family, was shown to be inhibited by cooling (Maingret et al., 2000), therefore making it a candidate molecule for underling the I_{COLD} current. Additionally, TREK-2 and TRAAK, also members of the K_{2P} family, have also been shown to be active at the resting membrane potential and sensitive to temperature (Kang et al., 2005).

Viana et al (2002) discovered that 30 % of cold insensitive trigeminal neurons expressed a slowly inactivating outward K^+ current, which could be fully inhibited by 4-AP (Viana et al., 2002). They named this current I_{KD} and hypothesised that it could have a role in reducing the excitability of cold insensitive neurons during cooling. They went on to demonstrate that application of 100 μM 4-AP to cold insensitive cells induced a novel cold response in 40 % of previously cold insensitive neurons. Thus expression of I_{KD} in a proportion of cold insensitive neurons effectively acts to counteract the effective inhibition of any leak current during cooling, preventing these neurons from reaching firing threshold. Cabanes et al (2003) also found that 29% of previously unresponsive neurons from guinea pig trigeminal ganglia developed a cold sensitivity after application of 100 μM 4-AP (Cabanes et al., 2003).

Aims of the present study

The role of voltage gated K^+ channels (VGKCs) in cold transduction has been previously investigated using DRG/TG neurons in culture. The present study aims to investigate the role of VGKCs in cold transduction in the periphery, using the in vitro skin nerve preparation. Thermal and mechanical responses of individual, characterised afferents will be re-investigated after direct application of 4-AP or TEA to their receptive terminals, to see whether blockade of VGKCs induces or modulates cold sensitivity. New findings from this study will further

our understanding of the role of voltage gated potassium currents in mediating cold sensitivity in different populations of primary afferent neurons.

6.2 Methods

6.2.1. Skin-nerve in vitro preparation and recording technique

The saphenous nerve with the skin of the of the hind paw attached was dissected from adult female Sprague Dawley rats as described in chapter 2. The skin was mounted corium inside up in an organ bath and superfused with warm oxygenated SIF. Receptive fields of individual primary afferent neurons were then identified with a mechanical search stimulus and characterised using electrical, mechanical and thermal stimuli as described in chapter 2.

6.2.2. Chemical stimulation

Fibres were tested with application of the broad spectrum potassium channel blockers 4-AP or TEA. Receptive fields of fibres were tested either with 4-AP or TEA application, but never both.
In experiments investigating the effects of 4-AP, the majority of receptive fields were tested with 300 μM and 3 mM 4-AP. In experiments investigating the effects of TEA, a single dose of 10 mM was applied onto the receptive fields of fibres. In a pilot study, fibres were initially investigated using lower concentrations of 4-AP or TEA, ranging from 10 μM -1 mM. A breakdown of the number of fibres tested is provided in Table.6.2.1 (see below). Most fibres investigated with 4-AP or TEA concentrations below 100 μM did not display any changes in receptive properties. Taking these findings into consideration, subsequent fibres were investigated using 4-AP in the range of 300 μM – 3 mM or TEA using 10 mM TEA.
Solutions were made on the day of the experiment by diluting a stock solution of 10 mM 4-AP or 1M TEA into warm SIF.

Experiments investigating the effects of 4-AP

After the fibres were fully characterised 4-AP was applied directly into the metal ring which sealed off the receptive field of the fibre. All fibres were tested with 3 mM 4-AP, while some were investigated with both the lower (300 μM) and

higher (3 mM) dose of 4-AP. The 4-AP solution was continuously gassed with oxygen. Application of 4-AP to the receptive field of the fibre lasted for 60 seconds. In some pilot studies lower concentrations of 4-AP were applied for up to 5 minutes on the receptive fields of C fibres (see table 6.2.1. below). However, applying the drug for longer did not appear to make any significant difference to the receptive properties of the fibres compared to application after 60 seconds. Therefore, 4-AP was applied for 60 seconds in all subsequent experiments.

At the end of the 60 seconds application time the 300 μM 4-AP solution was sucked away from the receptive field and the metal ring lifted to allow the flow of SIF to be briefly re-instated. This usually lasted between 15-20 seconds. The metal ring was then re-placed and the SIF was quickly evacuated from within the ring. The thermocouple was kept in its original location. A cold stimulus was then applied to the receptive field of the unit. The cold stimulus lasted for 60 s. A second cold stimulus was then re-applied after a couple of minutes. Following this, the SIF was evacuated from the ring and 3 mM 4-AP was then applied for 1 minute and the process described above repeated. In some recordings this was then followed by the application of a noxious heat stimulus and a mechanical stimulus (constant force stimulation or mechanical ramp).

In fibres which displayed changes to their receptive properties after application of 4-AP, the duration of this action was not investigated. Therefore, it is not known if there is washout of 4-AP from the preparation and whether the fibres response profile returns to pre-4-AP baseline activity after a longer duration wash-out.

The same skin area, of the same preparation was avoided in subsequent recordings to avoid re-exposure to noxious or chemical stimuli to avoid possible sensitisation or desensitisation of units.

For quantitative analysis all fibres were included if they were either activated by adequate mechanical, thermal or electrical stimuli at the end of the stimulus protocol. This was done as a precaution in order not to obtain and artificially lowered percentage because of the fact that fibres were lost during deteriorating recording conditions.

Experiments investigating the effects of TEA

Experiments investigating the effects of TEA were carried out in the same way to those investigating the effects of 4-AP, except that only a single dose of 10 mM TEA was applied to the receptive fields of fibres. A cold stimulus was then applied to the receptive field of the fibre after removal of the TEA. In most cases another two cold stimuli were applied onto the receptive field, separated by a couple of minutes. In fibres which displayed changes to their receptive properties after application of TEA, the duration of this action was not investigated. Therefore, it is not known whether there is washout of TEA from the preparation and whether the fibres response profile returns to pre-TEA baseline activity after a longer duration wash-out.

All statistical analyses were carried out as described in Chapter 2.

TABLE.6.2.1. Breakdown of the number of fibres tested with 4-AP and TEA

Fibre Type	Total tested with 4-AP (300 µM – 3 mM, 60 s)	Total tested with 4-AP (10 µM – 1mM, 300 s)	Total tested with TEA (10 µM-1mM, 60 s)	Total tested with TEA (10 mM, 60 s)	Total
Aβ	23	0	9	17	49
Aδ	13	0	0	12	25
C	29	21	1	18	69
Total	65	21	10	47	143

4-AP/TEA application to whole saphenous nerve trunk

In a different set of experiments, a combination of 3 mM 4-AP and 10 mM TEA was directly applied to the whole nerve trunk for 5 minutes. Recordings were then carried out from fibres in the periphery, whose receptive fields did not overlap with the site of 4-AP/TEA application. A schematic diagram representing this experiment is shown below (Fig.6.2.1).
These experiments were carried out to investigate whether 4-AP/TEA application onto the whole nerve would have any effect on activity evoked in the receptive terminals.

3 to 4 units were characterised in a single recording (Fig.6.2.1, step 1). This was carried out by placing multiple thin filaments onto the recording electrode. Once the fibres had been characterised using electrical, mechanical and thermal stimulation (step 2), the nerve trunk was isolated using a metal ring and 4-AP/TEA was applied for 5 minutes (step 3). At the end of 5 minutes, the 4-AP/TEA was removed, and the ring was lifted to allow SIF to flow. This lasted between 15-20 s and the ring was resituated over the whole nerve. A cold stimulus was then applied for 1 minute to see whether any spontaneous activity developed from the fibres in the periphery (step 4). If there was no activity, a cold stimulus was then applied directly onto the individual receptive fields (step 5).

If there was no induced response, the combination of potassium channel blockers was applied directly onto the receptive fields of the fibres for 1 minute. This was carried out to investigate whether the effect of the potassium channel blockers was specific to the receptive endings of the fibre. A cold stimulus was then re-applied to see whether a novel response to cold developed (step 6).

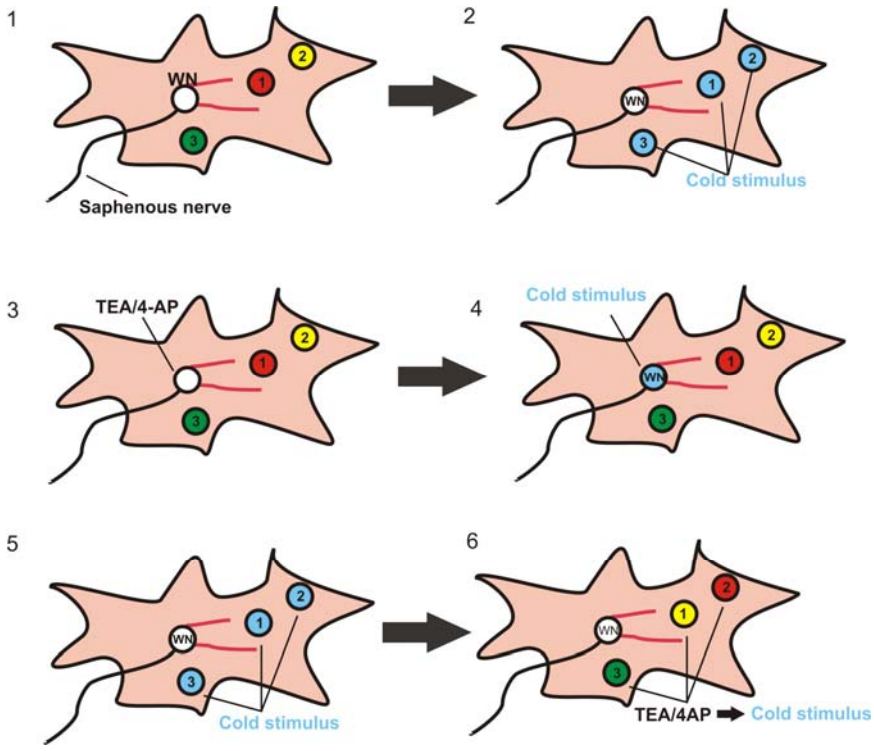

Fig.6.2.1. Schematic representation of an experiment investigating the effects of 4-AP/TEA onto the whole nerve
Areas numbered 1, 2 and 3 represent receptive fields of individually characterised units on the skin. WN represents the area of whole nerve on which TEA/4-AP was applied.

6.3 Results

Single unit recordings were made from a total of 143 afferent fibres from 42 animals. Of those 49 were classified as Aβ fibres, 25 as Aδ fibres and 69 as C fibres.

The mean conduction velocity of Aβ fibres was 18.2 ± 0.9 m/s. The mean conduction velocity of Aδ fibres was 6.7 ± 0.8 m/s. The mean conduction velocity of C fibres was 0.46 ± 0.02 m/s. General properties of fibres tested with 4-AP are provided in Table 6.3.1 and those tested with TEA are provided in Table 6.3.2. Fig.6.3.1 and 6.3.2 are schematic diagrams representing the proportion of fibres which displayed either a novel or increased response to a cold stimulus after application of 4-AP and TEA, respectively.

TABLE 6.3.1. Conduction velocity, von Frey thresholds and cold sensitivity of fibres tested with 4-AP

Fibre Type	n	Conduction velocity, m/s	Von Frey threshold, mN	Cold responsive before 4-AP	Novel/ increased cold response after 4-AP
RA	14	15.1± 0.8	1.4 (0.35-4)	0/14	7/14
SA	5	16.0 ± 2.5	5.6 (4-11.2)	0/5	5/5
SA (c)	4	15.6 ± 1.6	0.75 (0.37-1.5)	4/4	3/4
DH	4	7.0 ± 1.1	0.65 (0.28-1.0)	0/4	1/4
AM	5	7.8 ± 1.9	11.3 (11.2-11.3)	0/5	1/5
AMC	4	3.2 ± 0.8	6 (2.7-12)	4/4	4/4
CM	4	0.40 ± 0.02	21.7 (9.7-32)	0/4	0/4
CMC	5	0.44 ± 0.05	16 (16-22)	5/5	4/5
CMH	10	0.39 ± 0.02	19.3 (11.3-32)	0/10	2/10
CMCH	5	0.45 ± 0.01	22.6 (16-32)	5/5	1/5
CLTM-C	2	0.56 ± 0.03	1 (1-1)	2/2	1/2
CC	3	0.58 ± 0.11	-	3/3	0/3

Values are means ± SEM; except those for von Frey thresholds which are median and first (Q_1) and third (Q_3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; SA (c), cold sensitive slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMC, high threshold mechano-cold A fibre; AMH, high threshold mechano-heat A fibre; DH, D-hair receptors; CM, C mechano-sensitive nociceptor; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM, C low-threshold mechano-sensitive fibre; CC, C mechano-insensitive cold fibre.

TABLE 6.3.2. Conduction velocity, von Frey thresholds and cold sensitivity of fibres investigated with TEA

Fibre Type	n	Conduction velocity, m/s	Von Frey threshold, mN	Cold responsive before TEA	Novel/ increased cold response after TEA
RA	13	21.9 ± 2.0	1.4 (0.85-1.4)	0/13	4/13
SA	4	20.7 ± 4.5	0.44 (0.38-0.5)	0/4	3/4
DH	2	7.7 ± 0.3	0.85 (0.71-1)	0/2	1/2
AM	8	8.5 ± 1.4	16.7 (6.8-16)	0/8	0/8
AMH	2	2.2 ± 0.2	24 (16-32)	0/2	0/2
CMC	5	0.55 ± 0.13	18.4 (11.4-16)	5/5	3/5
CMH	4	0.43 ± 0.05	33.7 (19.3-48)	0/4	3/4
CMCH	4	0.31 ± 0.03	13.9 (8.5-19.3)	4/4	1/4
CLTM-C	1	0.45	0.71	1/1	0/1
CC	4	0.52 ± 0.06	-	4/4	0/4

Values are means ± SEM; except those for von Frey thresholds which are median and first (Q_1) and third (Q_3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMH, high threshold mechano-heat A fibre; DH, D-hair receptors; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM, C low-threshold mechano-sensitive fibre; CC, C mechano-insensitive cold fibre.

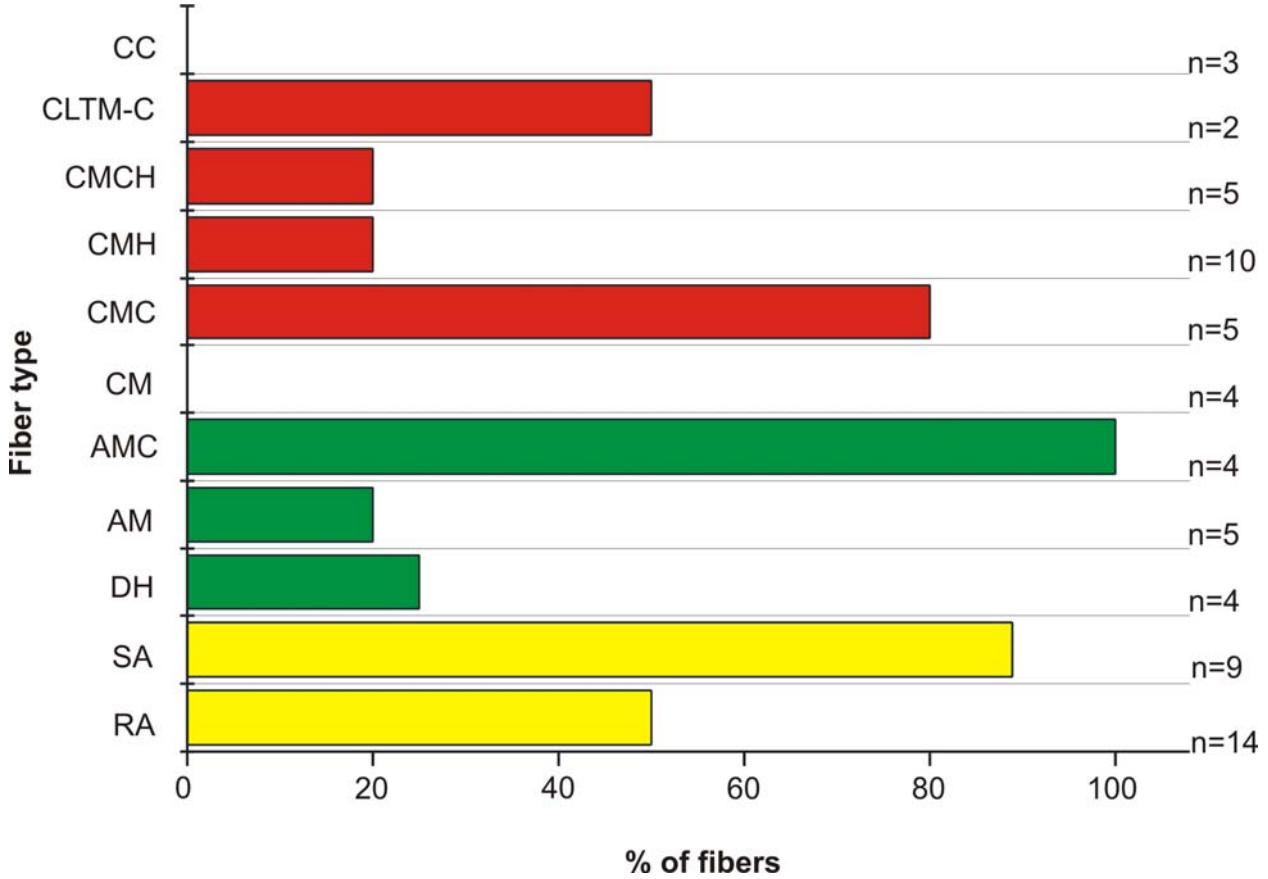

Fig.6.3.1 Percentage of fibres which displayed either a novel cold response or increased sensitivity to cold after application of either 300 µM or 3 mM 4-AP for 60 s

Aβ fibres are shown in yellow, Aδ fibres in green and C fibres in red. Overall, application of 4-AP was able to either induce a novel cold sensitivity or increase cold responses in a proportion of both A fibre mechanoreceptors and C fibre nociceptors.

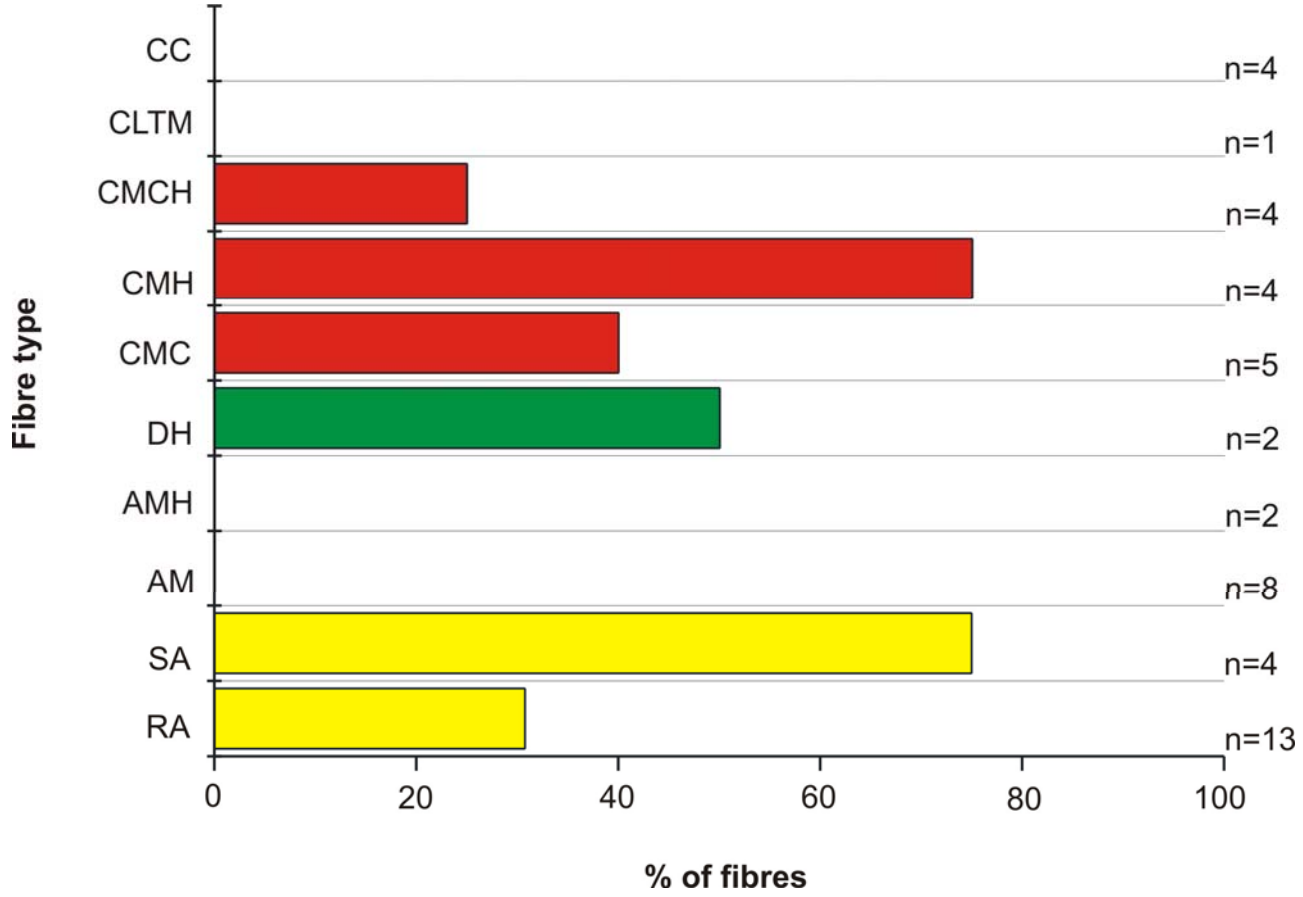

Fig.6.3.2 Percentage of fibres which displayed either a novel cold response or increased sensitivity to cold after application of 10 mM TEA for 60 s

Aβ fibres are shown in yellow, Aδ fibres in green and C fibres in red. Overall, application of TEA was able to either induce a novel cold sensitivity in a proportion of A fibre mechanoreceptors and C fibre nociceptors. Increased responses to cold were observed in a proportion of cold sensitive nociceptors.

Aβ fibres

Rapidly adapting (RA) mechanoreceptors

A total of 27 RA fibres was recorded. None responded to a cold or heat stimulus before application of potassium channel blockers. None were initially spontaneously active.

4-AP in concentrations of 300 μM and 3 mM was applied onto the receptive fields of 14 RA units in total. One RA unit began to respond during the application of 3 mM 4-AP. No fibres discharged during the application of 300 μM 4-AP.

After application of each dose of 4-AP, fibres were re-tested for their response to a cold stimulus. Subsequently, a novel cold sensitivity developed in 2 out of 5 fibres tested with 300 μM 4-AP and 7 out of 14 (50 %) of fibres after application of 3 mM 4-AP. An example of one such RA unit is shown in Fig.6.3.3. The average response threshold to cold after 300 μM 4-AP was 18 °C, ranging from 16.6-19.4 °C (n=2). The average response threshold to cold after 3 mM 4-AP was 21.2 ± 1.8 °C, ranging from 15-29.5 °C (n=7, see table 6.3.3).

Plotting the mean discharge rate during a cold stimulus revealed that after application of 4-AP, RA fibres typically discharged in a regular manner, displaying an early peak discharge (Fig.6.3.4A). Some fibres, particularly at higher doses also began to respond in bursts of action potentials (see Fig.6.3.3B). There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean response to a cold stimulus after application of 3 mM 4-AP, as illustrated in Fig.6.3.4B. There appeared to be a trend of fibres to display a greater response to cold after 3 mM 4-AP compared to after 300 μM 4-AP.

RA units were then re-tested for their response to a noxious heat stimulus, but none became sensitive to heat (see Fig.6.3.3B for an example).

Mechanical sensitivity of 6 RA units was re-investigated after exposure to 4-AP, using a constant force stimulus of 100 mN. 5 out of 6 (83 %) of these units began to fire at low frequencies during the constant force stimulation. An

example of one such unit is displayed in Fig.6.3.5A. Their firing pattern was studied by plotting the mean discharge rate as a function of stimulus force before and after 4-AP application (Fig.6.3.5B). This revealed that as well as a change in adaptation property, there was a slight increase in the total mean number of impulses during the CFS after 4-AP application. However, this difference did not reach statistical significance ($p>0.1$, Wilcoxon matched pairs test, Fig.6.3.5C). Interestingly, 4 out of the 5 units which displayed an increase in firing frequency to constant force stimulation also became cold sensitive after 4-AP application.

TEA was applied onto the receptive fields of a total of 13 RA units. None of the units responded directly during the application of TEA. Subsequently, fibres were re-tested for their response to a cold stimulus. Since the time for which TEA was applied for (one or five minutes) did not significantly affect the cold response or cold threshold of fibres (see Table.6.3.4), only the responses after one minute application of TEA are presented from here onwards.

Novel cold sensitivity developed in 4 out of 13 (30 %) RA fibres after application of TEA. An example is presented in Fig.6.3.6. The average response threshold to cold after TEA was 20.2 ± 2.3 °C, ranging from 18-25.9 °C (n=4, see table.6.3.4). Fig.6.3.7A shows the mean discharge rate of these fibres during a cold stimulus over time and a clear trend of an ($p=0.07$, Wilcoxon matched pairs test, Fig.6.3.7B) Fibres never became heat responsive after TEA application.

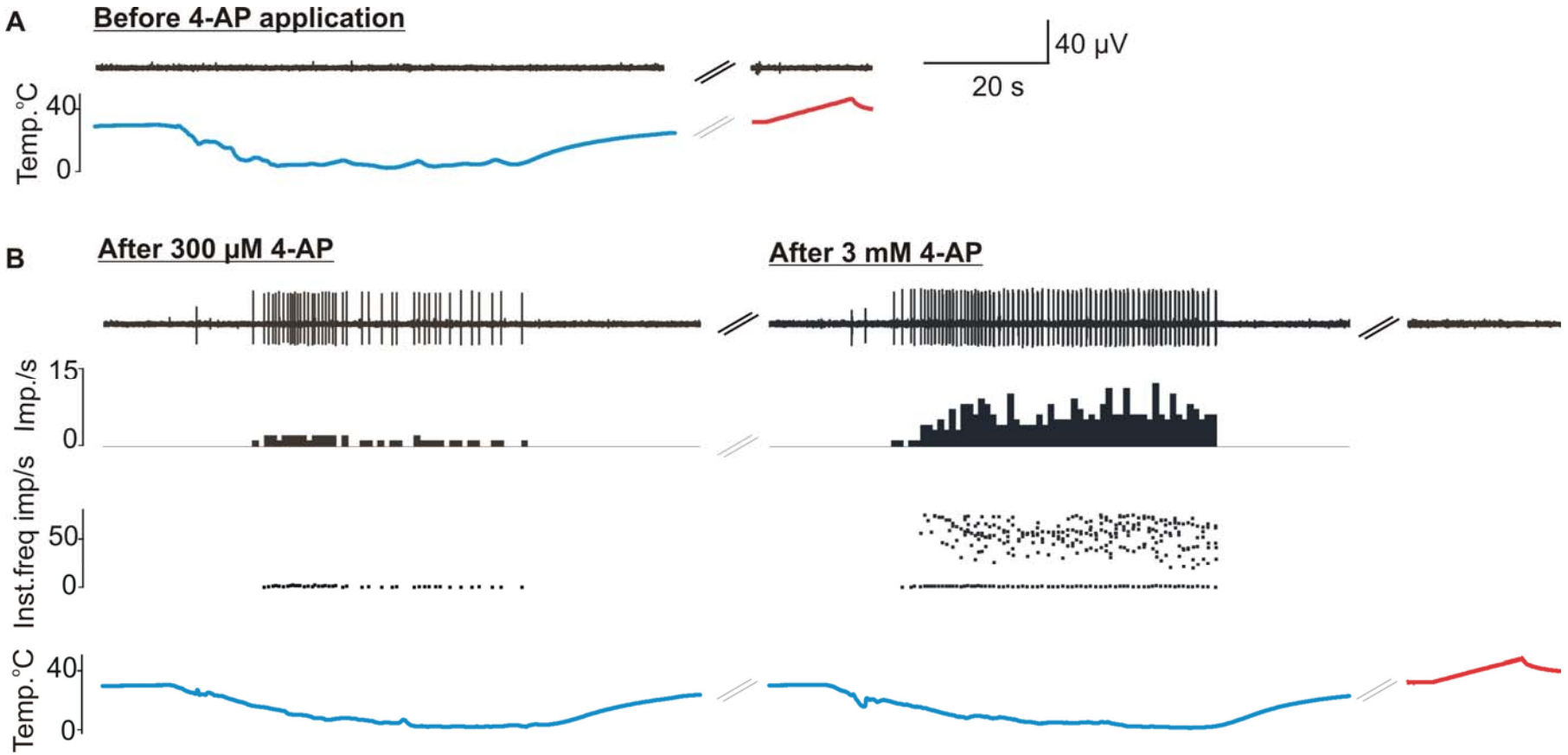

Fig.6.6.3 Example of a rapidly adapting (RA) fibre responding to thermal stimuli before and after application of 4-AP
(A) The fibre was insensitive to both a noxious cold and heat stimulus. (B) After application of 300 µM 4-AP (60 s) the fibre responded to the cold stimulus firing in a regular manner. After application of 3 mM 4-AP, the fibre discharged at high frequency bursts during cold. The fibre remained insensitive to a heat stimulus.

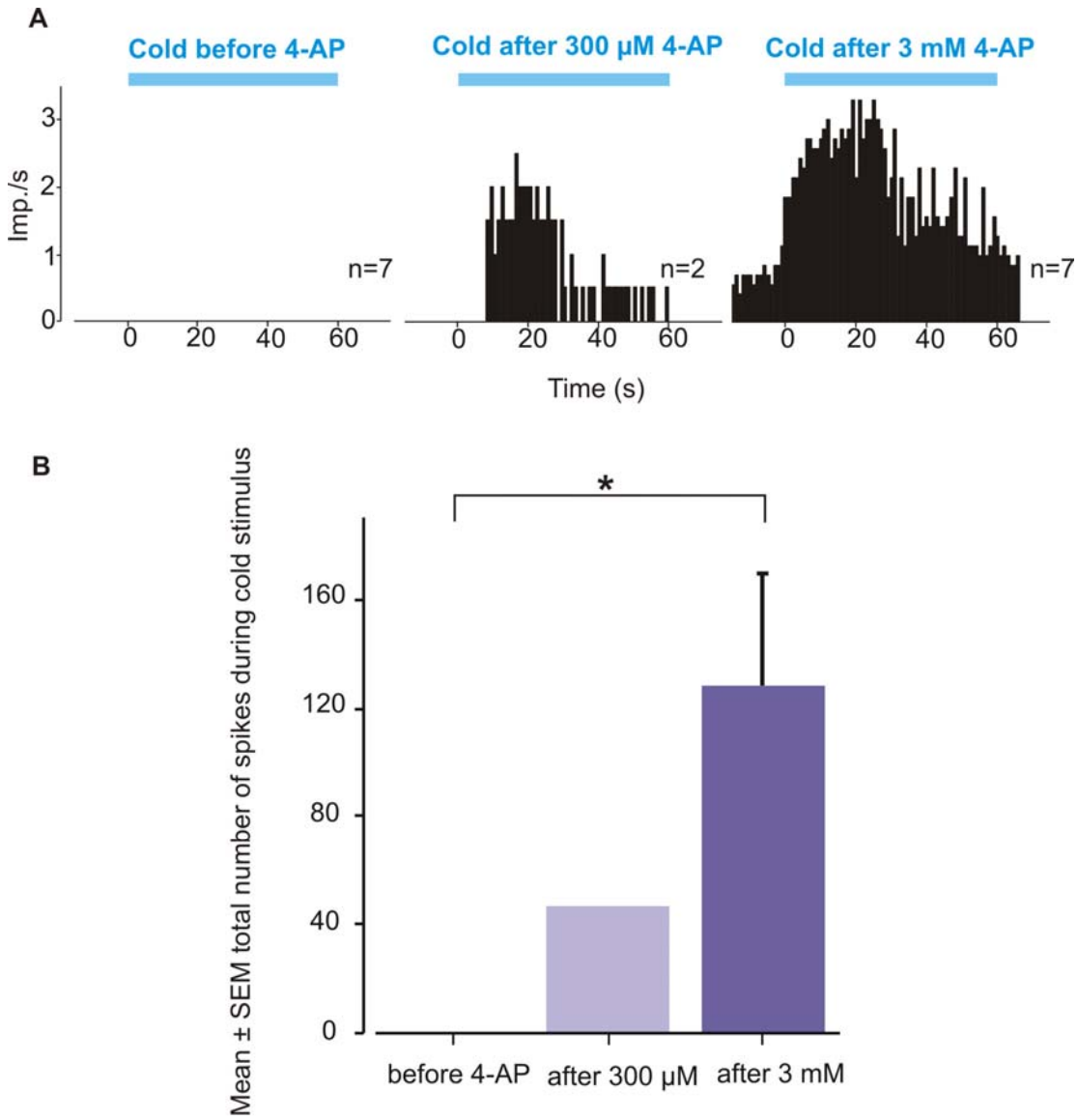

Fig.6.3.4 Mean response of RA fibres to a cold stimulus before and after 4-AP application (n=7)

(A) Units were not activated by cold stimuli before the application of 4-AP. Subsequently they responded to cold in a regular manner. (B) There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean number of impulses generated during a cold stimulus after application of 3 mM 4-AP.

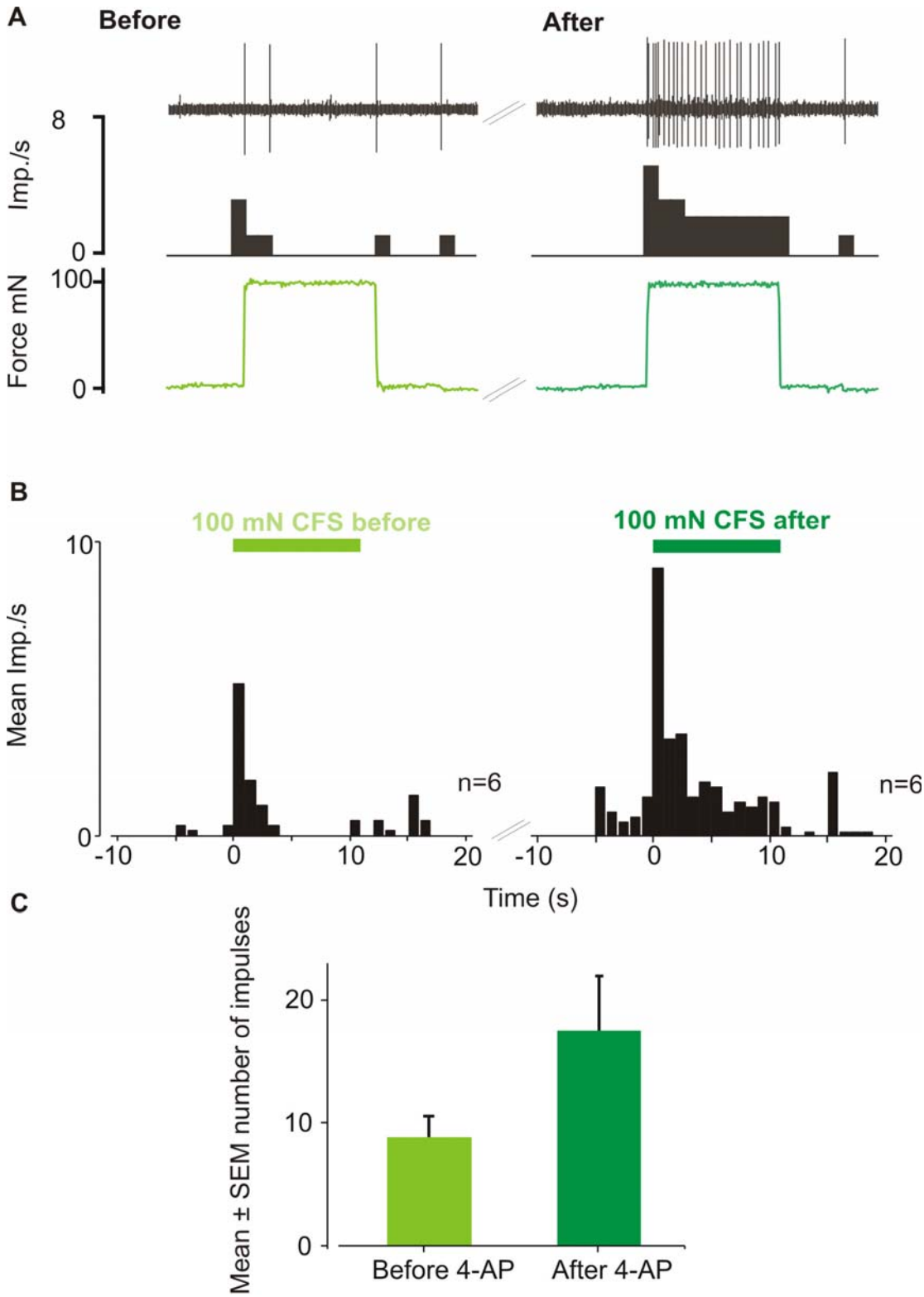

Fig.6.3.5 Mean response of rapidly adapting (RA) units to constant force stimulation (CFS) before and application of 4-AP

(A) An example of a RA fibre is shown. The fibre adapted rapidly to the CFS, responding at the beginning and end of the stimulus only. Subsequently the unit fired continuously during the CFS. (B and C) On average, there was an increase in the peak discharge rate after application of 4-AP. There was an increase in the mean total number of impulses generated during the CFS after 4-AP application. However, this difference did not reach statistical significance ($p>0.1$, Wilcoxon matched paired test).

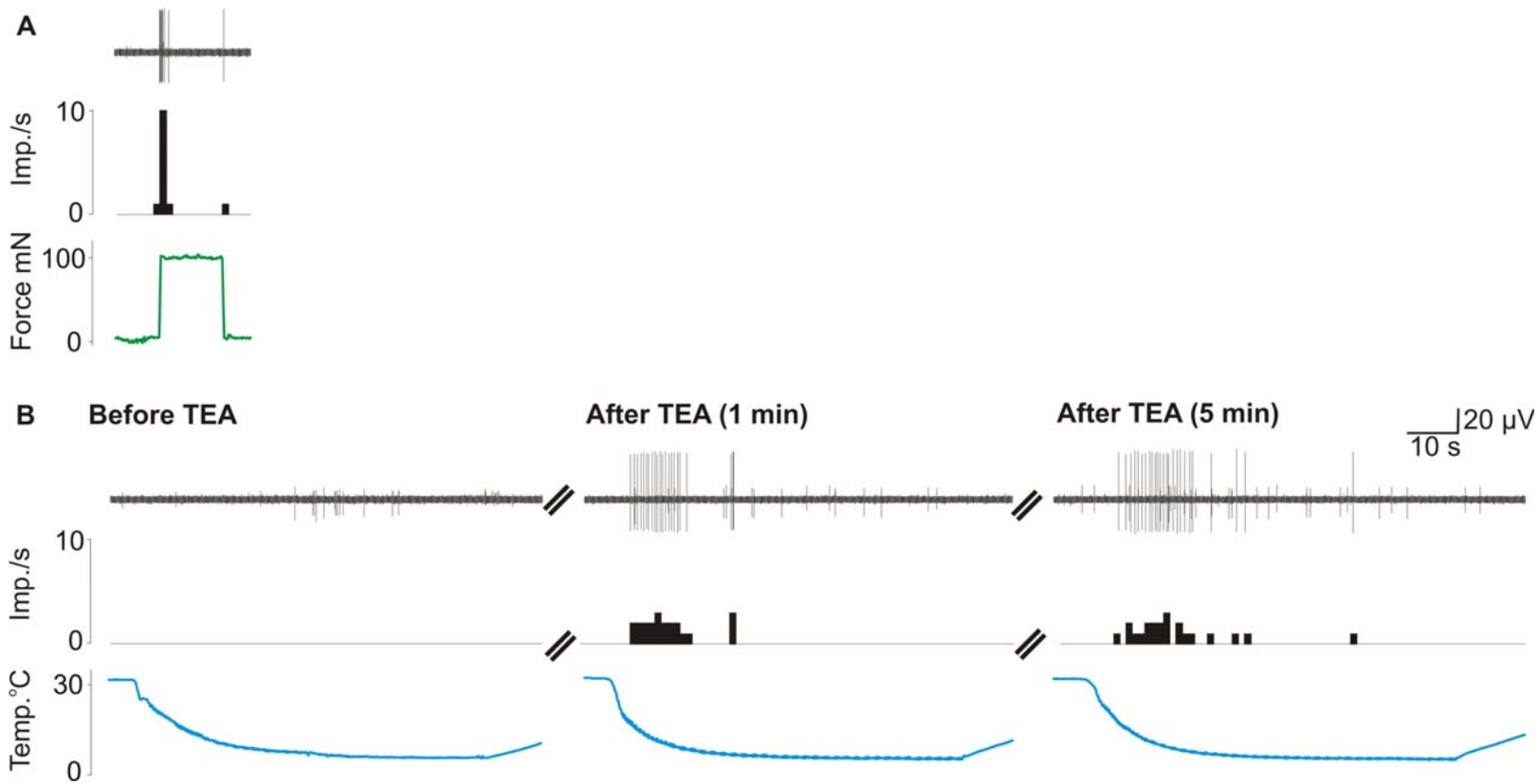

Fig.6.3.6 Example of a rapidly adapting (RA) fibre responding to cold before and after application of 10 mM TEA
(A) The unit adapted rapidly to a constant force stimulus of 100 mN. (B) The fibre was unresponsive to a cold stimulus. After the application of 10 mM TEA (60 s), the fibre responded briefly to a cold stimulus. There appeared to be no difference in the cold response subsequent to the application of 10 mM TEA for 5 minutes.

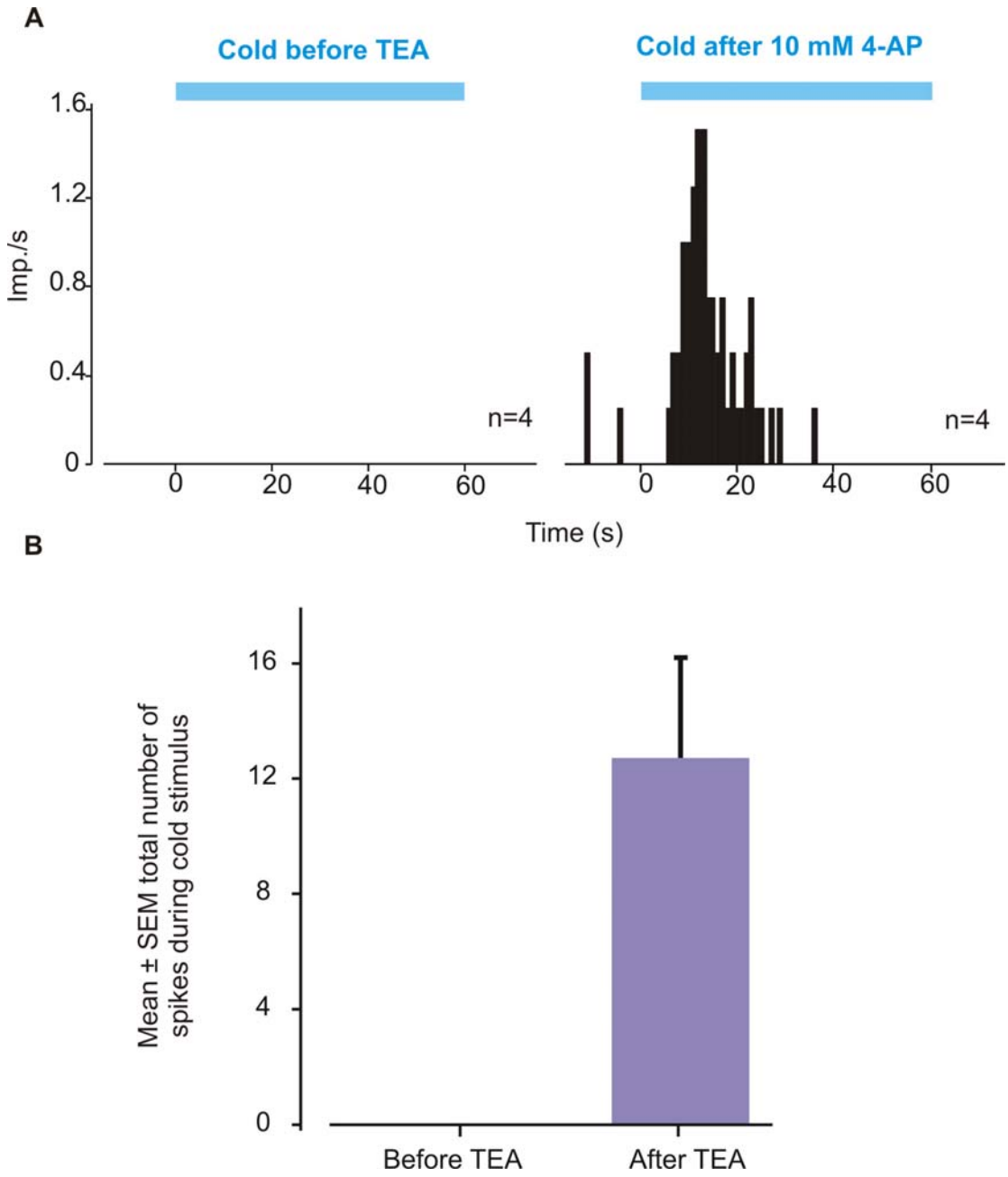

Fig.6.3.7 Mean response of RA fibres to a cold stimulus before and after 10mM TEA application (n=4)

(A) Units were not activated by cold stimuli before the application of TEA. Subsequently they responded to cold, displaying an early peak discharge. On average the fibres did not respond throughout the cold stimulus. (B) There was a clear trend (p=0.07, Wilcoxon matched paired test) for an increased mean response to cold after the application of TEA in this small sample.

Slowly adapting (SA) mechanoreceptors

A total of 13 SA fibres were recorded. 4 out of 13 (30 %) of fibres responded briefly during a cold stimulus. None responded to a heat stimulus. None were initially spontaneously active.

4-AP was applied onto the receptive fields of 9 SA fibres. 4 out of 9 of these fibres were cold sensitive, with an average response threshold of 19 ± 3.6 °C, ranging from 10.2-27.3 °C. Interestingly, 3 out of 4 (75 %) of these fibres displayed an increase in firing frequency during a cold stimulus after application of 4-AP. One unit began to discharge in bursts of action potentials during cold after 4-AP application. The mean response threshold to cold post 4-AP was 18.7 ± 1.7 °C which did not differ significantly ($p>0.6$, paired students t-test) from before 4-AP application (see Table.6.3.3). 2 of these units began to discharge directly during the application of 4-AP (see Figs.6.3.8 and 9 for an example). Plotting the mean response to cold over time revealed that after 4-AP application, fibres developed a sustained response during cold, whereas previously they had only responded briefly (Fig.6.3.10A). Although there was a greater response to cold after 4-AP (Fig.6.3.10B), statistically this increase was insignificant ($p>0.2$, Wilcoxon matched paired test).

All previously cold insensitive SA fibres (100 %, n=5) displayed a novel response to cold after application of 3 mM 4-AP. The average response threshold to cold after 3 mM 4-AP was 21.4 ± 2.7 °C, which did not differ significantly from that of cold induced RA units (21.2 ± 1.8 °C, n=7, $p>0.5$, unpaired students t-test). Following 4-AP application, 3 out of 5 units began to fire in bursts of action potentials during cold. 2 SA units which were tested with 300 µM 4-AP also became cold responsive (see Fig.6.3.11 for an example). The average response to a cold stimulus before and after 4-AP is shown in Fig.6.3.12. There was a significant increase ($p<0.05$, Wilcoxon matched paired test) in the mean response to cold after application of 3 mM 4-AP (see Fig.6.3.12B). SA units never became sensitive to heat after 4-AP application.

Mechanical sensitivity of 3 SA units was re-investigated after exposure to 4-AP, using a constant force stimulus of 100 mN. The mean response to mechanical stimuli before and after 4-AP is displayed in Fig.6.3.13. There did not appear to be any obvious changes in firing frequency or adaptation properties in the 3 fibres studied. Although there was a small increase in the mean total response after 4-AP application, this difference was statistically insignificant ($p>0.1$, Wilcoxon matched paired test). Interestingly all of these fibres displayed either a novel or an increased cold response after 4-AP.

TEA was applied onto the receptive fields of 4 SA units. None responded directly during the application period. Subsequently 3 out of 4 (75 %) units displayed a novel response to a cold stimulus. An example is shown in Fig.6.3.14. The average response threshold to cold was 22.9 ± 3.4 °C, which did not differ significantly ($p>0.6$, unpaired students T-test) from that of RA fibres with induced cold sensitivity (20.2 ± 2.3, $n=4$). Fig.6.3.15 shows the mean response to a cold stimulus before and after application of TEA. Although there was clearly a greater response to cooling after application of TEA statistically this difference was not significant ($p>0.1$, Wilcoxon matched paired test).

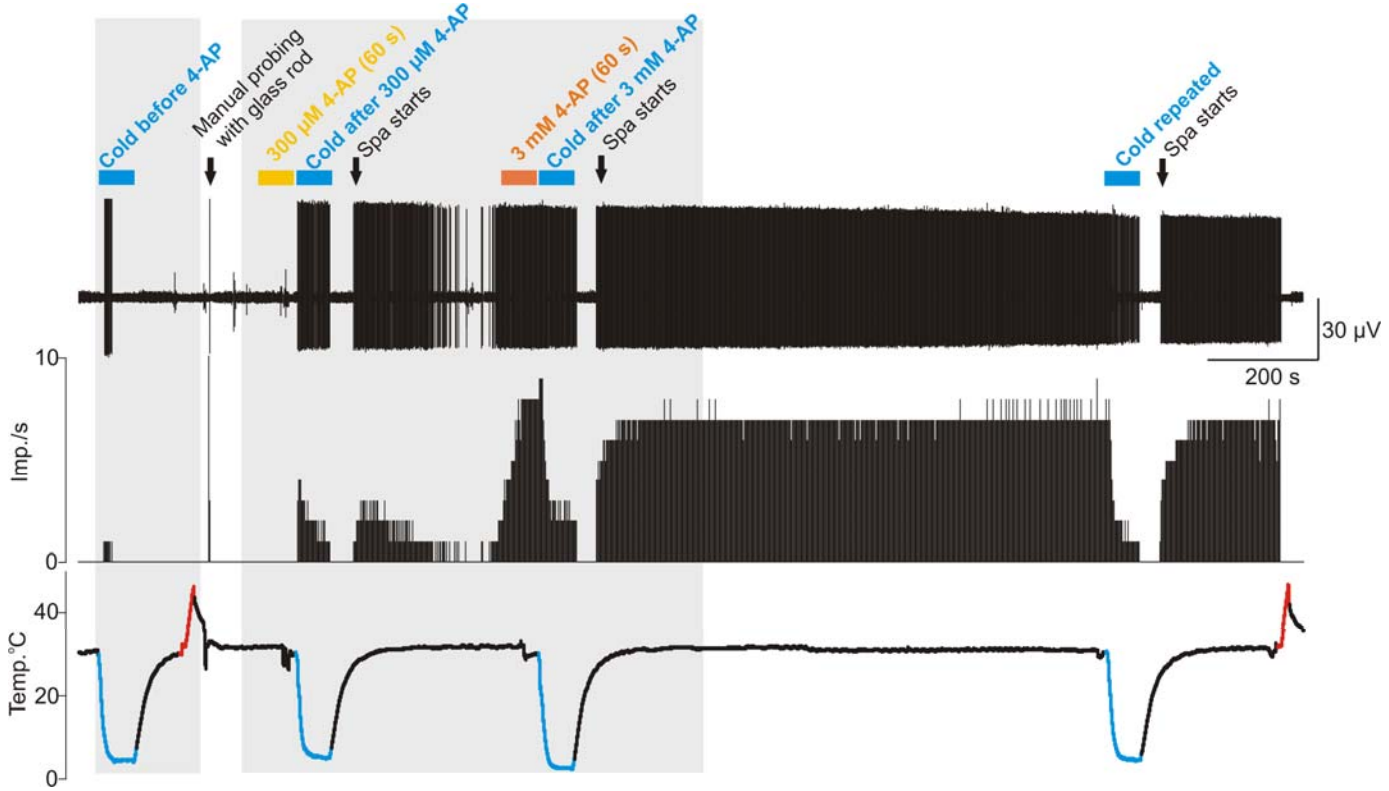

Fig.6.3.8 Response of a cold sensitive, slowly adapting (SA) mechanoreceptor to thermal stimuli before and after 4-AP application

The fibre responded briefly during the dynamic phase of the cold ramp. Following the application of 300 µM 4-AP, the fibre developed a sustained response to cold and displayed a greater firing frequency. The fibre then began to fire spontaneously (Spa) during the re-warming phase following the cold ramp and continued to discharge at a low frequency. Subsequently, when 3 mM 4-AP was applied there was an increase in firing frequency. Thereafter the ongoing activity was reduced during the cold stimulus. The fibre again began to fire during the re-warming phase and continued to discharge thereafter. The fibre did not become responsive to noxious heating. The areas highlighted in light grey are shown in Fig.6.3.9.

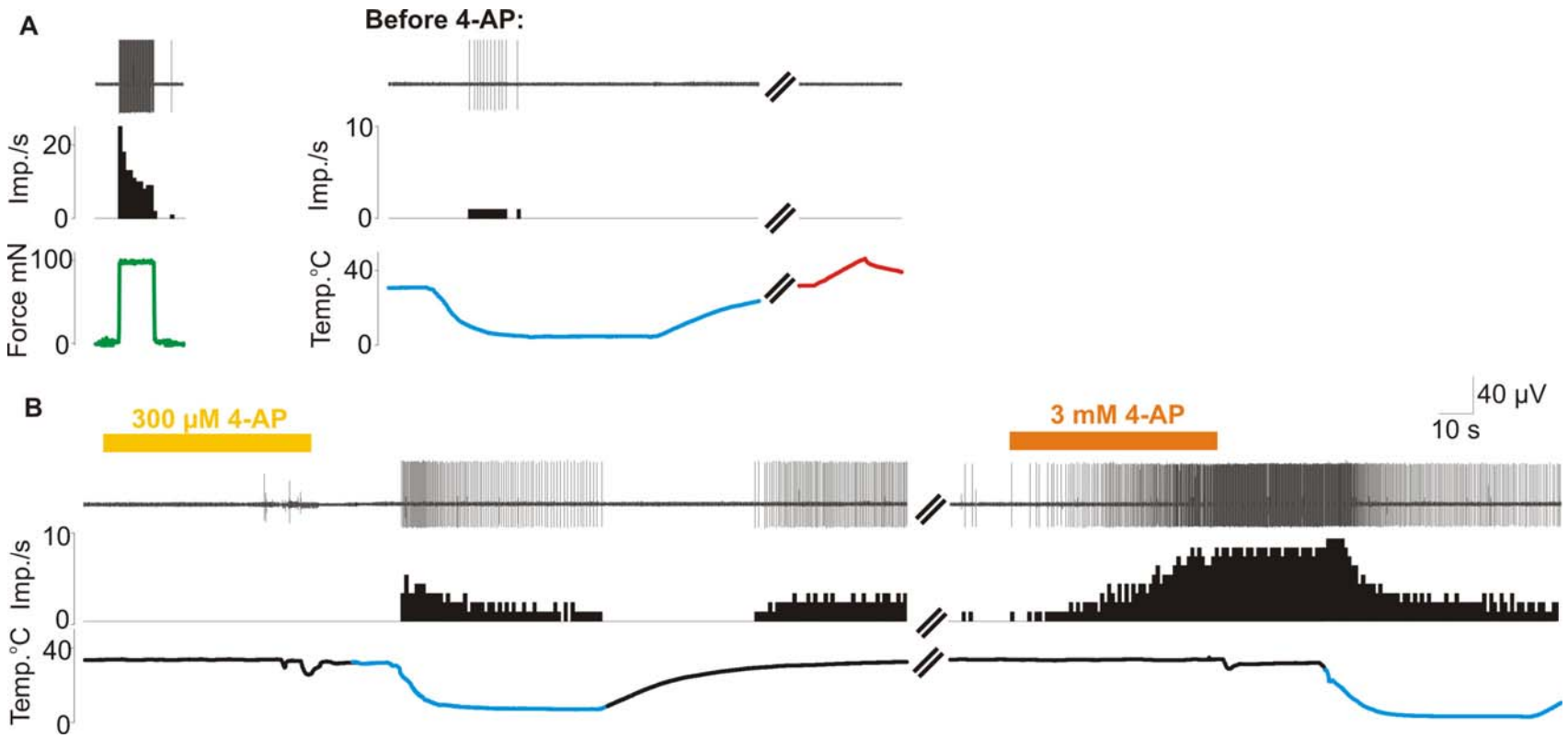

Fig.6.3.9 Example of a cold sensitive slowly adapting (SA) fibre responding to thermal stimuli before and after application of 4-AP (same as fibre shown in Fig.6.3.8)

(A) The fibre displayed a slowly adapting response to constant force stimulation. It responded very briefly during the ramp phase of the cold stimulus. (B) There was no activity from the fibre during the application of the lower dose of 4-AP. Subsequently, it responded throughout the cold stimulus and also displayed a novel response during the re-warming phase of the ramp. During application of the higher dose of 4-AP a clear increase in firing frequency was observed. During cooling, firing frequency dropped and lower level of firing was maintained throughout the stimulus.

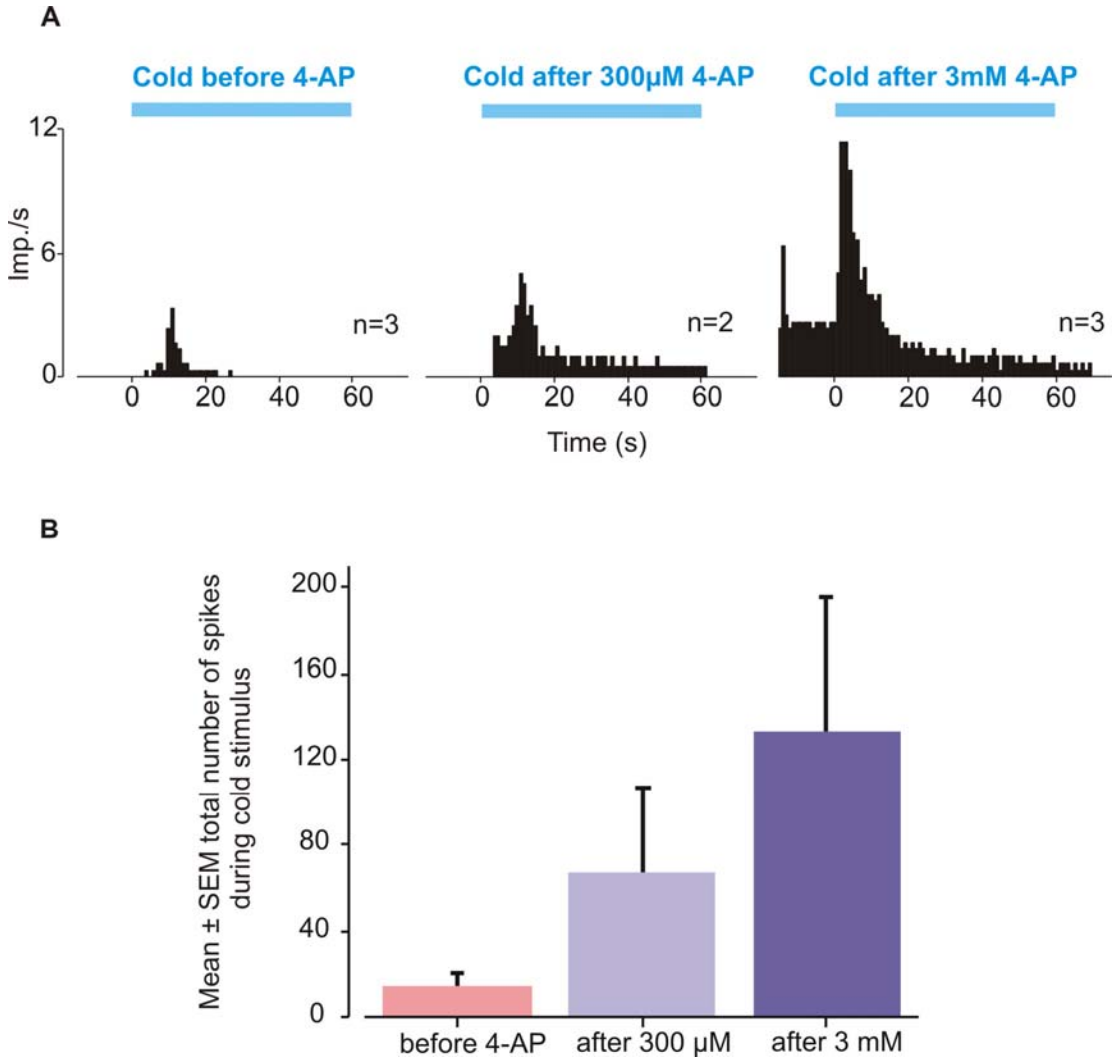

Fig.6.3.10 Mean response of cold sensitive slowly adapting (SA) fibres to a cold stimulus before and after application of 4-AP

(A) Before application of 4-AP, fibres responded briefly during the ramp phase of the cold stimulus. After application of 300 µM 4-AP, fibres developed a sustained response during cold. After application of 3 mM 4-AP, fibres displayed a clear increase in firing frequency. Note the fibre activity before cold is applied; this is due to direct activation of fibres during application of 4-AP. (B) Although there was a clear increase in the total response to cold after application of 3 mM 4-AP, this difference was statistically insignificant (p>0.2, Wilcoxon matched paired test).

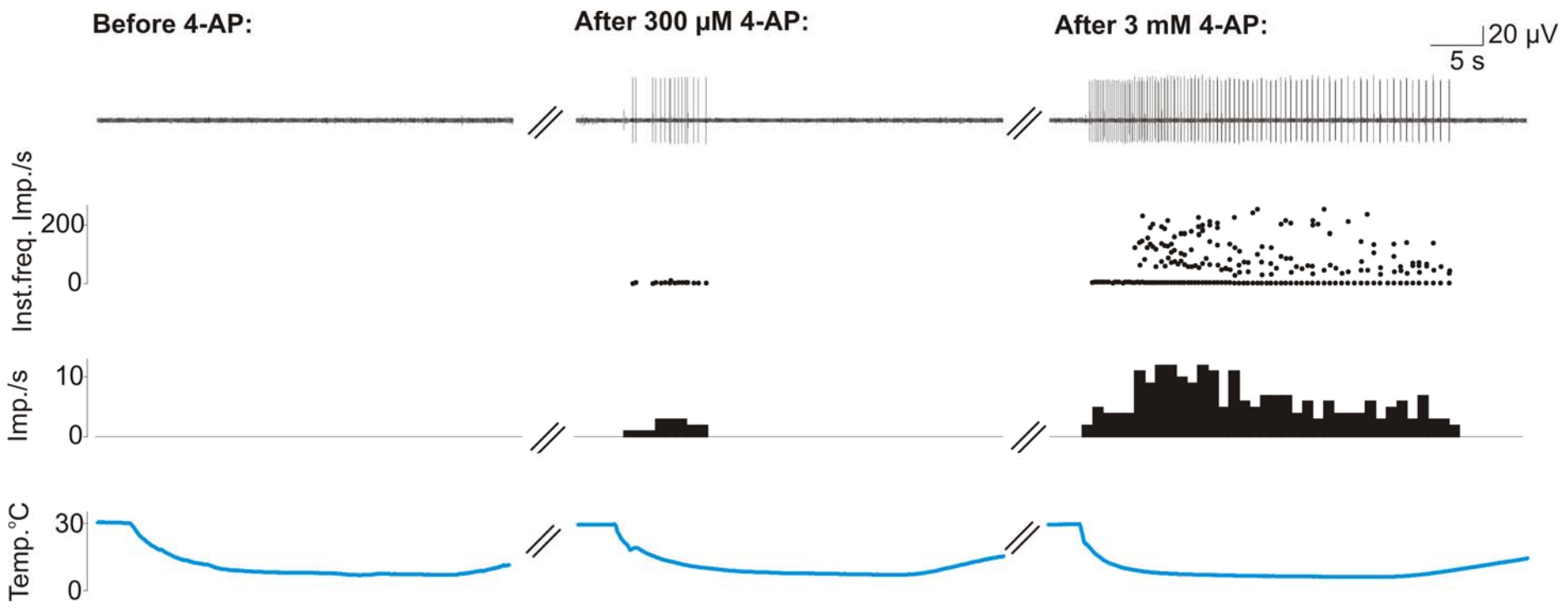

Fig.6.3.11 Example of a slowly adapting (SA) fibre responding to a cold stimulus before and after application of 4-AP
The SA fibre was previously unresponsive to a cold stimulus. After the application of 300 µM 4-AP, the fibre developed a brief response during cooling. Following the application of 3 mM 4-AP, the fibre discharged throughout cooling, in a high frequency bursting manner.

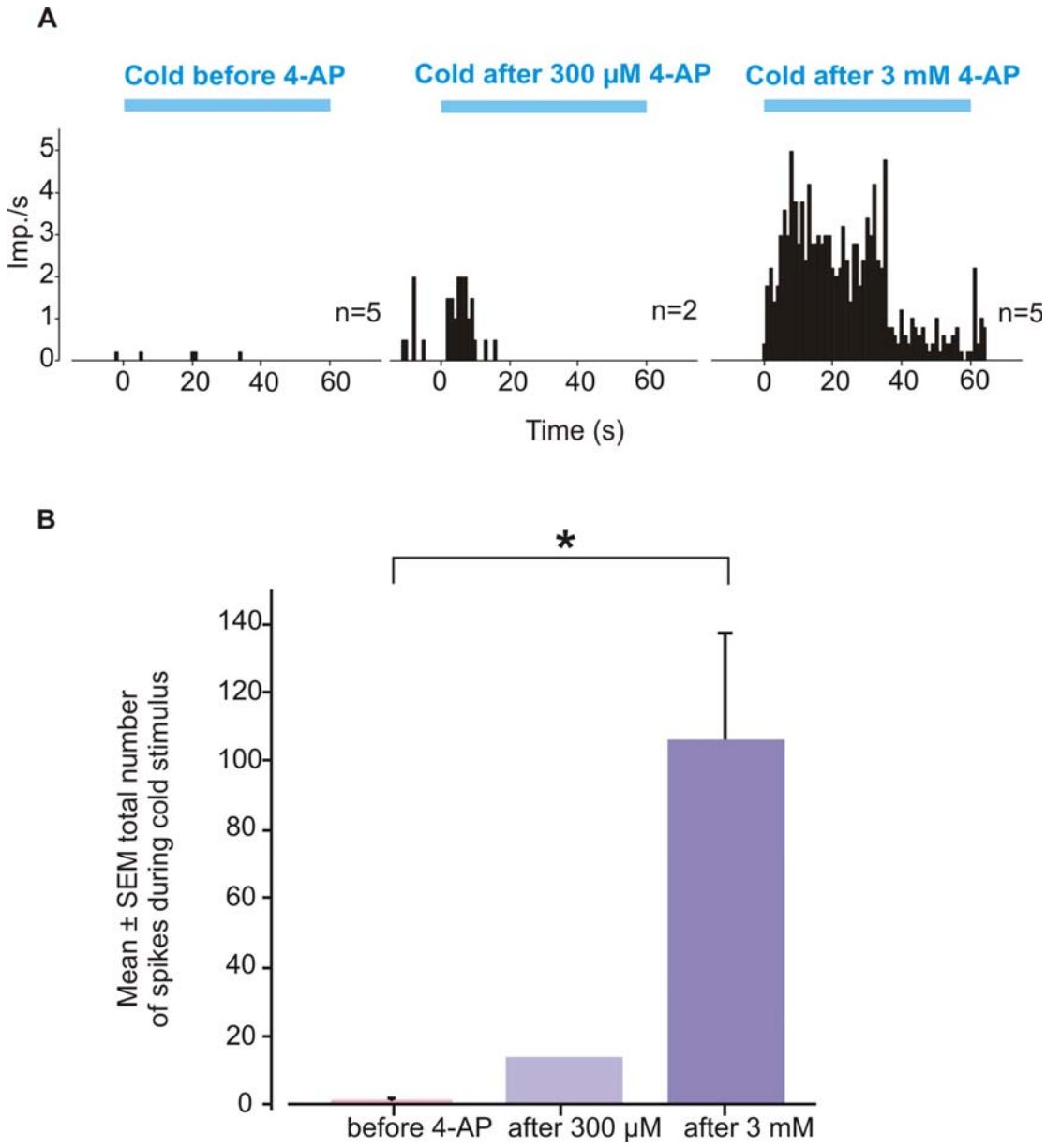

Fig.6.3.12 Mean response of SA fibres to a cold stimulus before and after 4-AP application (n=5)

(A) Units were not activated by cold stimuli before the application of 4-AP. Subsequently they responded briefly to cold after application of 300 µM 4-AP. Fibres developed a much greater response to cold after application of the higher dose of 4-AP. (B) There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean number of impulses generated during a cold stimulus after application of 3 mM 4-AP.

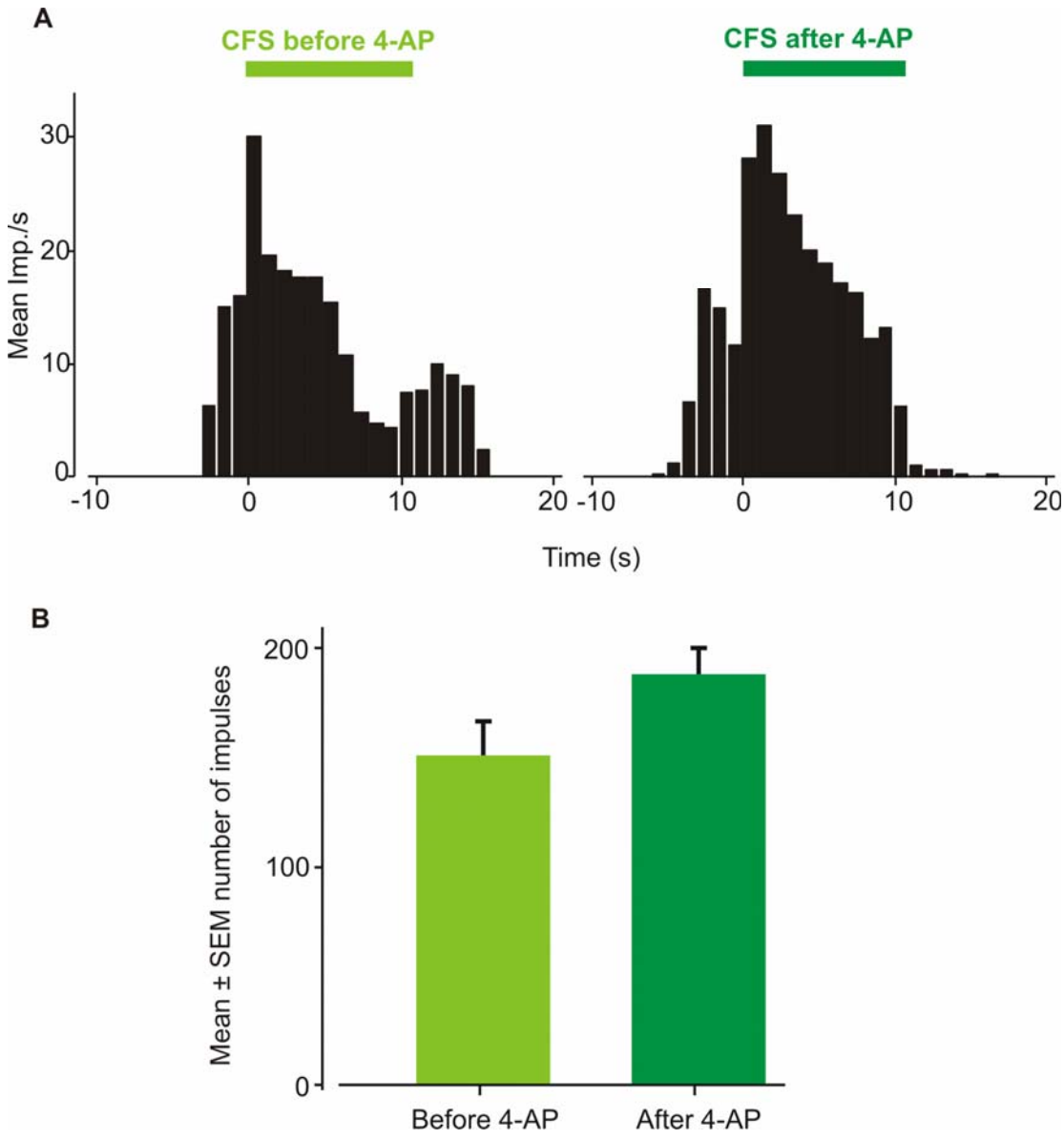

Fig.6.3.13 Mean response of slowly adapting (SA) mechanoreceptors to a constant force stimulus before and after application of 4-AP (n=3)
(A) Overall, there appeared to be no major difference in firing pattern or peak discharge rate during the constant force stimulus (CFS) after application of 4-AP. (B) There was a slight increase in the total response during the CFS, but statistically this difference was insignificant (p>0.1, Wilcoxon Matched paired test).

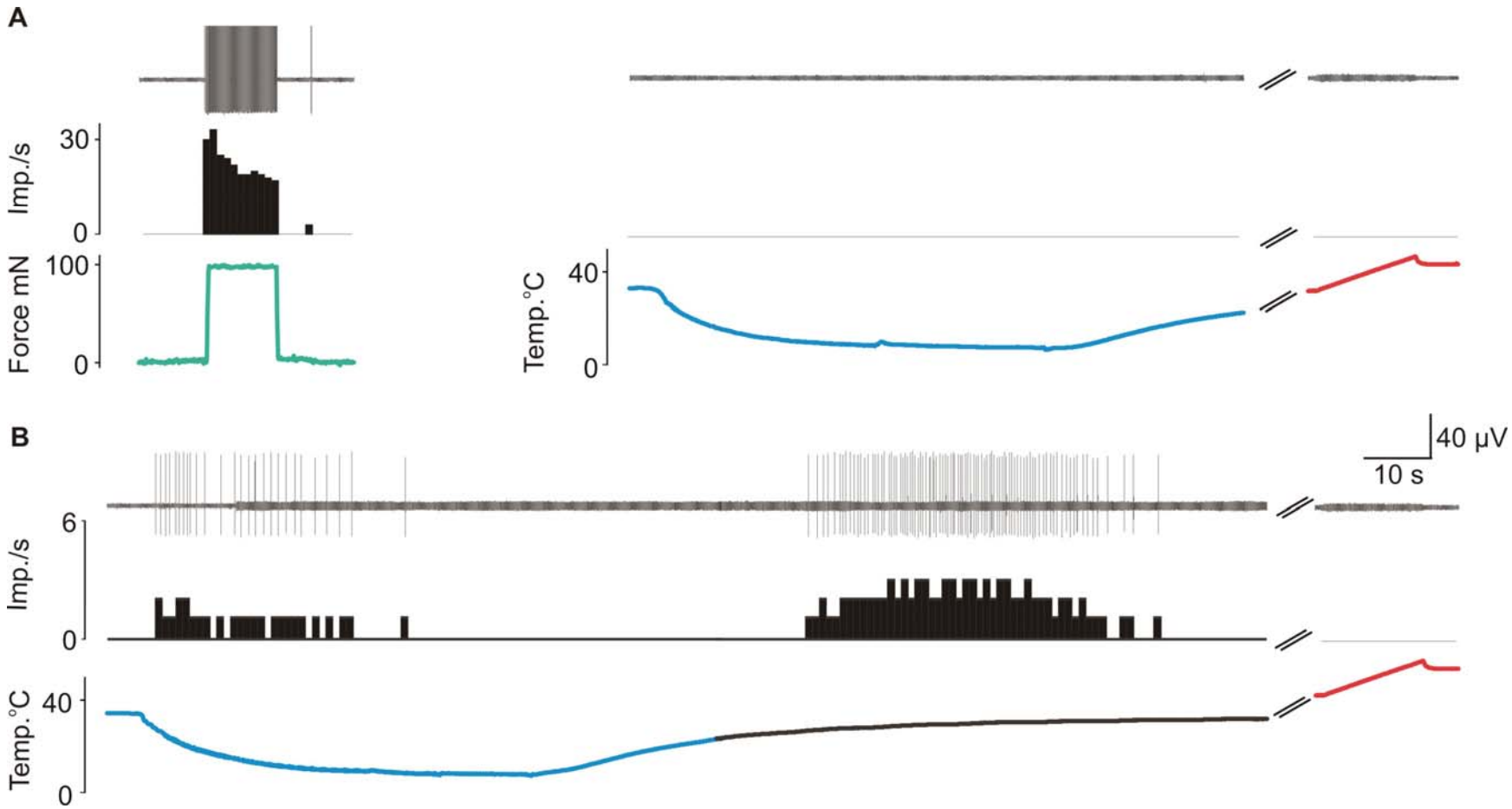

Fig.6.3.14 Example of a slowly adapting (SA) fibre responding to thermal stimuli before and after application of 10 mM TEA
(A) The fibre adapted slowly to constant mechanical stimulation of 100 mN. Subsequently, it did not respond to cold or heat stimuli. (B) After application of 10 mM TEA to the receptive field of the fibre, it developed a brief response to cold. A novel response was observed during the re-warming phase of the cold ramp. The fibre remained insensitive to noxious heating.

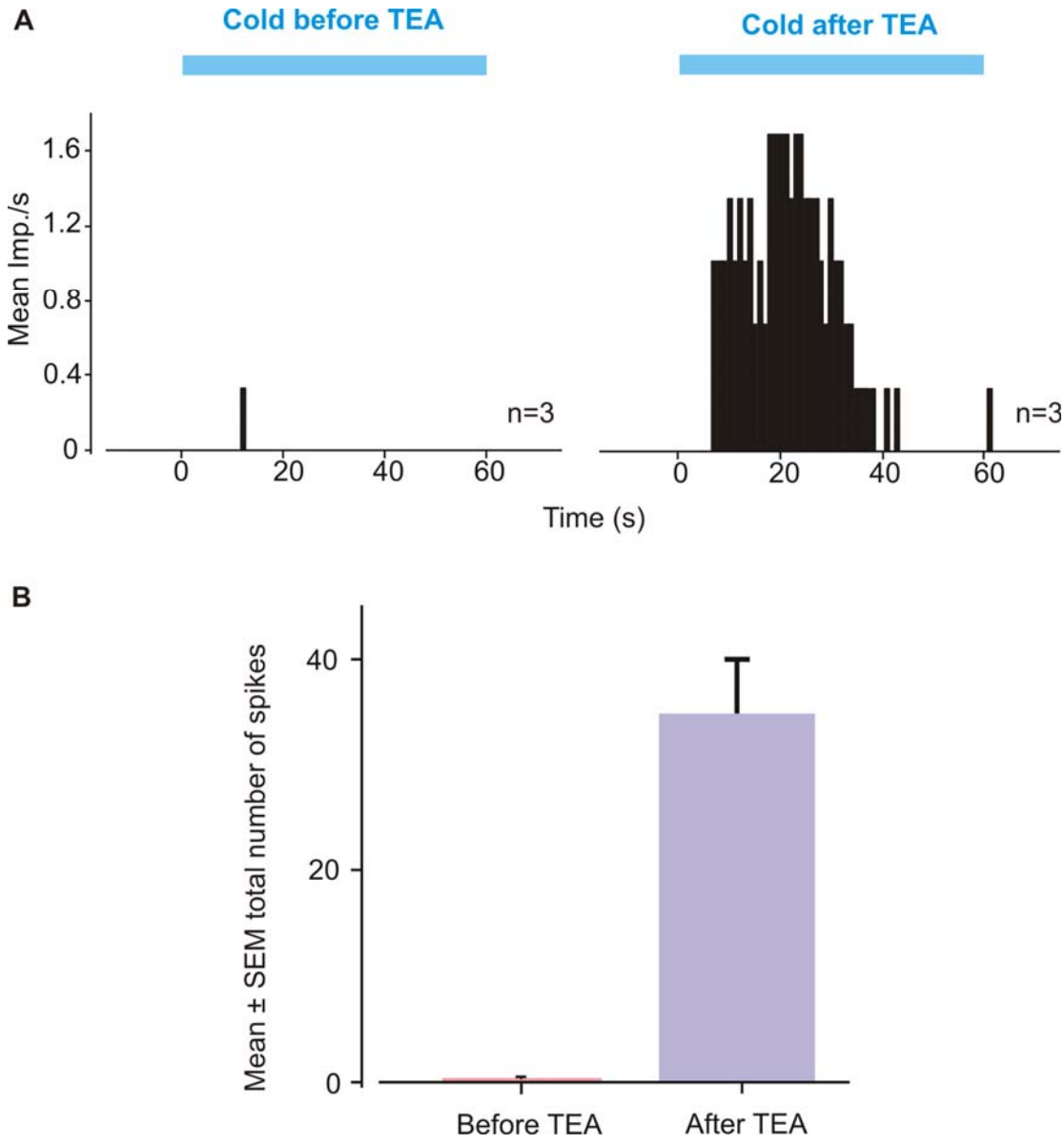

Fig.6.3.15 Mean response of slowly adapting (SA) fibres to a cold stimulus before and after application of TEA (n=3)
(A) Fibres developed a novel cold sensitivity after application of TEA. However, they did not discharge throughout the stimulus. (B) Although there was clearly a greater response to cooling after application of TEA, this difference was not statistically significant (p>0.1, Wilcoxon Matched paired test).

Comparison of TEA and 4-AP sensitivity in RA and SA fibres

Overall, application of 4-AP was able to induce a novel cold response in a total of 12 out of 19 (63 %) Aβ fibres. This did not differ significantly ($p>0.1$, χ^2 test) from the proportion of Aβ fibres which became cold responsive after application of TEA (7/17, 41 %).

RA fibres which became cold responsive after 3 mM 4-AP (n=7) displayed a significantly greater response to cold ($p<0.05$, Mann Whitney U test) compared to those RA fibres which became cold responsive after 10 mM TEA (n=4). However, there was no significant difference ($p=0.3$, Fishers exact test) in the proportion of RA fibres which became cold responsive after 4-AP (7/14) or TEA (4/13).

Statistically there was no significant difference ($p>0.1$, Mann Whitney U test) in cold response between previously cold insensitive SA fibres treated with 4-AP (n=5) or TEA (n=3). However, there was a trend of SA fibres to respond more vigorously to cold after they had been treated with 4-AP (mean response: 98 ± 4 spikes) compared to treatment with TEA (mean response: 35 ± 4 spikes). There was no significant difference ($p>0.4$, Fishers exact test) in the proportion of SA fibres which became cold responsive after 4-AP (5/5) or TEA (3/4) application.

Aδ fibres

D-hair fibres

A total of 6 DH fibres was recorded. None responded to a cold or heat stimulus. None were initially spontaneously active.
1 out of 4 (25 %) DH fibres became cold responsive after application of 4-AP (Fig.6.3.16), with a response threshold of 26.4 °C (see Table 3). 1 out of 2 (50 %) DH fibres became cold responsive after application of TEA with a response threshold of 19.1 °C. Fibres remained unresponsive to noxious heating after application of 4-AP and TEA.

High threshold mechanically sensitive A (AM) fibres

A total of 19 AM fibres was recorded. 4 out of 19 (21 %) fibres responded during a noxious cold stimulus (AMC) with an average response threshold of 13.8 ± 1.9 °C. 2 out of 19 (10.5 %) fibres responded to noxious heat with an average response threshold of 35.4 °C, ranging from 34.4-36.4 °C.
4-AP was applied onto a total of 9 AM units (5 thermo-insensitive AM units and 4 AMC units). 1 out of 5 (20 %) AM fibres became cold sensitive after application of 4-AP (Fig.6.3.17). Interestingly, this fibre displayed a greater response to cold after the application of 300 µM 4-AP compared to after 3 mM 4-AP (see table.6.3.3).
All of the AMC fibres (100 %, n=4) displayed a greater response during a cold stimulus after application of 4-AP. The mean response threshold to cold after application of 3 mM 4-AP was significantly lower (22.8 ± 2.9 °C, $p<0.01$, paired students t-test, n=4), compared to before application of 4-AP (13.8 ± 1.9 °C, n=4). An example of one such AMC unit is shown in Fig.6.3.18. The mean response to cold before and after application of 4-AP is illustrated in Fig.6.3.19. On the whole the firing pattern to cold remained unchanged (Fig.6.3.19A) and, although there was a trend of the units to respond greater to cold after application of 4-AP, the difference was statistically insignificant ($p=0.07$, Wilcoxon Matched paired test, Fig.6.3.19B). AM fibres did not develop sensitivity to heat after 4-AP application.
Mechanical sensitivity of 6 AM (including all the AMC fibres) units was re-investigated after exposure to 4-AP using a mechanical ramp stimulus from 0-200 mN. On average there did not appear to be any change in the encoding of the mechanical stimulus, but there was a slight decrease in the total response after application of 4-AP (Fig.6.3.20). However, statistically this difference was insignificant ($p>0.1$, Wilcoxon matched paired test).

TEA (10 mM) was applied onto a total of 10 AM units (8 thermo-insensitive AM and 2 AMH). None of the units developed cold sensitivity post TEA application.

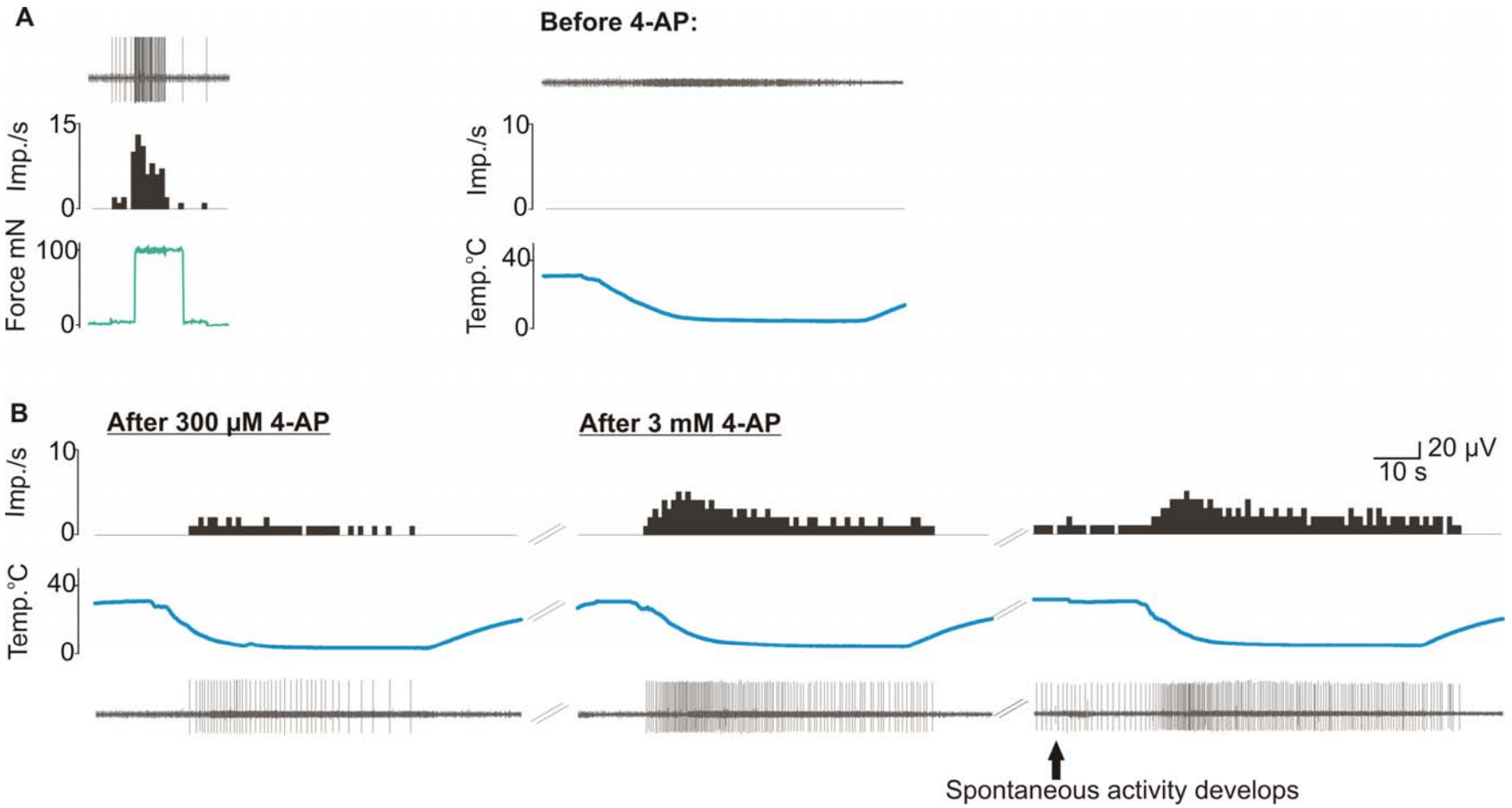

Fig.6.3.16 Example of a D-hair fibre responding to a cold stimulus before and after application of 4-AP
(A) The fibre was previously insensitive during a cold stimulus. (B) After the application of 300 µM 4-AP, the fibre discharged in a regular manner during a cold stimulus. Post 3 mM 4-AP, the fibre displayed a greater response to cold. The fibre then began to discharge spontaneously and continued to do so until the next cold stimulus was applied.

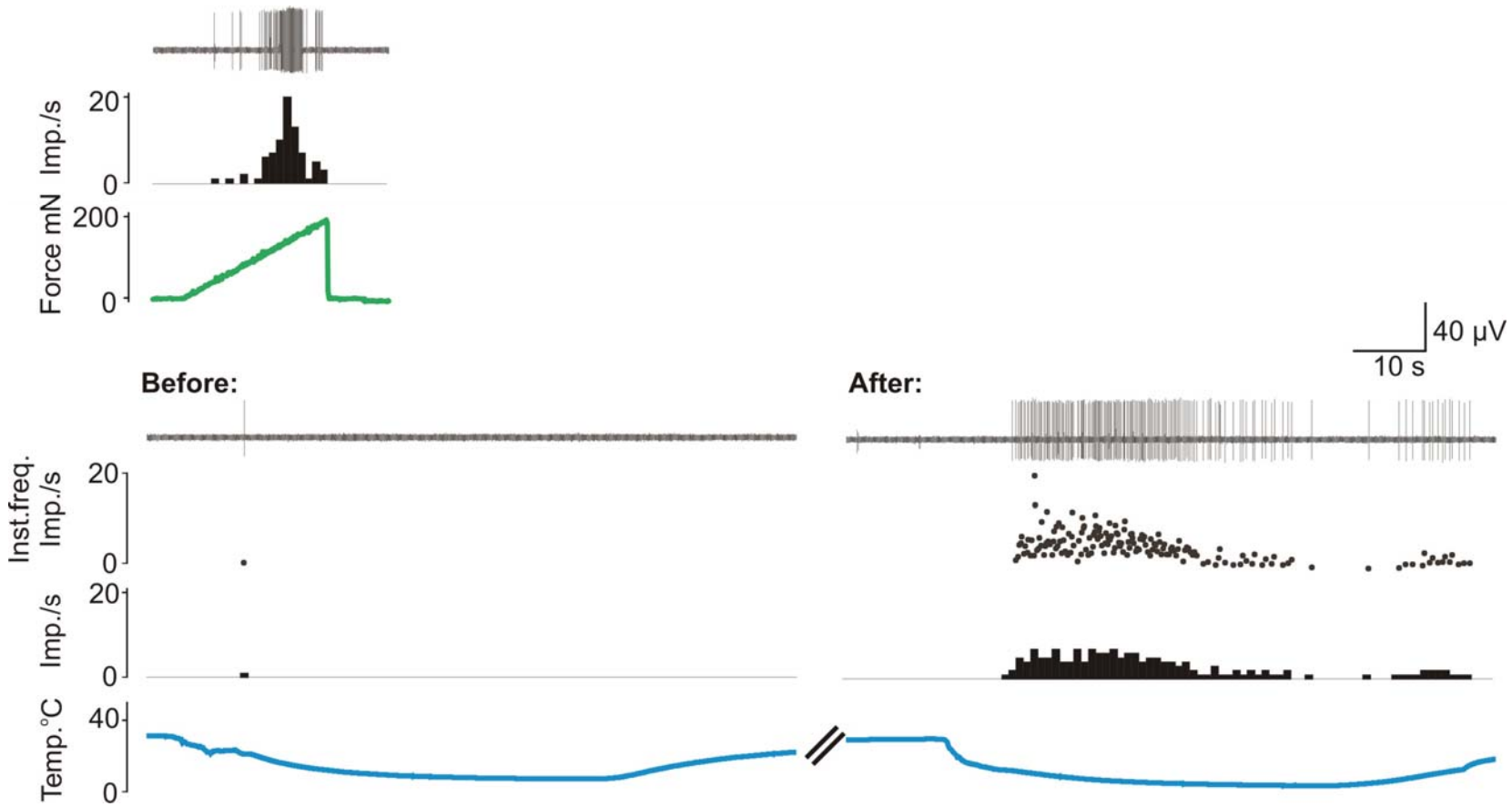

Fig.6.3.17 Example of a high threshold Aδ nociceptor (AM fibre) responding to a cold stimulus before and after 4-AP application

The fibre was previously insensitive to cold. After the application of 300 µM 4-AP, an irregular bursting discharge developed during a cold stimulus.

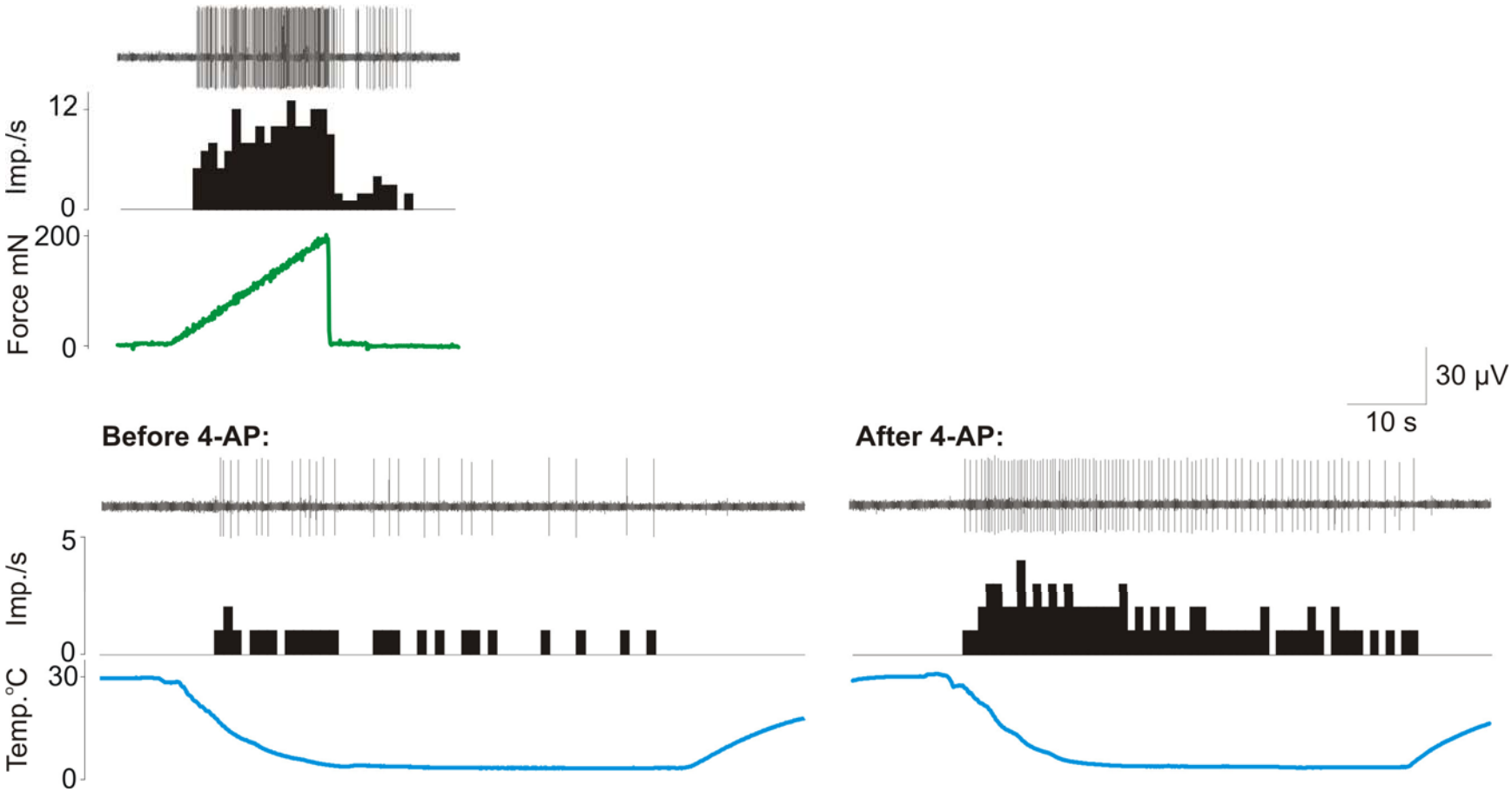

Fig.6.3.18 Example of cold sensitive Aδ nociceptor (AMC fibre) responding to a cold stimulus before and after application of 4-AP

After the application of 3 mM 4-AP the fibre discharged in a more regular manner and displayed a clear increase in firing frequency during a cold stimulus.

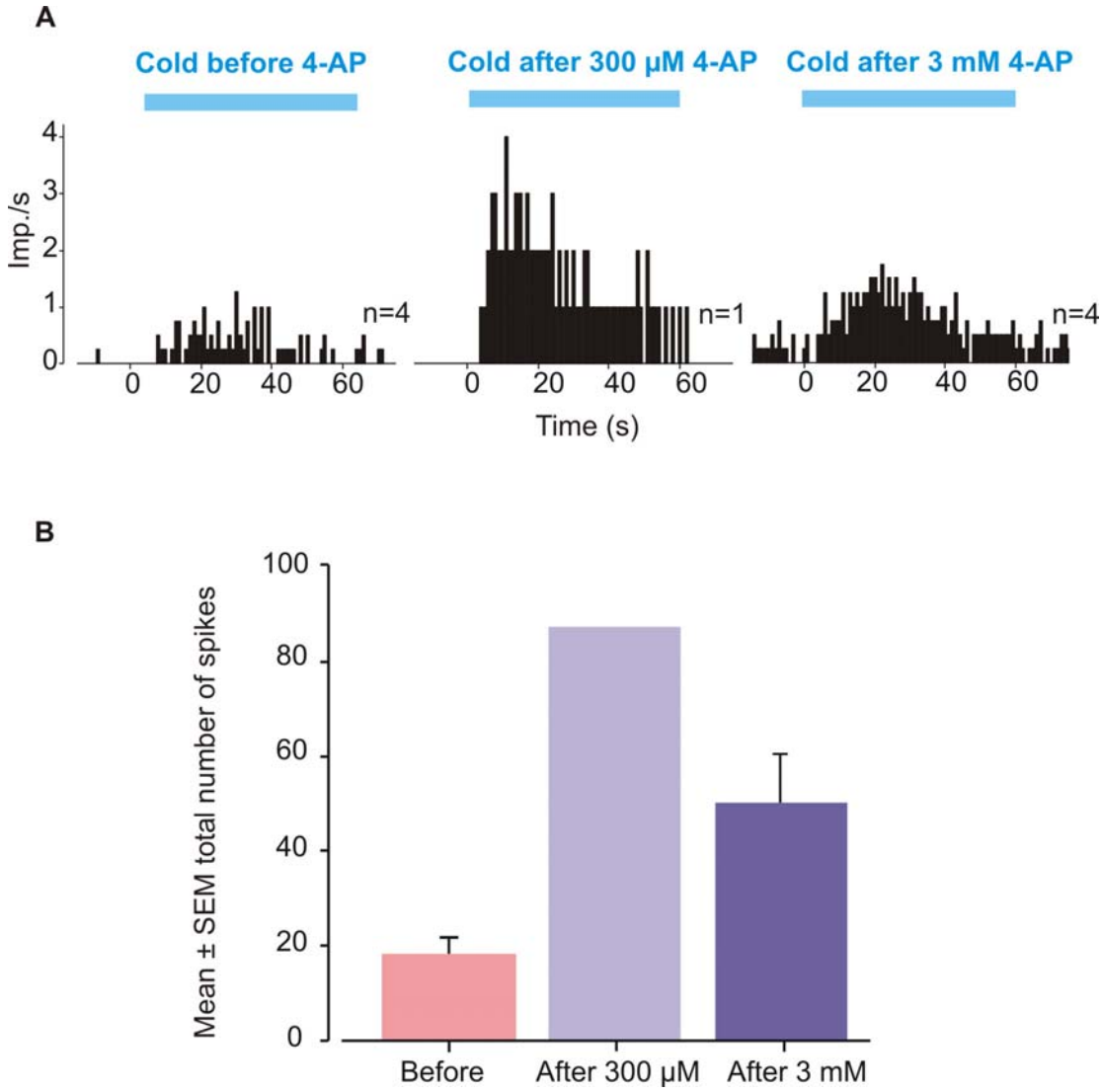

Fig.6.3.19 Mean response of cold sensitive Aδ nociceptors to a cold stimulus before and after application of 4-AP

(A) After application of 4-AP, there was an increase in the mean firing frequency during cold, although there was no change in the firing pattern. (B) The mean total response to cold after application of 3 mM 4-AP was greater (before 4-AP: 18 ± 3 spikes, after 4-AP: 50 ± 9 spikes). However, this difference did not reach statistical significance (p=0.07, Wilcoxon matched paired test).

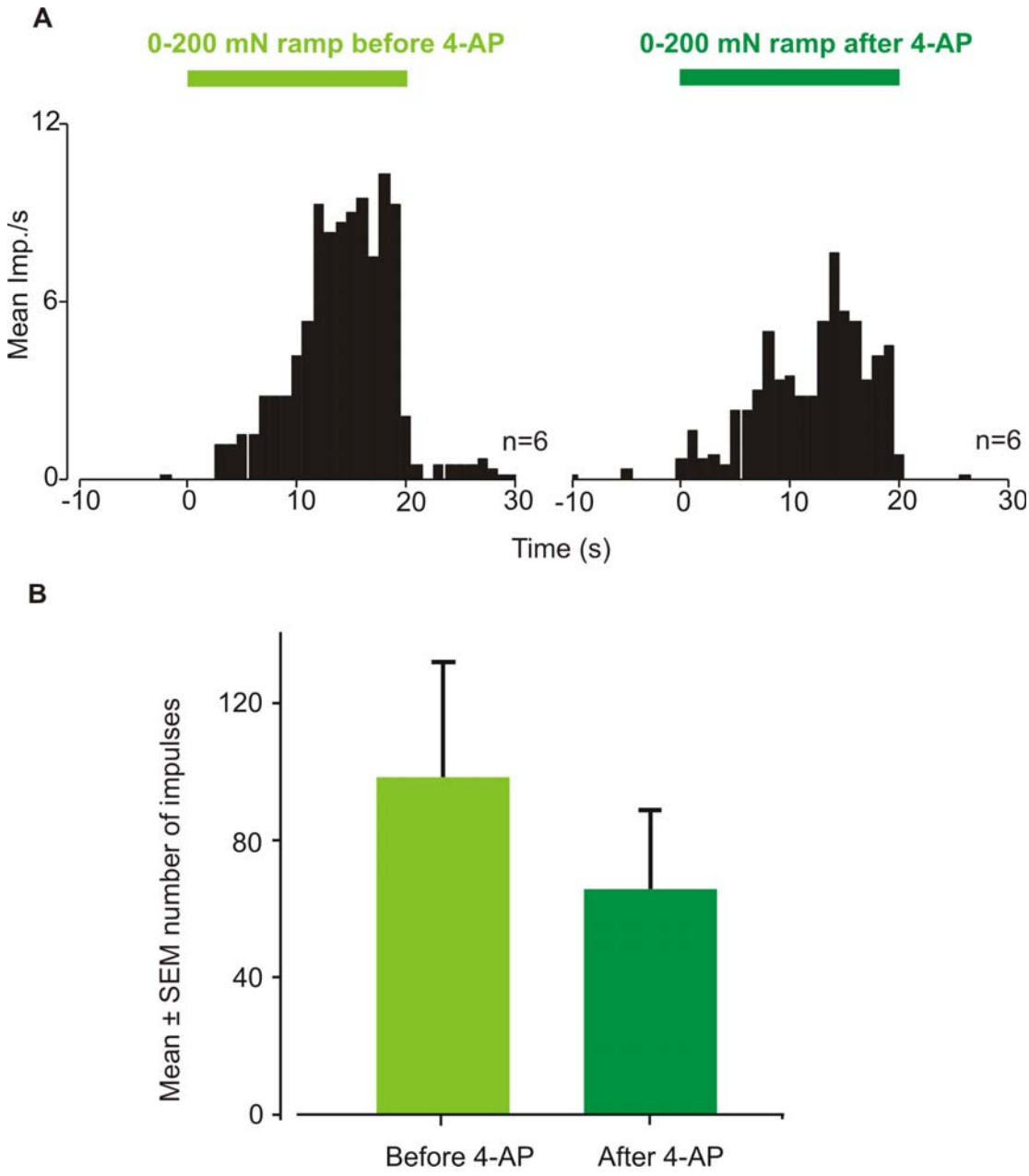

Fig.6.3.20 Mean response of Aδ nociceptors (AM fibres, n=6) to a mechanical ramp stimulus before and after application of 3 mM 4-AP
(A) On average, AM fibres were less responsive during a mechanical ramp stimulus after application of 4-AP. (B) Although there was decrease in mean total number of spikes elicited during the mechanical ramp stimulus after application of 4-AP, this difference was statistically insignificant (p>0.1, Wilcoxon matched paired test).

C fibres

A total of 69 C fibres were recorded. Of these, 22 C (both low threshold and nociceptive C fibres) fibres were investigated initially in a pilot study using lower concentrations of 4-AP, ranging from 10 μM -1 mM. The majority of fibres investigated with 4-AP concentrations below 100 μM did not display any changes in receptive properties. Some C fibres began to fire in doublets after application of 4-AP ranging between 100 μM-1 mM. Taking these findings into consideration, subsequent fibres were investigated using 4-AP in the range of 300 μM – 3 mM.
The following analysis is of 47 C fibres; 29 C fibres which were investigated with 4-AP (300 μM – 3 mM) and 18 C fibres which were investigated with 10 mM TEA.

C fibre nociceptors

A total of 37 C-fibre nociceptors were recorded. 24 C fibres were tested with 4-AP and 13 were tested with TEA.
The mean response to a cold stimulus before and after application of 4-AP or TEA, in C fibre nociceptors which either displayed a novel or increased sensitivity to cold is shown in Fig.6.3.21.

4-AP was applied on 4 CM fibres; novel cold sensitivity did not develop in any of these fibres. TEA was not applied on any CM fibres.
5 out of 10 (50 %) cold sensitive C fibre nociceptors (4/5 CMC and 1/5 CMCH fibres) displayed an increased response during a cold stimulus after application of 4-AP. An example of a CMC fibre is shown in Fig.6.3.22. Fibres did not fire in bursts after application of 4-AP, unlike some Aβ fibres.
Fig.6.3.23 illustrates the mean response of these fibres during cold stimulation before and after application of 4-AP. There was a significant increase ($p<0.05$, Wilcoxon matched paired test) in the response to cold after the application of 3 mM 4-AP (Fig.6.3.23B). Response thresholds to cold did not differ significantly ($p>0.4$, paired students t-test) before (13.5 ± 3.3 °C, n=5) and after (15.8 ± 1.6 °C, n=5) application of 3 mM 4-AP.

4 out of 9 (44 %) cold sensitive C fibre nociceptors displayed a greater response to cold after application of 10 mM TEA (3/5 CMC and 1/4 CMCH fibres). This did not differ significantly ($p>0.9$, Fishers exact test) from the proportion of cold sensitive C nociceptors which displayed an increase in cold sensitivity after application 4-AP. Plotting the mean response during cold revealed that after TEA application, there was a greater response (Fig.6.3.24A), but this increase was not statistically significant ($p=0.07$, Wilcoxon matched paired test, Fig.6.3.24B). After application of TEA there was a trend of the mean cold threshold to shift to warmer temperatures, from 13.8 ± 4.9 °C to 23 ± 3.1 °C, but statistically this difference was insignificant ($p>0.1$, paired students t-test). This trend was also observed in cold sensitive nociceptors after application of 4-AP.

2 out of 10 (20 %) CMH fibres developed novel cold response after application of 4-AP, with a mean response threshold of 16.9 °C, ranging from 5.9-27.9 °C. Subsequent to 4-AP application, one CMH fibre began to discharge in bursts of action potentials during the re-warming phase of the cold ramp (see Fig.6.3.25). The average response to a cold stimulus before and after application of 4-AP is shown in Fig.6.3.26. Both fibres developed a low level of firing during noxious cold.
3 out of 4 (75 %) CMH fibres became cold responsive after TEA application. This did not differ significantly ($p>0.08$, Fishers exact test) from the proportion of CMH fibres which came cold responsive after application of 4-AP. The average response threshold to cold was 17.7 ± 6.5 °C. All of these fibres developed a low level of regular firing during a cold stimulus after TEA application. 2 out of 3 CMH fibres began to fire directly during application of TEA. An example of one such unit is shown in Fig.6.3.27. The mean response of these fibres during a cold stimulus before and after TEA is shown in Fig.6.3.28. Although there was an increase in the response to cold after application of TEA (Fig.6.3.28B), statistically this difference was insignificant ($p>0.1$, Wilcoxon matched paired test).

Mechanical sensitivity was re-investigated in 12 CM fibres post application of 4-AP. The mean response of these fibres to a mechanical ramp stimulus of 0-

200 mN is shown in Fig.6.3.29. After application of 4-AP, there was a reduction in both the peak discharge rate (Fig.6.3.29B) and the total number of spikes elicited during the mechanical ramp (Fig.6.3.29C).

Non-nociceptive C fibres

4-AP was applied on 2 cold sensitive low threshold C (CLTMC) fibres. Subsequently 1 out of 2 of the fibres displayed an increased response to cold. The fibre displayed a reduction in cold threshold after application of 4-AP (see table 3). TEA was applied onto 1 CLTM fibre, which subsequently did not display any changes in receptive properties.

3 mechanically insensitive cold thermoreceptors (CC fibres) were investigated with 4-AP. The average response to a cold stimulus before and after 4-AP application is shown in Fig.6.3.30A. 4-AP did not appear to affect the cold sensitivity of these fibres and overall there was a slight decrease in the total response to cold after 4-AP application (mean response before: 345 ± 94 spikes, n=3. Mean response after: 326 ± 75 spikes, n=3, Fig.6.3.30B). However, statistically this difference was insignificant ($p>0.2$, Wilcoxon matched paired test). There was no significant difference in the response threshold to cold between before (30.5 ± 0.2 °C, n=3) and after (30.8 ± 0.3 °C, n=3) application of 4-AP ($p>0.3$, paired students t-test).
4 CC fibres were investigated with TEA. Similar to the results found with 4-AP, application of TEA did not affect the cold response of these fibres. There appeared to be no change in firing pattern and there was no significant difference in the mean response to cold after TEA application (before: 319 ± 75 spikes, after: 273 ± 43 spikes, $p>0.3$, Wilcoxon matched paired test, Fig. 31). There was no significant difference ($p>0.7$, paired students t-test) in the response threshold to cold before (29.4 ± 0.9 °C) and after (29.0 ± 1.0 °C) application of TEA.

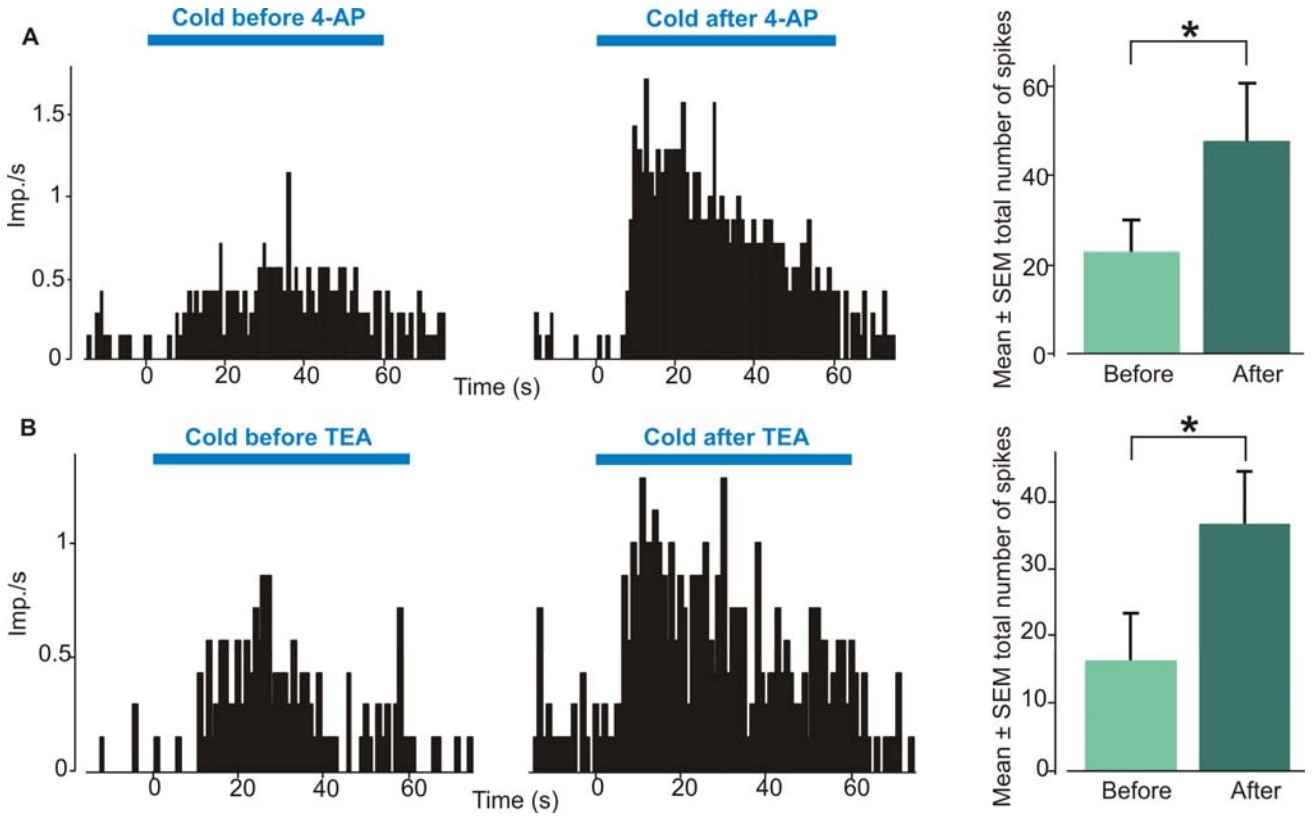

Fig.6.3.21 C fibre nociceptors which developed a novel or increased cold sensitivity after application of 4-AP or TEA
(A) 7 out of 24 (29 %) CM fibres displayed a novel or increased response to a cold stimulus after application of 3 mM 4-AP. The average response of these 7 fibres (4 CMC, 1 CMCH and 2 CMH) to a cold stimulus is shown above. There was a significant increase (before: 21 ± 7 spikes, after: 48 ± 13 spikes) in the total number of spikes elicited during cold after application of 4-AP ($p<0.05$, Wilcoxon matched paired test). (B) 7 out of 13 (53 %) CM fibres (3 CMC, 1 CMCH and 3 CMH) displayed a novel or increased response after application of 10 mM TEA. There was a significant increase (before: 16 ± 7 spikes, after: 36 ± 7 spikes) in the total response to cold after application of TEA ($p<0.05$, Wilcoxon matched paired test).

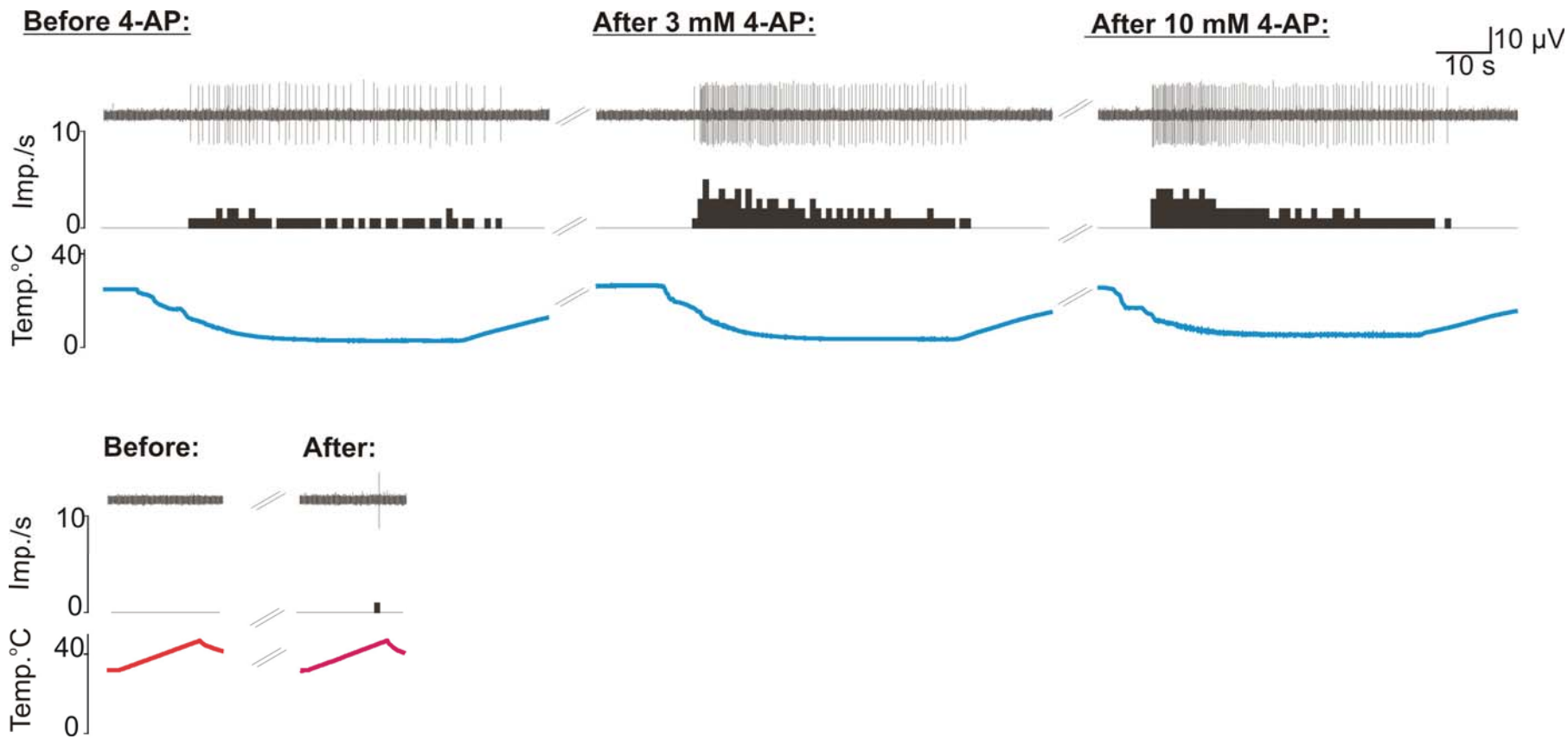

Fig.6.3.22 Example of a cold sensitive C fibre nociceptor (CMC) responding to thermal stimuli before and after 4-AP application

After application of 3 mM 4-AP, the fibre displayed an increased response during cold. The fibre continued to fire in a regular manner, but never in bursts. The response did not increase further after application of 10 mM 4-AP. The fibre remained insensitive to noxious heating and discharged only one action potential.

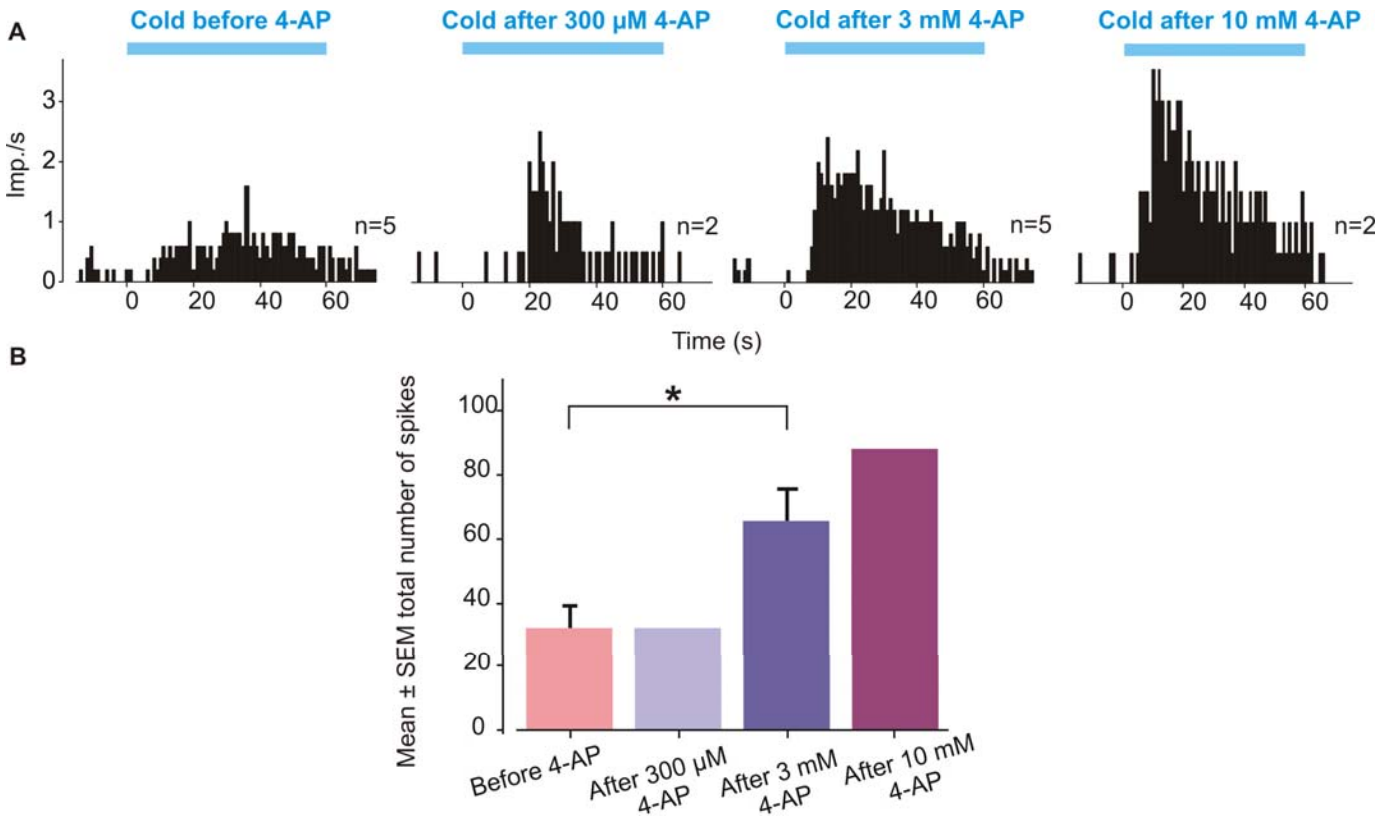

Fig.6.3.23 Mean response of cold sensitive C fibre nociceptors (CMC and CMCH fibres, n=5) to a cold stimulus before and after application of 4-AP

(A) 50 % of cold sensitive C fibre nociceptors displayed an increased response to cold after application of 4-AP. There appeared to be a slight change in the average firing pattern after 4-AP; on average fibres appeared to reach peak discharge rate earlier on in the stimulus. (B) There was a significant increase (before 4-AP: 30 ± 8 spikes, after 3 mM 4-AP: 61 ± 12 spikes) in the mean total response to cold after application of 3 mM 4-AP ($p<0.05$, Wilcoxon matched paired test).

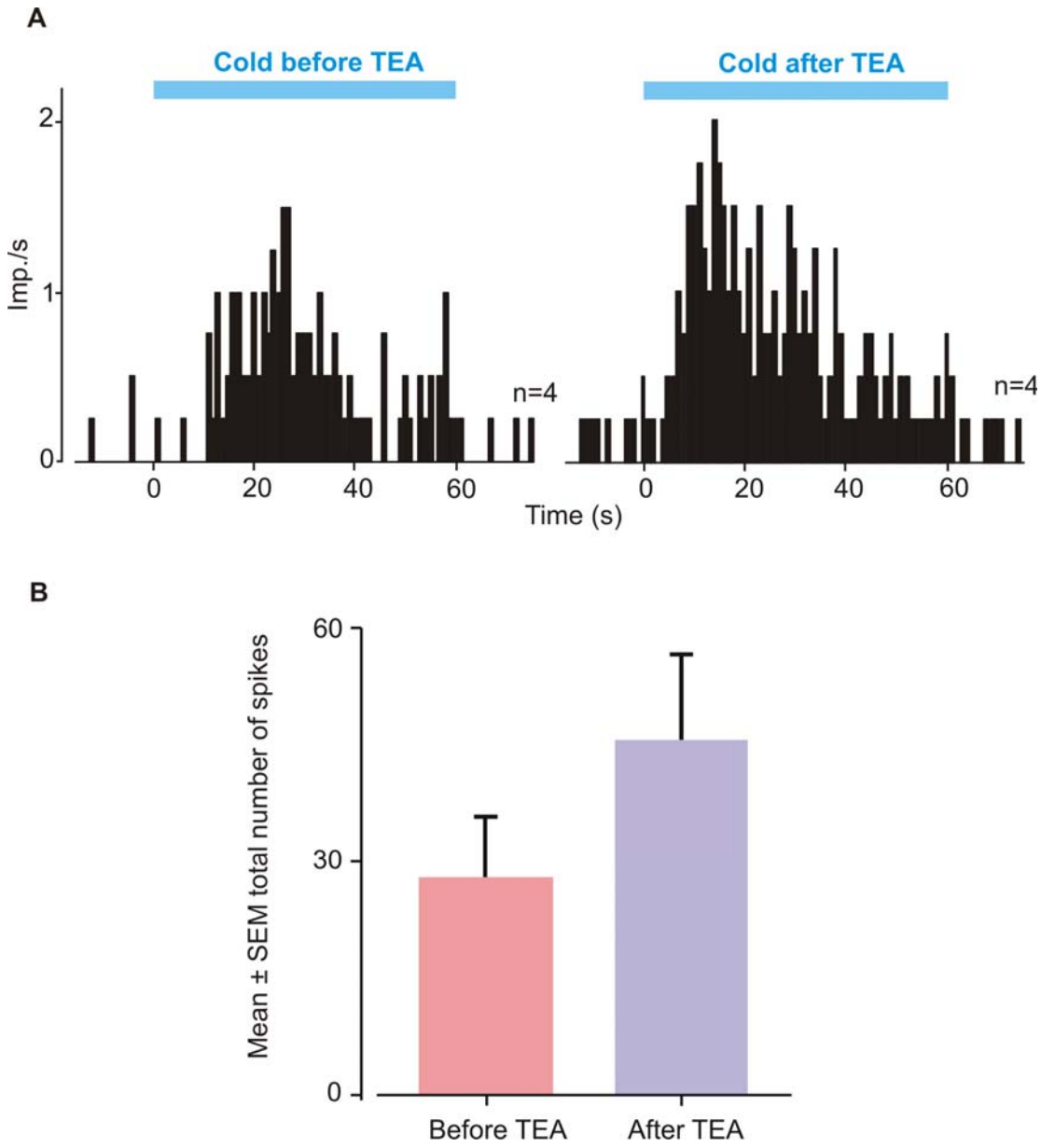

Fig.6.3.24 Mean response of cold sensitive C fibre nociceptors (CMC and CMCH fibres, n=4) to a cold stimulus before and after application of 10 mM TEA

(A) After application of TEA, there was an increase in firing frequency during a cold stimulus. There was a tendency of fibres to display an earlier peak discharge after application of TEA. (B) Although the mean total response during a cold stimulus was greater after application of TEA (before TEA: 28 ± 8 spikes, after TEA: 46 ± 10spikes), statistically this difference was insignificant (p=0.07, Wilcoxon matched paired test).

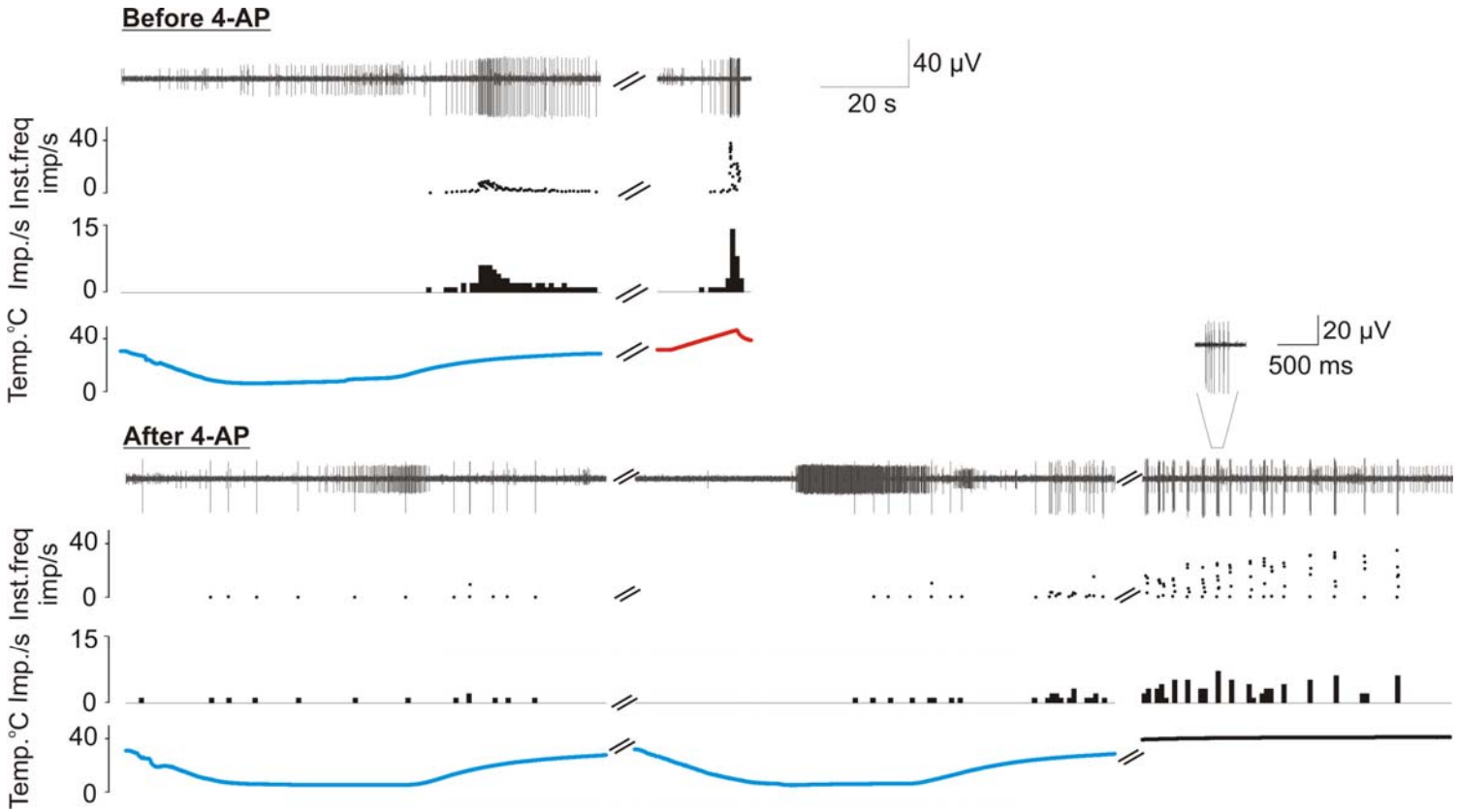

Fig.6.3.25 Example of a heat sensitive C fibre nociceptor (CMH fibre) fibre which became cold responsive after application of 3 mM 4-AP

Before application of 4-AP, the fibre was unresponsive during a noxious cold stimulus and only discharged during the re-warming phase of the cold ramp. This behaviour is characteristic of many CMH fibres. After application of 3 mM 4-AP, the fibre became responsive to cold, firing in a regular manner but at a very low firing frequency. This was shown to be reproducible during application of another cold stimulus. The fibre then began to discharge in bursts of action potentials during the re-warming phase of the second cold stimulus.

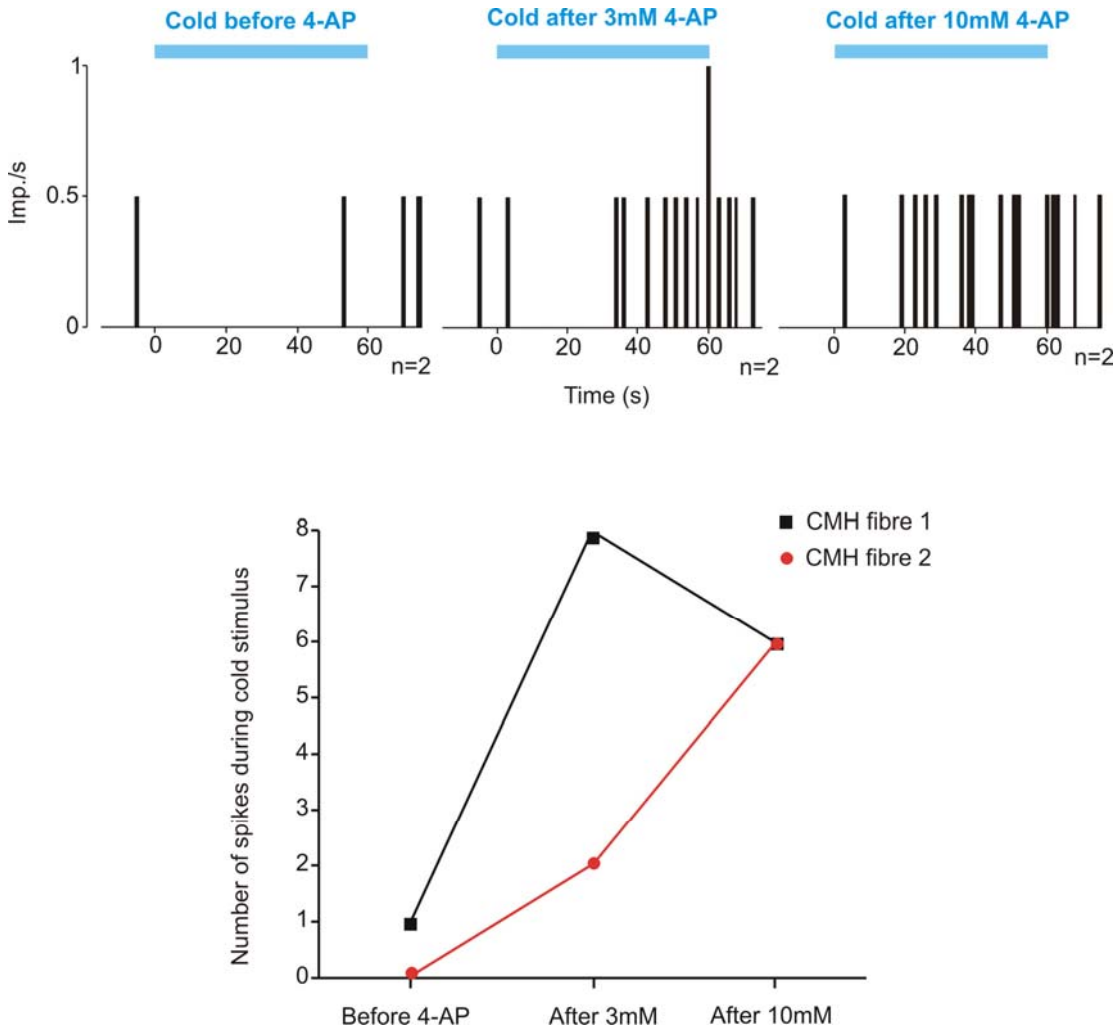

Fig.6.3.26 Mean response of heat sensitive C fibre nociceptors (CMH fibres, n=2) to a cold stimulus before and after application of 4-AP
20 % of CMH fibres developed a novel response to cold after application of 4-AP. If any on-going activity was present previously, this was suppressed during cooling. However after application of 4-AP, fibres displayed a low frequency discharge during the application of cold.

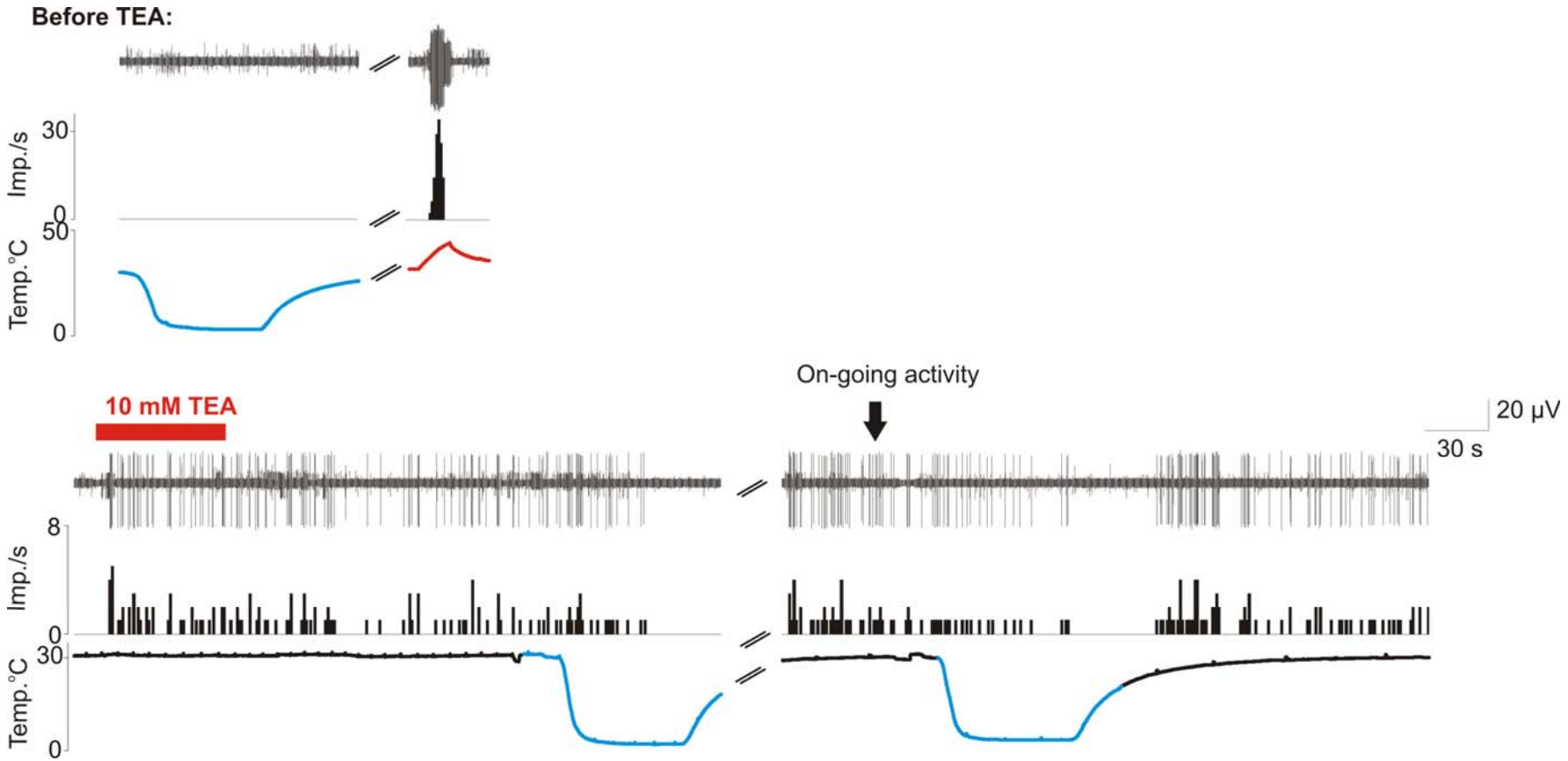

Fig.6.3.27 Example of a heat sensitive C fibre nociceptor (CMH fibre) which became responsive to cold after application of 10 mM TEA

The fibre was previously unresponsive during the cold stimulus. The fibre then began to discharge directly during the application of 10 mM TEA. Upon cooling the receptive field, the fibre continued to fire and then abruptly stopped near the end of the stimulus. Subsequently, the fibre began to fire during the re-warming phase of the cold ramp and continued to do so throughout the second cold stimulus applied.

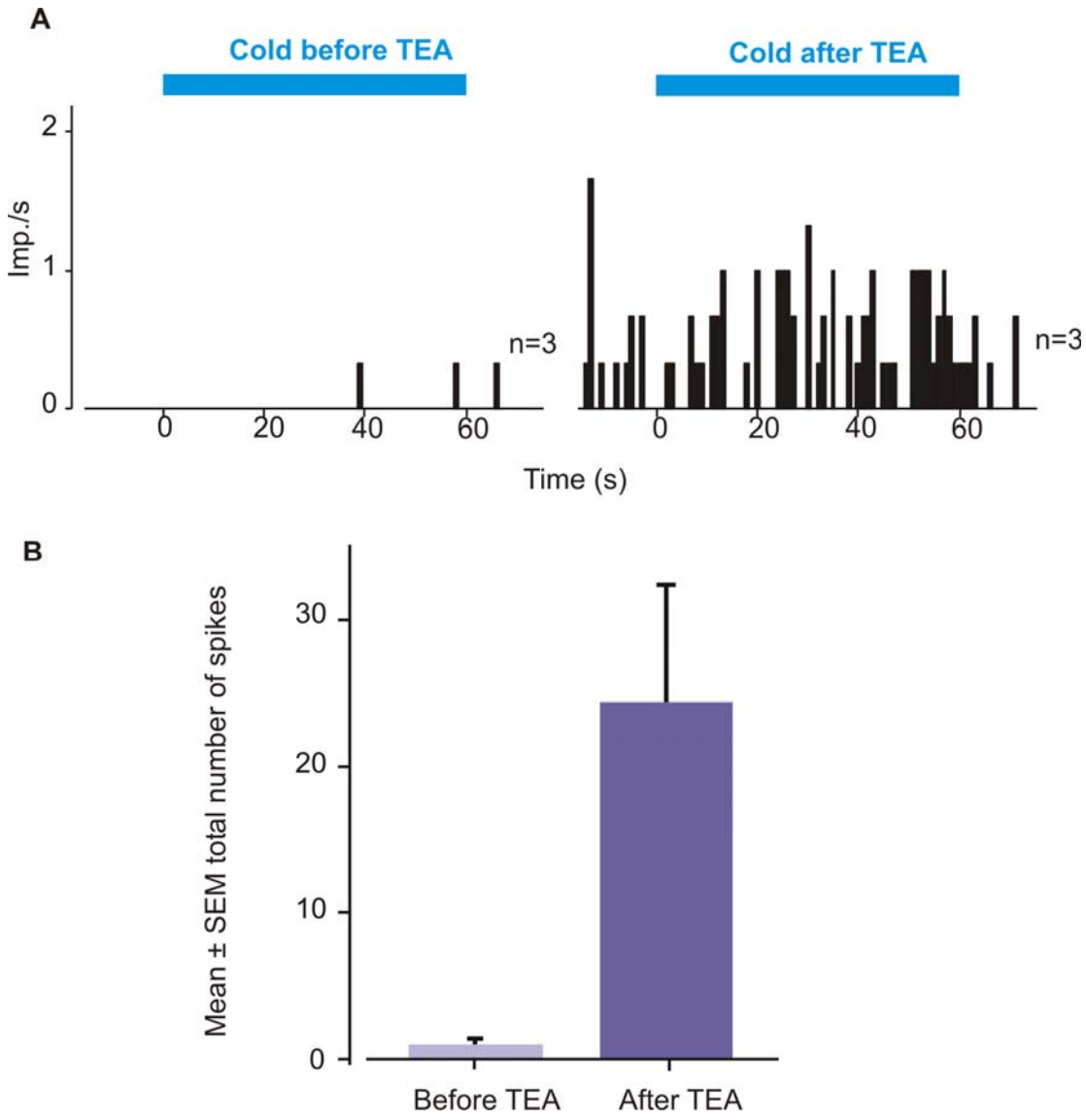

Fig.6.3.28 Mean response of heat sensitive C fibre nociceptors (CMH fibres, n=3) to a cold stimulus before and after application of 10 mM TEA *(A) 3 out of 4 CMH fibres developed a novel response to cold after application of 10 mM TEA. On average they discharged at low frequencies, in an irregular manner. (B) Although there was an increase in the total response to a cold stimulus after application of TEA, statistically this difference was insignificant (p=0.11, Wilcoxon matched paired test).*

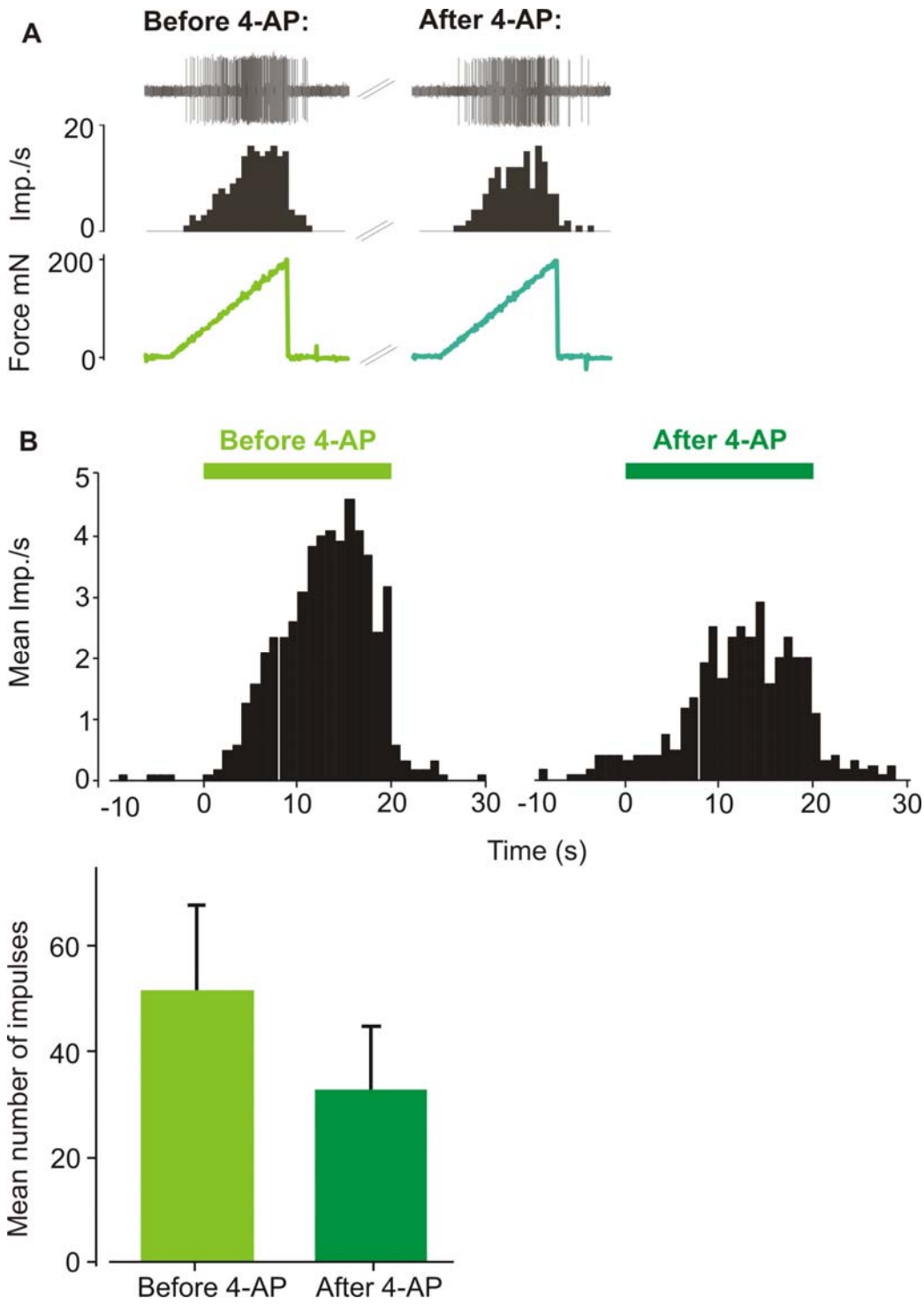

Fig.6.3.29 Mean response of mechanically sensitive high threshold C (CM) fibres to a mechanical ramp stimulus before and after application of 4-AP (n=12)

(A) A CMC fibre is presented. There did not appear to be any difference in the firing pattern or frequency in response to a mechanical stimulus after the application of 3 mM 4-AP. (B) Plotting the mean response of 12 CM fibres revealed that after application of 4-AP, there was a decrease in the peak discharge rate and response during a mechanical ramp stimulus. (C) However, statistically this difference was insignificant (p=0.07, Wilcoxon matched paired test).

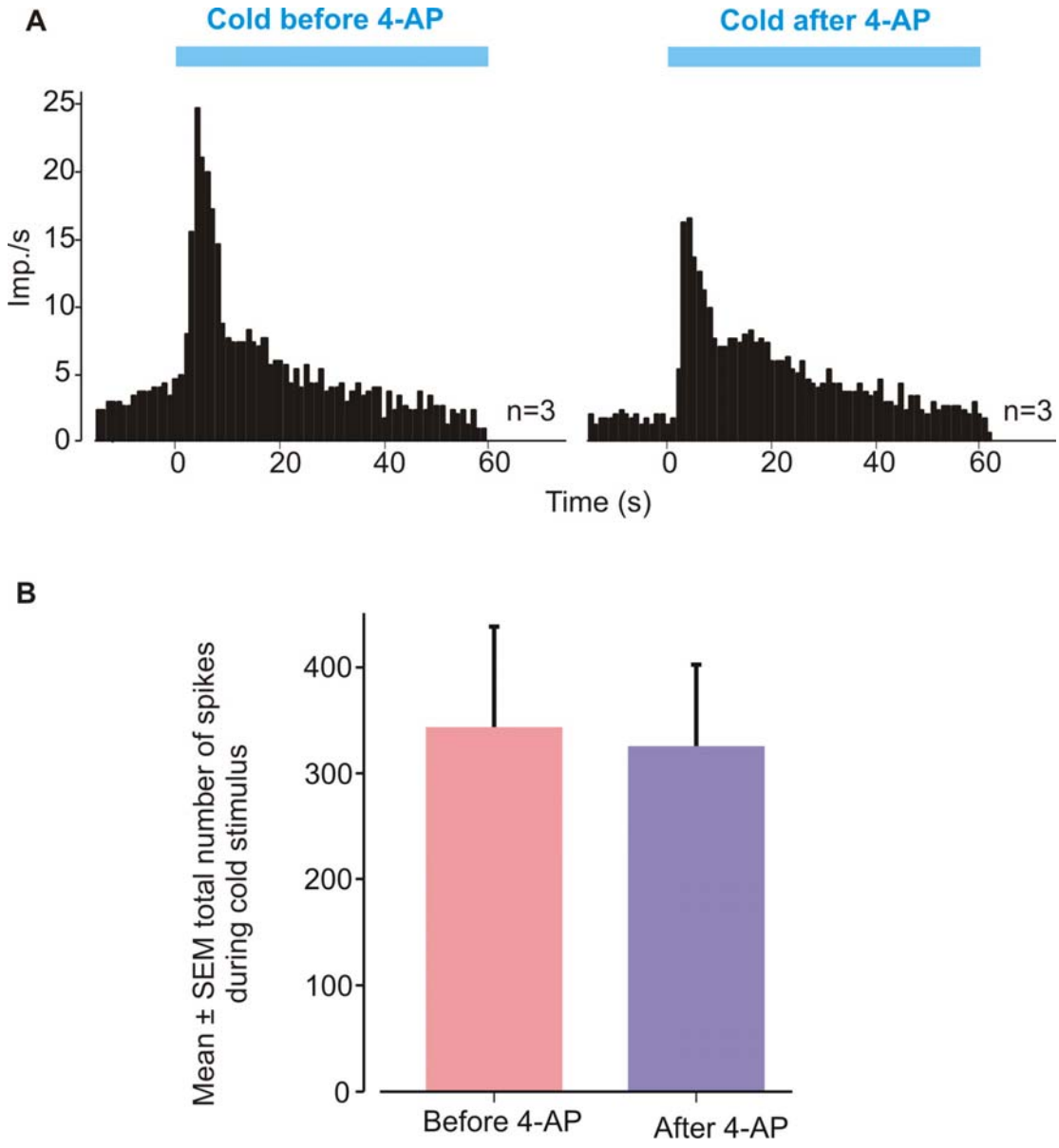

Fig.6.3.30 Mean response of mechanically-insensitive cold (CC) fibres response to a cold stimulus before and after application of 3 mM 4-AP (n=3)

(A) Overall, there was a decrease in the peak discharge rate during a cold stimulus after application of 3 mM 4-AP. No other obvious changes were evident. (B) Statistically there was no significant difference in the total spikes elicited during a cold response after application of 4-AP (p>0.2, Wilcoxon matched paired test).

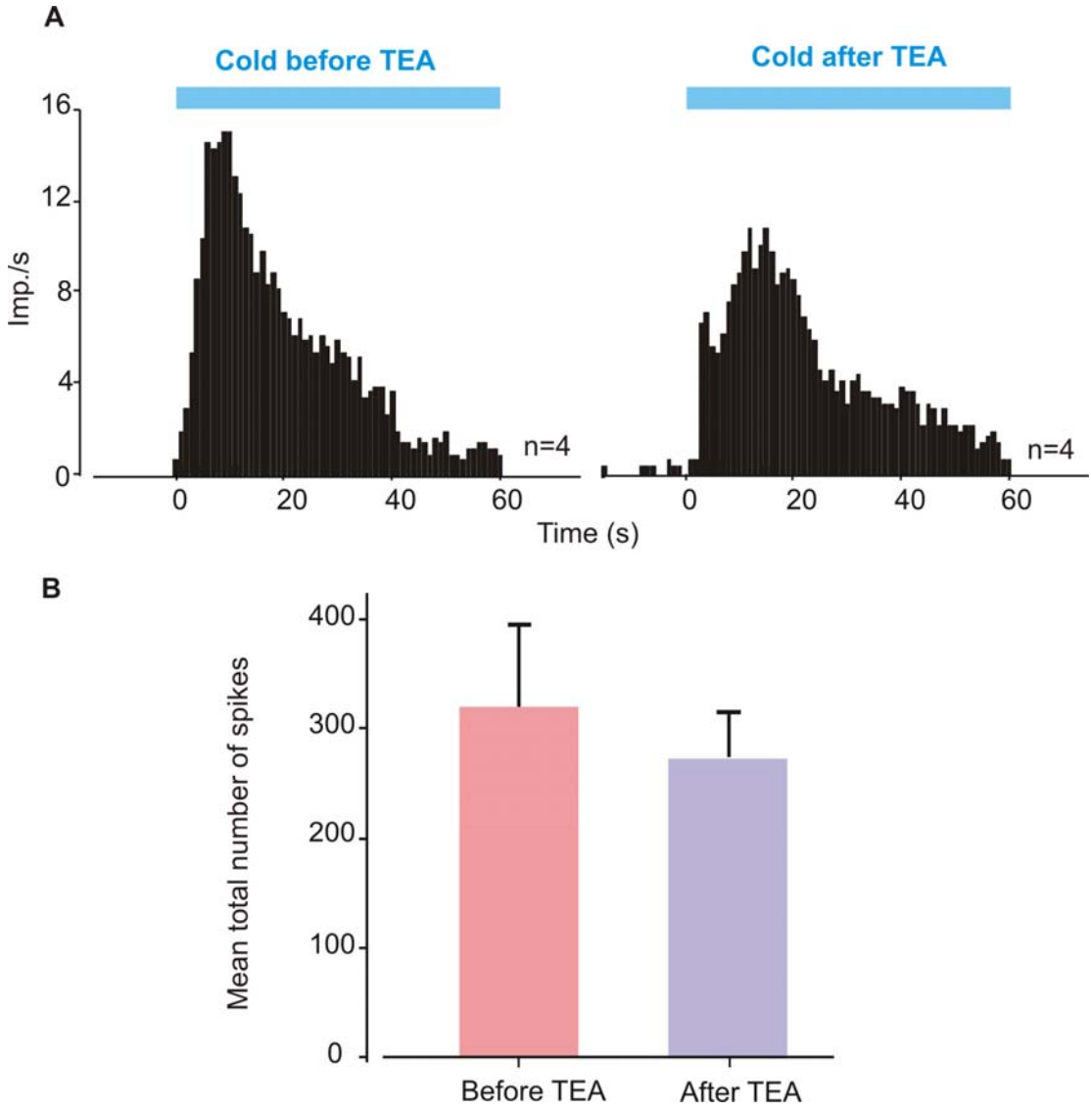

Fig.6.3.31 Mean response of mechanically-insensitive cold (CC) fibres response to a cold stimulus before and after application of 10 mM TEA (n=4)

(A) Overall, there was a decrease in the peak discharge rate during a cold stimulus after application of 10 mM TEA. (B) However, statistically there was no significant difference in the total spikes elicited during a cold response after application of TEA (p>0.3, Wilcoxon matched paired test).

TABLE 6.3.3. Summary of the fibres with induced cold sensitivity: Mean number of spikes to a cold stimulus and cold thresholds (CT) before and after 4-AP

Fibre Type	n	Total spikes during cold before 4-AP	CT, ˚C	n	Total spikes during cold after 300 µM 4-AP	CT, ˚C after 300 µM 4-AP	n	Total spikes during cold after 3 mM 4-AP	CT, ˚C after 3 mM 4-AP	n	Total spikes during cold after 10 mM 4-AP	CT, ˚C after 3 mM 4-AP
RA	7	0	-	2	46 (43-50)	18 (16.6-19.4)	7	128 ± 41	21.2 ± 1.8	0	-	-
SA	5	0	-	2	14 (12-16)	21.6 (18.8-24.4)	5	98 ± 34	21.4 ± 2.7	0	-	-
SA (c)	3	15 ± 4	16.3 ± 3.2	2	67 (27-107)	20.9 (17.8-24)	3	128 ± 60	18.7 ± 1.7	0	-	-
DH	1	0	-	1	42	15.3	1	126	26.4	0	-	-
AM	1	0	-	1	150	15.1	1	4	8	0	-	-
AMC	4	18 ± 3	13.8 ± 1.9	1	87	27.5	4	50 ± 9	22.8 ± 2.9	3	47 ± 8	20.2 ± 4.6
CMC	4	34 ± 9	14.6 ± 4.0	2	29 (6-53)	16.9 (13.7-20.1)	4	65 ± 15	16.4 ± 1.9	2	88 (79-97)	17.2 (16.7-17.7)
CMH	2	0	-	0	-	-	2	5 (2-8)	(5.9-27.9)	2	6 (6-6)	(5.3-26.8)
CMCH	1	17	9.3	0	-	-	1	46	13.4	1	26	9.7
CLTMC	1	15	21.1	0	-	-	1	41	29.1	0	-	-

Values are means ± SE. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; SA (c), cold sensitive slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMC, high threshold mechano-cold A fibre; DH, D-hair receptors; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM, C low-threshold mechano-sensitive fibre.

TABLE 6.3.4. Summary of the fibres with induced cold sensitivity: Mean number of spikes to a cold stimulus (CT) and cold thresholds before and after TEA

Fibre Type	n	Number of spikes during cold stimulus before TEA (1 min)	CT, ˚C	n	Number of spikes during cold stimulus after 10mM TEA 4AP (1 min)	CT, ˚C after 10mM TEA	n	Number of spikes during cold stimulus after 10mM TEA 4AP (5 min)	CT, ˚C after 10mM TEA
RA	4	0	-	4	14 ± 4	20.2 ± 2.3	4	12.8 ± 3.5	18.7 ± 2.1
SA	3	0	-	3	35 ± 4.9	22.9 ± 3.4	3	71.3 ± 36.5	22.9 ± 5.6
DH	1	0	-	1	22	19.1	1	9	18.0
CMC	3	26 ± 11	15.1 ± 6.8	3	41.7 ± 14.1	20.4 ± 2.4	2	54.5 (44-65)	17.1 (15.4-18.7)
CMH	3	0	-	3	24.3 ± 8.1	17.7 ± 6.5	3	17 ± 8.2	9.2 ± 2.1
CMCH	1	34	9.7	1	59	30.8	1	44	31.1

Values are means ± SE. There was no significant difference in the cold response between after 1 or 5 minutes application of TEA ($p>0.4$ for RA fibres, $p>0.2$ for SA fibres and $p>0.1$ for CMH fibres, Wilcoxon matched paired test). There was no significant difference in cold thresholds after 1 or 5 min application of TEA ($p>0.05$ for RA fibres, $p>0.9$ for SA fibres, $p>0.1$ for CMH fibres, Wilcoxon matched paired test). RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; DH, D-hair receptors; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor.

Comparison of 4-AP and TEA sensitivity in C fibre nociceptors

Overall, application of 4-AP was able to induce a novel cold response in a total of 7 out of 24 (29 %) C fibre nociceptors. This did not differ significantly ($p>0.1$, χ^2 test) from the proportion of C-fibres which became cold responsive after application of TEA (7/13, 53 %).
There was no significant difference in the cold response of mechanosensitive C fibres which became cold responsive after 3 mM 4-AP (n=7) or 10 mM TEA (n=7) ($p>0.5$, Mann Whitney U test).

Investigating the combined effect of 4-AP and TEA application on the whole nerve trunk

In a different set of 2 experiments a mixture of 3 mM 4-AP and 10 mM TEA was applied to the saphenous nerve trunk of the whole nerve. Recordings were then carried out from fibres which were located in the periphery, distally to the 4-AP/TEA treated area.

In each experiment a multi-unit recording was carried out, in which 3 or 4 fibres were characterised. This was then followed by the application of 4-AP/TEA to the whole nerve for 5 minutes. A cold stimulus was then directly applied to the whole nerve, where the 4-AP/TEA had been applied, to see if any activity could be evoked from the units in the periphery. All the receptive fields in the periphery were then individually re-tested for their response to a cold stimulus (see Fig.6.2.1.)
A total of 7 fibres were characterised in the experiments; 5 RA and 2 DH fibres. None of the units recorded from the periphery were initially cold sensitive. None of these fibres became spontaneously active after a cold stimulus was applied onto the 4-AP/TEA treated area of the whole nerve. When a cold stimulus was applied directly onto their receptive fields, they remained unresponsive.
4-AP/TEA was then re-applied for 5 mins, but this time to the individual unit receptive fields in the periphery. A cold stimulus was then re-applied directly to those receptive fields. After this, 5 out of 7 (71 %) of units (3 RA and 2 DH

fibres) then displayed a novel cold response. An example of one such multi-unit recording is shown in Fig.6.3.32.

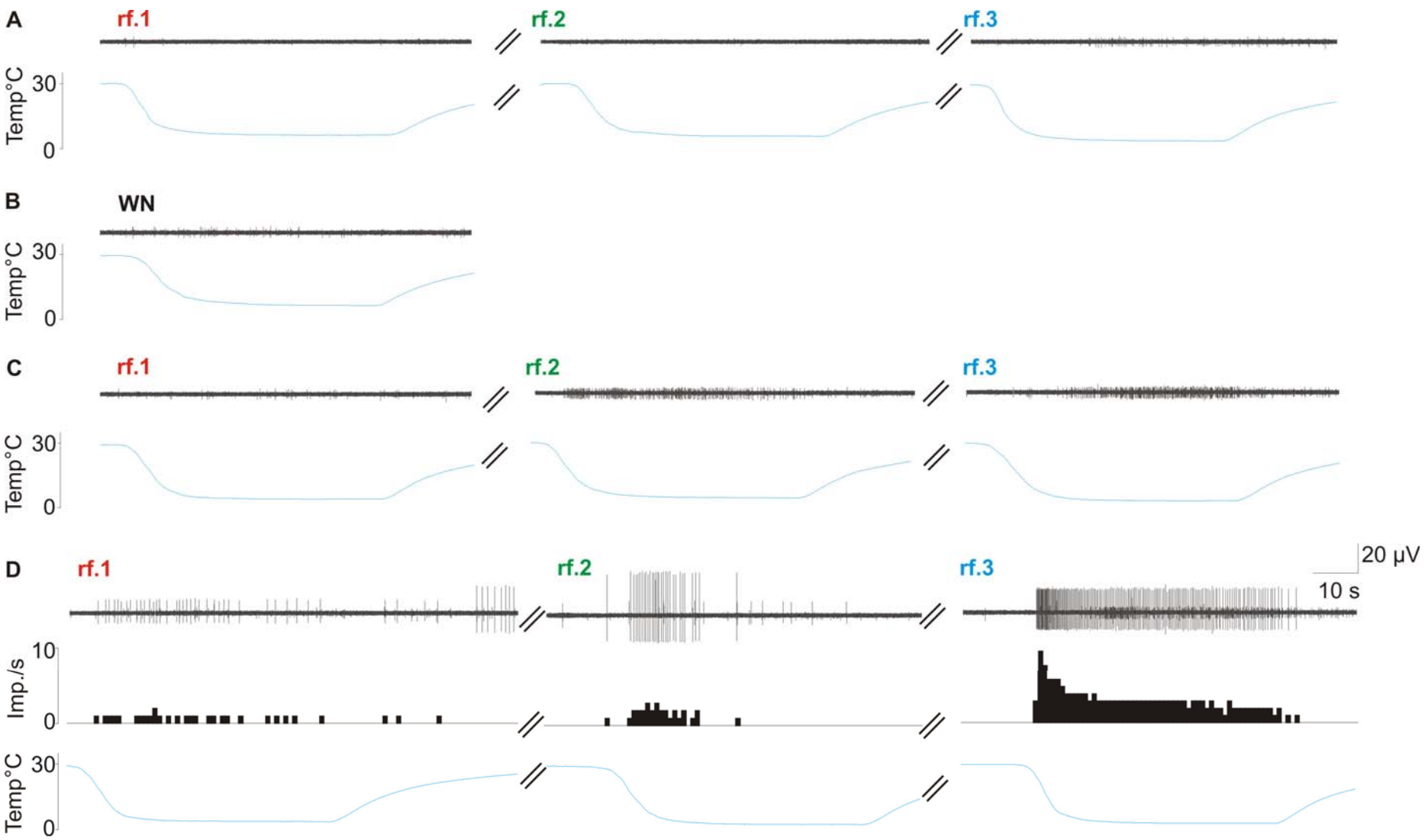
A
rf.1
rf.2
rf.3
Temp°C
30
0
B
WN
Temp°C
30
0
C
rf.1
rf.2
rf.3
Temp°C
30
0
D
rf.1
rf.2
rf.3
20 µV
10 s
Imp./s
10
0
Temp°C
30
0

Fig.6.3.32. Example of an experiment in which combination of 3 mM 4-AP and 10 mM TEA (5 min) was applied to the saphenous nerve trunk

(A) A cold stimulus was applied to the receptive fields (rf) of 3 characterised units in the periphery; rf.1 was identified as a DH unit, rf.2 and 3 were characterised as RA units. None were initially cold sensitive. (B) 4-AP/TEA was then applied onto the whole nerve trunk. A cold stimulus was then applied directly onto the whole nerve (WN). No activity was evoked from the periphery. (C) The fibres were then re-tested with a cold stimulus. All 3 units remained unresponsive. (D) 4-AP/TEA was then applied directly onto the receptive fields of the fibres. Subsequently all units displayed a novel cold sensitivity.

6.4 Discussion

Research has shown that several mechanisms are likely to contribute to the transduction of cold temperatures in primary sensory neurons. In addition to the cold and menthol activated TRPM8 receptor, at least two other mechanisms have been shown to potentially be involved in cold transduction in rodent cultured dorsal root ganglion (DRG) or trigeminal (TG) neurons. First, cold sensitive neurons inhibits a background K^+ current, which is largely resistant to 4-AP and TEA (Reid and Flonta, 2001). Second, in a proportion of cold insensitive TG neurons, the presence of a 4-AP sensitive K^+ current has been shown to act as an excitability break, preventing these neurons from reaching their firing threshold during cooling (Viana et al., 2002).
Most studies have used neurones in culture as a model of otherwise inaccessible receptor terminals to investigate the role of potassium conductances in the transduction of cold. Using the in-vitro skin nerve preparation, the present study shows for the first time that application of 4-AP or TEA directly to the receptive fields induces a novel cold sensitivity in a proportion of cold insensitive neurons, consistent with the above finding. In addition to this, this study also show that application of 4-AP or TEA increases cold responses in a proportion of cold sensitive neurons. Interestingly 4-AP or TEA had no effect on the cold responses of innocuous cold thermoreceptors. Application of a mixture of 4-AP and TEA onto the whole nerve did not induce changes in receptive properties of fibres recorded from the periphery.

Almost half of all neurons become cold responsive after application of 4-AP or TEA

Overall, application of 4-AP induced a novel cold sensitivity in 42 % (16/38) of all fibres tested. TEA induced a novel cold sensitivity in 48 % of all fibres tested (11/23).
Our findings are in agreement with those of Viana et al (2002) who demonstrated that application of 100 μM 4-AP to cultured TG neurons from mice induced a novel cold sensitivity in 40 % (8/20) of cold insensitive neurons (Viana et al., 2002). This has also been shown to be the case in mice DRG neurons, where following application of 300 μM 4-AP, 39 % of previously

unresponsive DRG neurons became cold responsive (Munns et al., 2007). In addition to this, this study has shown for the first time that following application of 4-AP, 65 % of all cold sensitive neurons displayed an increased response to cold. Similarly, 44 % of cold sensitive fibres displayed an increase in cold sensitivity after TEA application.

Munns et al (2007) showed that of those cells which displayed an induced cold response, 34 % were sensitive to capsaicin but only 1% were sensitive to menthol. Consistent with this observation, we found that some heat sensitive C fibre nociceptors (CMH fibres) displayed a novel cold response after application of 4-AP (2/10 fibres) or TEA (3/4 fibres) and we have previously shown that the majority of these fibres are capsaicin sensitive (see chapter 4). Other fibre types which developed a novel cold sensitivity after application of 4-AP included RA, SA, DH and AM fibres. We have shown that these fibre populations lack sensitivity to menthol (chapter 4) and therefore our results are consistent with the finding from Munns et al (2007) who reported a lack of menthol sensitivity in many DRG neurons with induced cold sensitivity.

We observed that half of the cold sensitive nociceptors (CMC fibres) displayed a significant increase in cold sensitivity after application of 4-AP or TEA and we have previously shown that the majority of these fibres are menthol sensitive (see chapter 4). Therefore these neurons are likely to represent the menthol and 4-AP sensitive neurons in culture.

Kirchhoff and colleagues investigated the effects of 4-AP and TEA on sensory nerve endings in the same preparation used in present study (Kirchhoff et al., 1992). However, they did not systematically investigate the effects of thermal and mechanical stimuli after application of 4-AP or TEA and only studied the effects of drug application alone. They demonstrated that 74 % of all fibres tested responded directly to application of 10 mM 4-AP. In the present study, we also observed a small proportion of fibres which responded directly during 4-AP or TEA application. Kirchhoff et al (1992) found that cooling the receptive field of some fibres during application of 4-AP always caused a decrease in firing frequency and in some cases a complete cessation in firing. They concluded that sensory properties of the nerve terminals were not obviously affected during application of 4-AP. This is in contrast to our findings, since we

have shown a change in cold sensitivity and also mechanical sensitivity in some fibres. This discrepancy is likely to be due to the higher drug concentrations used by Kirchhoff et al (1992), since in the majority of cases we only applied 3 mM 4-AP as the highest dose. They state that the high frequency discharges initiated by the application of high concentrations of 4-AP or TEA obscured the responses to thermal or mechanical stimulation carried out and therefore a quantitative analysis was difficult.
Furthermore, the present study has shown that cold can have different effects depending on the concentration of potassium channel blocker used. This became particularly apparent in a recording of an Aβ SA fibre (see Fig.6.3.8 and 9). 4-AP at low concentration specifically induced cold sensitivity in this fibre. Application of a higher dose of 3 mM 4-AP induced activity in the fibre and upon cooling there appeared to be a reduction in fibre activity.

Half of the low threshold mechanically sensitive Aβ fibres became cold responsive after application of 4-AP or TEA. Some Aδ D-hair fibres also displayed a novel cold response.

Application of 300 µM 4-AP resulted in 57 % of previously cold insensitive Aβ fibres to become cold responsive. Application of 3 mM 4-AP and 10 mM TEA was able to induce a novel cold sensitivity in a total of 17 of 36 (52 %) previously cold insensitive Aβ mechanoreceptors (4-AP; 12/19 fibres and TEA; 7/17 fibres).
This is a similar to the proportion of Aβ fibres (11/20, 55 %) which were excited by application of 4-AP to their receptive fields (Kirchhoff et al., 1992). Kirchoff et al found that the majority of fibres investigated with TEA were also sensitive to 4-AP but there was no fibre which was insensitive to both 4-AP and TEA, indicating that fibres expressed at least one dominant potassium current. This suggests that the proportion of afferents which developed a novel cold sensitivity is potentially higher than that of the present study since a fibre which did not become cold sensitive after application of 4-AP may have done so after application of TEA and vice versa.
Kirchhoff et al (1992) found that a characteristic bursting discharge developed in Aβ fibres exposed to high concentrations of 4-AP. We also found that some

RA and SA fibres developed a bursting discharge during cooling after application of 3 mM 4-AP.
Viana et al (2002) showed that a 4-AP sensitive potassium current, named IK_D was present in approximately 40 % of cold insensitive neurons. Interestingly, when they applied a combination of 4-AP and TEA, the percentage of neurons which developed a cold sensitivity increased to 58 % (Viana et al., 2002). 4-AP in the micromolar range is thought to block the slowly inactivating transient component of the potassium current, which corresponds to IK_D described by Viana et al (2002). In the millimolar range it also blocks fast inactivating A type current. Based on this, our results indicate that IK_D is present in approximately 50 % (or possibly more) of low threshold Aβ mechanoreceptors and that during cooling this current prevents these fibres from reaching their firing thresholds despite the inhibition of a background K^+ current. 4-AP in the millimolar range acts to reduce the interspike interval, thereby promoting repetitive firing. This would explain the development of bursting discharges commonly observed during a cold stimulus in some Aβ fibres after application of 3 mM 4-AP.

Interestingly, our results indicate that expression of IK_D is not homogeneous among Aβ fibres. The proportion of slowly adapting (SA) fibres which displayed a novel cold response after application of 4-AP or TEA (90 %) was significantly greater compared to the proportion of rapidly adapting (RA) fibres (40 %), indicating that the expression of IK_D is much greater among SA fibres. This is in agreement with the findings from Waddell et al (1986). They found that only half of DRG neurons in which current injection evoked a single spike, i.e., a rapidly adapting response, displayed sensitivity to 4-AP, whereas those which fired multiple spikes, i.e., slowly adapting neurons, were all sensitive to 4-AP (Waddell et al., 1989). However, it contrasts to the findings by Kirchhoff et al (1992) who demonstrated equal sensitivity to 4-AP among SA and RA fibres.
In the present study, application of 4-AP or TEA was also able to induce a novel cold sensitivity in previously cold insensitive D-hair fibres, suggesting that a proportion of these fibres also express IK_D which acts to reduce their excitability during cooling.

Cold sensitive slowly adapting (SA) mechanoreceptors become more cold sensitive after application of 4-AP

75 % of cold sensitive SA (3/4) fibres displayed an increase in response to cold after application of 3 mM 4-AP. Cold sensitivity among SA mechanoreceptors has been reported previously (Hensel, 1974;Konietzny, 1984;Leem et al., 1993;Spray, 1986), but the molecular mechanism underlying the transient cold responses of these fibres remains unknown. We have previously shown that these fibres do not respond to menthol, therefore it is unlikely that their cold responses are mediated via the TRPM8 receptor (see chapter 4).
Reid and Flonta (2001) demonstrated that cooling increased the input resistance in all DRG neurons, indicating that it inhibited an outward potassium current. They went on to show that there was a greater increase in input resistance in cold sensitive cells. Taking these findings into consideration it is possible that cold sensitive SA fibres express a greater amount of background K^+ current than cold insensitive SA fibres and therefore upon cooling they reach firing threshold, resulting in a brief discharge. We found that after application of 4-AP these fibres fired throughout the cold stimulus, whereas previously they had only fired very briefly. Expression of the IK_D current in these fibres appears to therefore limit their response during cooling. There was a reduction in the mean cold threshold from 16.3 to 18.7 °C post 4-AP application, although this difference did not reach statistical significance. The expression of IK_D may be involved in modulating the temperature threshold in cold sensitive SA fibres.

K_V1.1 and K_V1.2 are candidate subunits underlying 4-AP/TEA sensitivity in large myelinated A fibres

Several studies have investigated the expression of K_v channel subunits in DRG neurons. Using immunostaining, Ishikawa et al (1999) showed that rat DRG neurons in culture expressed moderate to high levels of K_v1.1, 1.2, 1.3, 1.6 and lower levels of K_v 2.1, 1.4 and 1.5 (Ishikawa et al., 1999). Rasband and colleagues investigated the expression of K_v1 subunits in the adult rat DRG (Rasband et al., 2001). They showed that K_v1.1 and 1.2 were expressed in primarily large sized diameter (42-52 μm) DRG neurons. In some of the largest

diameter neurons, $K_v1.1$ was observed in the absence of any other K_v1 subunits. Rare expression of $K_v1.4$ was also seen in a few large diameter neurons. Chi and Nicol (2007) reported expression of $K_v1.1$ in a proportion of large, medium and small rat DRG neurons (Chi and Nicol, 2007).
Gold et al (1996) found that large diameter neurons in the adult rat expressed a transient current sensitive to 4-AP (Gold et al., 1996). Pearce and Dunchan (1994) showed that large sized DRG neurons from mice expressed a current similar to the A-type potassium current, except it had a very slow inactivation rate. This slow inactivating current is likely to correspond to the I_D current described by other studies and possibly also IK_D. Slowly and rapidly inactivating currents in large myelinated afferents are likely to be mediated by different combinations of $K_v1.1$, 1.2 and 4.1 subunits.
Interestingly, using the phrenic nerve diaphragm preparation, hyperexcitability at the nerve terminal was induced by cooling in mice lacking $K_v1.1$ (Zhou et al., 1998). This provides further support of the involvement of $K_v1.1$ in cold induced responses observed in the Aβ fibres in the present study.

Mechanical sensitivity increases in some Aβ mechanoreceptors after exposure to 4-AP

Over 80 % of rapidly adapting Aβ mechanoreceptors showed a change in adaptation property in response to constant force stimulation after application of 4-AP. Fibres began to discharge throughout the stimulus, whereas previously they had only discharged at the beginning of the stimulus. Both RA and SA fibres became mechanically more responsive after application of 4-AP. Interestingly the average response of nociceptive AM and CM fibres to a mechanical stimulus did not increase after 4-AP application. This supports the notion of a differential expression of potassium channels in the DRG neurons and their functional importance.
The ionic basis underling the mechanical adaptation properties displayed by the tactile spine neuron, a type of mechanoreceptor found in the cockroach has been investigated by French and Torkkeli (French and Torkkeli, 1994). They showed that these neurons adapt rapidly in response to constant force stimulation and that this is partly dependent on an inward sodium current.

Additionally they found that blockade of an A-type potassium current by 4-AP, resulted in a dramatic increase in firing rate so that the neuron continued to fire throughout the constant force stimulation. This is a similar finding to ours. Interestingly, they report that application of TEA makes the action potential much wider, but does not effect the adaptation. The findings of this and the present study suggest that expression of a 4-AP sensitive potassium current may be responsible for determining the mechanical adaptation properties in Aβ mechanoreceptors.

Down regulation of A-type potassium channels expressed on nociceptors has been implicated in the development of mechanical hypersensitivity in rats in models of neuropathic pain (Chien et al., 2007). The effect of spinal nerve ligation was investigated on the expression pattern of two A-type channels, $K_v3.4$ and $K_v4.3$, which were shown to be predominantly expressed in non-peptidergic C fibre neurons. Mechanical sensitivity of the rat hind paw was assessed behaviourally by recording the von Frey hair threshold required to produce a withdrawal response. Seven days after spinal nerve ligation an increase in mechanical sensitivity was observed and immunohistochemical analysis revealed a reduction in $K_v3.4$ and $K_v4.3$ in the DRG. In addition, suppression of $K_v3.4$ and $K_v4.3$ by intrathecal injection of antisense-oligonucleotides was able to downregulate the channel expression and induce mechanical hypersensitivity.

In the present study an increase in mechanical sensitivity in C fibre nociceptors post 4-AP application was not found. One possible reason for this could be that the mechanical stimulus was always applied at the end of the testing protocol, often after the application of several cold stimuli, by which time the effect of the 4-AP may have been washed out. Therefore, future investigations could involve applying the mechanical ramp stimulus during application of 4-AP. Alternatively, the increase in mechanical sensitivity which correlated with a downregulation of K_v3/ K_v4 potassium channels are only observed in behavioural experiments.

Some A and C fibre nociceptors display a novel cold response after application of 4-AP or TEA

One high threshold A (AM) fibre displayed a novel cold response after application of 4-AP, compared to none (0/10 fibres) after application of TEA. In this study a greater proportion of low threshold mechanically sensitive A (RA, SA and DH) fibres displayed a novel cold response after application of 4-AP or TEA compared to nociceptive A fibres. This indicates that there is a greater expression of IK_D in low threshold A fibre mechanoreceptors compared to A fibre nociceptors. However, a lower expression of IK_D does not confer A fibre nociceptors cold sensitivity, since these fibres are also likely to express less background K^+ current.

None of the CM fibres (0/4) displayed a cold response after application of 4-AP. A proportion of heat sensitive C fibre nociceptors (CMH) developed sensitivity to cold after application of 4-AP or TEA. This is consistent with the finding from Munns et al (2007) who reported that a proportion of mouse DRG neurons which displayed a novel cold sensitivity after 4-AP application were also sensitive to capsaicin (Munns et al., 2007). However, 4-AP induced cold responses were always much greater in A fibres compared to C fibres, indicating that A fibres express a greater amount of 4-AP sensitive current than C fibres.

Many cold sensitive A and C fibre nociceptors become more cold responsive after application of 4-AP or TEA

All of the cold sensitive A fibre nociceptors (AMC fibres) displayed a greater response to cooling after application of 4-AP. This was accompanied by a significant decrease in the temperature required to elicit a response, i.e., there was a shift in the threshold to warmer temperatures. This suggests that expression of a 4-AP sensitive current, IK_D, acts to reduce excitability during cooling in these neurons and regulates their temperature threshold. Blockade of IK_D in AMC fibres results in increased excitability, therefore less stronger cooling is required to generate a response.

Similarly, half of cold sensitive C fibre nociceptors became more responsive to cold after application of 4-AP (50 % of fibres) or TEA (44 % of fibres) and there was a shift in the cold thresholds to warmer temperatures. This indicates that IK_D is also expressed in a proportion of cold sensitive C fibre nociceptors and acts to limit their response during cooling and modulate their temperature threshold.

K_V1.4 is a candidate subunit to form 4-AP sensitive A-type current in C fibre nociceptors

In adult rat DRG neurons, Rasband et al (2001) demonstrated that K_v1.4 was mainly expressed in small diameter neurons (Rasband et al., 2001). Vydyanathan et al (2005) found that application of 4-AP produced a significantly greater reduction in K_V currents in small diameter IB4 positive compared to IB4 negative rat DRG neurons (Vydyanathan et al., 2005). In contrast they showed that all the IB4 negative neurons displayed a large proportion of TEA sensitive current and a small 4-AP sensitive, A type current. Double immunofluorescence labelling revealed that IB4 positive neurons stained positively for K_v1.4, but not K_v1.1 or K_v1.2. Binzen et al found that 74 % of rat DRG positive for K_v1.4 also expressed TRPV1 (Binzen et al., 2006). 4-AP sensitive currents in C fibre nociceptors are therefore likely to be mediated by K_v1.4. Vydyanathan et al (2005) showed that in 34 % of IB4 positive cells, the 4-AP sensitive K_v currents displayed both fast, I_A and slow I_D inactivating components. C fibres which express the slowly inactivating current (which is likely to correspond to IK_D) are likely to become cold sensitive upon blockade of this current. Some expression of K_v1.1 or K_v1.2 was found in small diameter DRG neurons between 15-30 μM (Vydyanathan et al., 2005). Chi and Nicol (2007) also showed that some small sized DRG neurons were positively stained for K_v1.1 (Chi and Nicol, 2007). K_v1.1 and K_v1.2 channels are likely to constitute TEA sensitive currents in IB4 negative C fibres.

4-AP or TEA application had no affect on the cold responses of cold thermoreceptors

Application of 4-AP or TEA on the receptive fields of innocuous cold thermoreceptors (CC fibres) had no effect on their cold responses or thresholds. This suggests that voltage gated potassium channels do not play a role in modulating cold sensitivity in these fibres. We have previously reported that the majority of these fibres are sensitive to menthol (see chapter 4) therefore indicating that TRPM8 must be the principle cold transducer in these fibres. Viana et al (2001) reported that neurons most sensitive to cooling had a lower expression of $IK_{D,}$ than did cold sensitive neurons that were only excited by stronger cooling (Viana et al., 2002). The results of the present study are in agreement with this finding, since we have shown that cold thermoreceptors, which are very sensitive to cooling, are insensitive to 4-AP/TEA, indicating that they express very low or no IK_D. In contrast, half of cold sensitive nociceptors, which are excited by stronger cooling and are less cold sensitive than CC fibres and express more IK_D.

Reduction in potassium currents may contribute to cold hyperalgesia and allodynia in patients with neuropathic pain

Nerve injury in rodents has been reported to result in over a 50 % reduction in potassium currents in injured DRG neurons (Everill and Kocsis, 1999;Rasband et al., 2001;Yang et al., 2004), suggesting a potential mechanism of hyperexcitability in these neurons.

Cold allodynia and hyperalgesia are common clinical findings in patients with neuropathic pain. Sensitisation of cold sensitive nociceptors and thermoreceptors has been proposed as a possible mechanism underlying the generation of cold hyperalgesia in these patients (Scadding and Koltzenburg, 2005). Our results indicate that a reduction in potassium currents in a proportion of cold sensitive A and C fibre nociceptors would increase cold sensitivity in these fibres and that this would contribute to the cold hypersensitivity observed in patients with neuropathic pain.

Blockade of voltage gated potassium channels with 4-AP has been shown to increase action potential duration and amplitude in areas of demyelination and improve action potential propagation in vitro. This suggested that potassium channel blockers might be clinically useful in demyelinating diseases such as multiple sclerosis (MS) (Bostock et al., 1981). However, the use of 4-AP was found to be limited by its side effects it caused; including paraesthesias (Bever, 1995). Kocsis et al (1986) demonstrated that application of 4-AP on sensory nerve fibres gave rise to hyperexcitability and bursting activity and they suggested that this could account for the paraesthesias observed in patients administered with 4-AP (Kocsis et al., 1986).

Blockade of potassium currents has also recently been implicated in the development of paraesthesias and dysesthesias associated with ciguatera poisoning. Birinyi-Strachan et al (2005) investigated the actions of the marine toxin pacific ciguatoxin-1 (PCTX-1) on rat DRG neurons using patch clamp techniques. They found that as well as effecting TTX sensitive sodium channels, PCTX-1 also blocked both sustained and rapidly inactivating A type currents (Birinyi-Strachan et al., 2005). Data from the present study suggests that blockade of potassium channels in a proportion of A fibre mechanoreceptors may account for the paraesthesias and dysesthesias experienced by these people.

Application of 4-AP and TEA to the whole nerve did not induce cold sensitivity in fibres recorded from the periphery

Application of a combination of 4-AP and TEA directly onto the whole nerve trunk for 5 minutes did not result in changes in receptive properties of fibres in the periphery.

A possible explanation is that the 4-AP and TEA was unable to diffuse through the connective tissue around the whole nerve within five minutes and therefore no effects in the periphery were seen. However, ongoing experiments in the laboratory using nerve excitability testing have shown that effects of 4-AP or TEA do occur within this time range.

Conclusions

This is the first study which has investigated the role of voltage gated potassium channels in thermosensitivity in identified primary afferent neurons innervating the skin. Blockade of voltage gated potassium channels using 4-AP and TEA was able to induce novel cold sensitivity in a proportion of mechanically low threshold A fibres as well as A and C fibre nociceptors. A summary diagram representing the proportion of fibres which displayed a novel or increased cold sensitivity after blockade of 4-AP/TEA is shown in Fig.6.4.1, below.

Application of the lower dose of 4-AP (in the micromolar range) induced a novel cold response in 2/3 of previously cold insensitive units. Fibres were never directly activated using this dose. In contrast, the higher dose of 4-AP (in the millimolar range) resulted in direct activation of some fibres and the development of bursting and repetitive firing. 4-AP in the micromolar range selectively blocks the slowly inactivating transient component of the potassium current, thereby allowing a proportion of cold insensitive fibres to become excited during cooling. The higher dose of 4-AP also blocks the fast inactivating A type current, resulting in repetitive firing and bursting discharges. Changes in mechanical adaptation properties observed in some RA fibres after application of 3 mM 4-AP suggests that the fast inactivating current may also play a role in regulating mechanical sensitivity in these fibres. TEA has been shown to block the sustained or non-inactivating component of the potassium current. Application of 10 mM TEA was able to induce a novel cold response in a similar proportion of fibres as 4-AP. However, qualitatively we observed that Aβ fibres which became cold responsive after 4-AP displayed a more sustained response throughout the cold stimulus than those fibres after TEA, suggesting that the 4-AP sensitive current contributes greater in regulating the cold responses in large myelinated A fibres than the TEA sensitive current.
Interestingly, a novel response to noxious heat was never observed after application of 4-AP or TEA, indicating that blockade of voltage gated potassium channels does not generically increase the excitability of fibres in response to all stimulus modalities.

We found that all cold sensitive A fibre nociceptors and approximately 50 % of cold sensitive C fibre nociceptors displayed an increase in cold response and a decrease in cold threshold after application of 4-AP or TEA. Interestingly our data suggest that voltage gated potassium channels do not mediate the cold responses and thresholds of innocuous cold thermoreceptors.
These findings therefore suggest that during nerve injury, down regulation of voltage gated potassium channels expressed on cold sensitive A and C fibre nociceptors and not cold thermoreceptors would contribute to the development of cold hyperalgesia observed in patients.
The abnormal cold responses observed in previously cold insensitive A fibre mechanoreceptors may contribute to the paraesthesias observed in MS patients administered with 4-AP and those with ciguatera poisoning.

In the present study, fibres investigated with 4-AP were not subsequently tested with TEA and vice versa. Therefore it is likely that some fibres which did not display a novel cold response after application of 4-AP may have done so after application of TEA and vice versa.

In summary, blockade of the slowly inactivating transient potassium current (I_D) appears to be specifically involved in the development of novel cold sensitivity in a proportion of Aβ mechanoreceptors. However, it is unlikely that simply blocking this current is enough to make these fibres cold responsive. In addition to I_D, these neurons are also likely to have a higher expression of background K^+ current and therefore during a cold stimulus, inhibition of both these currents promotes the neuron to reach its firing threshold. This may explain why very few cold insensitive, mechanically high threshold A fibres became cold responsive despite blockade of their voltage gated K^+ channels. Finally voltage gated K^+ channels appear to reduce the excitability of the majority of cold sensitive A fibre nociceptors and about 50 % of cold sensitive C fibre nociceptors during cooling, and these fibres may therefore be involved in the development of cold hyperalgesia observed in patients with neuropathic pain.

An abnormal cold hypersensitiviy and cold intolerance is observed in patients suffering from colorectal cancer shortly after infusion of the chemotherapeutic

agent oxaliplatin. The next and final results chapter (chapter 7) will investigate for the first time, the effect of oxaliplatin application to the receptive properties of afferents innervating the hairy skin in rat.

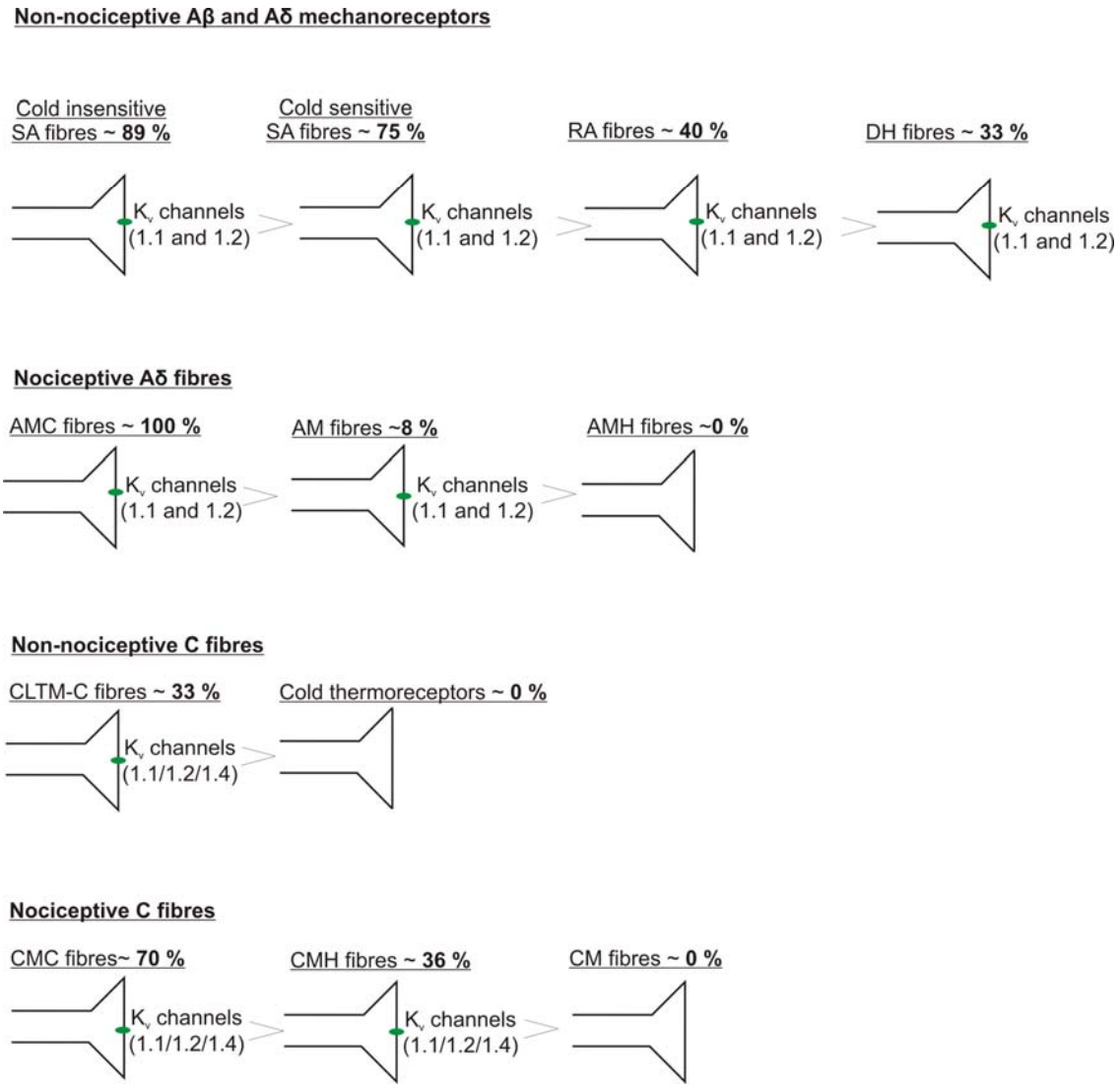

Fig.6.4.1 Schematic representation of voltage gated potassium channel subunit expression on the peripheral terminals of fibres proposed to be involved in regulating cold sensitivity of these fibres

The proportion of fibres which displayed either a novel or increased cold sensitivity after application of 4-AP/TEA to their receptive fields is shown as a percentage (%) next to the name of each fibre type. Within each fibre group, the sub-population of fibres which displayed the highest sensitivity to 4-AP/TEA is shown first and those which displayed the least sensitivity are shown last. For example, within the non-nociceptive Aβ and Aδ mechanoreceptors, 89 % of cold insensitive SA fibres displayed a novel cold response after application of 4-AP/TEA, compared to only 33 % of DH fibres. Therefore cold insensitive SA fibres are shown first and DH fibres are shown last.

Chapter 7

The effect of oxaliplatin on the receptive properties in rat primary afferent neurons

7.1 Introduction

Oxaliplatin ($C_8H_{14}N_2O_4Pt$, trans-1-diaminocyclohexane oxaliplatinum), is the first platinum based chemotherapeutic agent which has demonstrated significant efficacy against advanced colorectal cancer, a disease widely considered to be resistant to existing chemotherapy drugs, cisplatin and carboplatin. Efficacy against colorectal cancer has been demonstrated in many clinical trials (Extra et al., 1990;Machover et al., 1996;Raymond et al., 1998) and oxaliplatin has since been approved by the U.S Food and Drug Administration (FDA) and in the UK by the National Institute for Health and Clinical Excellence (NICE) for the treatment of advanced colorectal cancer. Oxaliplatin is commonly administered with two other chemotherapeutic drugs, 5-fluoroucil (5-FU) and leucovorin (LV). This combination, known as FOLOX has been shown to be more active against colorectal cancer than 5-FU/LV alone (Blieberg Harry et al., 2002;de Gramont et al., 2000). Studies have shown that oxaliplatin in combination with 5-FU/LV can prolong survival, shrink tumour mass, and delay the progression of cancer in patients with advanced colorectal cancer (Sanofi-Aventis US, 2006).

Oxaliplatin is a platinum containing compound in which the platinum atom is complexed with 1,2- diaminocyclohexane carrier ligand (DACH ligand) and with an oxalate ligand, also known as the 'leaving group'. Oxaliplatin is administered intravenously and is thought to enter cells via passive diffusion. In addition to this, some evidence suggests that copper influx transporters are involved in the influx of oxaliplatin into the cell (Kuo et al., 2007). It becomes activated intracellularly via a series of non-enzymatic conversions. This involves the substitution of the oxalate leaving ligand by molecules such as H_2O and Cl^- and HCO_3 ions. Several cytotoxic platinum products are formed which subsequently bind to DNA forming DNA adducts. This activates different signal transduction pathways involved in DNA damage recognition and repair, cell cycle arrest and

leads to programmed cell death (apoptosis) (Kelland, 2007). Oxaliplatin has an advantage over cisplatin since its DNA adducts are bulkier and less easily recognised by DNA repair proteins.
The decline in platinum levels following oxaliplatin administration is triphasic, characterised by two short distribution phases and a longer elimination phase. At the end of a two hour infusion, 15 % of the platinum is present in the systemic circulation. Here, platinum binds irreversibly to plasma proteins and erythrocytes, but this is not considered to be of clinical significance (Sanofi-Aventis US, 2006). The reactive oxaliplatin derivatives are present as unbound platinum in the plasma. The remaining 85 % is rapidly distributed into tissues or eliminated in urine.

Unlike other platinum derivatives, oxaliplatin does not result in significant renal toxicity or ototoxicity and there is less nausea and vomiting (Raymond et al., 1998). However, development of a peripheral sensory neuropathy is the major dose limiting toxicity for oxaliplatin. The neuropathy occurs in two stages. The first is an acute neuropathy occurring during or shortly after infusion in up to 90 % of patients. After several cycles of oxaliplatin treatment a chronic neuropathy can occur. The different spectrum of clinical activity and toxicity of oxaliplatin compared to cisplatin and carboplatin is believed to be due to its 1, 2-diaminocyclohexane carrier ligand (DACH ligand), which forms bulkier DNA adducts.

The acute sensory neuropathy recurs after each chemotherapy cycle, but is usually reversible within hours or days. The duration and intensity of the neuropathy has been shown to increase as the cumulative exposure to oxaliplatin increases (Extra et al., 1990;Machover et al., 1996).
Patients develop paraesthesias, defined as non-painful but abnormal sensations such as tingling and ‘pins and needles’ in the fingers, hands, feet and sometimes the lips. Patients also experience dyesthesias which are defined as painful or distressing sensations in the forearms, legs, trunk, mouth and throat (Extra et al., 1990). Interestingly, all of these symptoms are aggravated by cold temperatures (Besser et al., 2007;de Gramont et al., 2000;Extra et al., 1990;Krishnan et al., 2005;Krishnan et al., 2006;Lehky et al.,

2004;Leonard et al., 2005;Machover et al., 1996;Sanofi-Aventis US, 2006;Wilson et al., 2002). Patients experience a cold hypersensitivity (which is often described as painful) while eating, drinking or handling a cold object (Leonard et al., 2005). Breathing cold air can also cause unpleasant sensations (Sanofi-Aventis US, 2006).

As well as the development of a sensory neuropathy a motor neuropathy also exists. Painful involuntary masticatory spasms followed by a failure of muscle relaxation has also been observed in a group of patients, 6 to 24 hours after oxaliplatin administration (Santini et al., 2003). One of the most distressing symptoms reported by patients is the sensation of tightness and discomfort in the throat, often described as a poor awareness of breathing (Leonard et al., 2005;Sanofi-Aventis US, 2006).

The acute neurotoxicity induced by oxaliplatin is distinct from the chronic neuropathy which is associated with long term treatment and high cumulative doses of oxaliplatin. The development of a long term sensory neuropathy (Extra et al., 1990), distal sensory loss, loss of deep tendon reflexes (de Gramont et al., 2000) and ataxia occur after long term treatment and often result in drug discontinuation (Lehky et al., 2004). In some patients symptoms are very severe and interfere with activities of daily living such as writing, buttoning clothes, swallowing, difficulty walking and picking up objects (Extra et al., 1990;Sanofi-Aventis US, 2006).

This chronic neuropathy is thought to be due to the cumulative neurotoxic effects of platinum accumulation in the dorsal root ganglion (DRG) (Holmes et al., 1998). Similar to cisplatin, chronic treatment of oxaliplatin has been shown to reduce DRG neuronal cell body, nucleolus and nucleus size in rats (Cavaletti et al., 2001). In rats treated with oxaliplatin for 8 weeks, a decrease in the relative frequency of large sized DRG and a slowing of peripheral nerve conduction velocity in the hind limbs has also been shown (Jamieson et al., 2005). In patients, a reduction in amplitude of the sural sensory nerve action potential (SNAP) and superficial radial SNAP amplitude was reported after completion of their oxaliplatin therapy (Krishnan et al., 2005). Lehky et al (2004)

also found that by chemotherapy cycle 8 or 9 there was a reduction in SNAP amplitude in median, radial and sural nerves (Lehky et al., 2004). These findings are thought to correspond to loss of DRG neurons or its axons.

Oxaliplatin induced neuropathy is detrimental to patients in terms of unpleasant symptoms and the need to reduce the dose or discontinue treatment. However, currently there are no therapies available to treat or prevent the chemotherapy induced neuropathies. Since exposure to cold temperatures is problematic for most patients they are advised to avoid it and protect themselves, for example by wearing gloves when opening the freezer.

Some studies have investigated the role of neuromodulatory agents such as calcium-magnesium infusions, gabapentine, carbamazepine and glutathione to limit the neurotoxic effects of oxaliplatin. In patients who received calcium-magnesium infusions before and after oxaliplatin, symptoms such as distal paraesthesias were lower compared to the control group of patients (Gamelin et al., 2004). At the end of the treatment 20 % of patients in the calcium-magnesium group experienced neuropathy compared to 45 % in the control group. The calcium-magnesium infusions therefore appeared to reduce the occurrence and intensity of the neuropathy. Studies with smaller patient numbers have looked at carbamazepine; one study administered carbamazepine before oxaliplatin (Eckel et al., 2002), while the other administered after the first cycle of oxaliplatin treatment (Wilson et al., 2002). Wilson et al found that carbamazepine did not alter the oxaliplatin induced symptoms experienced by patients. Eckel et al (2002) found that when carbamazepine was administered before oxaliplatin, no neuropathy above grade 1 occurred. However, von Delius et al (2007) found that there was no clear difference in the intensity or duration of neurotoxcicty in a group of patients in which carbamezepine was administered 6 days before oxaliplatin compared to a control group (von Delius et al., 2007).
In order to develop a suitable therapy to treat or prevent the neuropathy, it is vital to define the mechanisms which are involved in the neuropathies underling both acute and chronic neuropathies.

In patients post oxaliplatin infusion, needle electromyography (EMG) revealed repetitive discharges in motor units, resembling neuromyotonia (Wilson et al., 2002). Oxaliplatin induced hyperexcitability in motor units has also been reported by Besser et al (2007), who using EMG showed that neuromyotonic like discharges developed in 16 out of 20 patients (Besser et al., 2007).

An increase in sodium channel activity and/or decrease in potassium channel activity could be a mechanism for generating neuronal hyperexcitability and repetitive discharges.

Based on this, Adelsberger and colleagues investigated the effects of 250 μM oxaliplatin on electrotronic responses of the sural nerve after pre-treatment of the nerve with TTX, a sodium channel blocker and 3 mM TEA and 4-AP, two broad potassium channel blockers in the rat (Adelsberger et al., 2000). They showed that oxaliplatin caused repetitive firing in response to depolarisation currents. When the nerve was pre-treated with 1 μM of TTX they found that oxaliplatin had no effect on the electrotonic responses. A block of fast and slow potassium channels was detected after TEA and 4-AP application, indicating that oxaliplatin did not block these channels. This suggested that oxaliplatin was most likely effecting sodium and not potassium channels.

These findings were also supported by Webster et al (2005), who showed that application of 1 μM TTX before the application of oxaliplatin was able to prevent multiple trains of action potentials induced by oxaliplatin in the phrenic nerve. They also demonstrated that apamin (10 μM) and 4-AP (0.3 mM) application failed to cause oxaliplatin like effects and that therefore SK channels nor delayed rectifier K^+ channels were unlikely targets for oxaliplatin at the neuromuscular junction (Webster et al., 2005). The above studies suggest that oxaliplatin induced acute neuropathy may be mediated via sodium channel abnormalities resulting in nerve hyperexcitability.

In an isolated nerve preparation, oxaliplatin has been shown to increase the A fibre compound action potential of rat sural and vagal nerves (Adelsberger et al., 2000). The C fibre compound action potential was shown to be relatively

unaffected. However the effects of oxaliplatin on single, identified A and C fibres remains unexplored. Aβ fibres are thought to be involved in chemotherapy induced paraesthesias, loss of vibration sensation and proprioceptive deficits. Aδ and C fibres are thought to be involved in producing sensations of burning pain and increased cold sensitivity (Mantyh, 2006) but direct evidence for this is currently lacking.

Penetration of intravenously administered oxaliplatin, through the blood brain barrier (BBB), has been shown to be extremely poor (Jacobs et al., 2005), but oxaliplatin has been shown to be accessible to DRG and peripheral nerves (Screnci et al., 2000). Research suggests that the DRG is the site via which the chronic effects of oxaliplatin are mediated. However, it is unclear which site or sites of the nerve are responsible for mediating the acute effects of oxaliplatin. It is unknown whether the acute effects of oxaliplatin are mediated through its action on the DRG, the axon of the sensory nerves or its receptive terminals.

Aim of the present study:

This present study aimed to firstly investigate the effects of direct application of oxaliplatin on the receptive properties of single, primary afferents which innervated the hairy skin on the hind limb of a rat. In particular the possible mechanisms behind the increased cold sensitivity observed in patients were explored. Secondly, the study aimed to identify which part or parts of the nerve are sensitive to acute application of oxaliplatin. To address this question, a comparison of the effects of oxaliplatin application on the whole nerve vs. receptive terminal were made.

7.2 Methods

7.2.1. Skin-nerve in vitro preparation and recording technique

The saphenous nerve with the skin of the of the hind paw attached was dissected from adult female Sprague Dawley rats as described in chapter 2. The skin was mounted corium inside up in an organ bath and superfused with warm oxygenated SIF. Receptive fields of individual primary afferent neurons were then identified with a mechanical search stimulus and characterised using electrical, mechanical and thermal stimuli as described in chapter 2.

7.2.2 Chemical stimulation

Fibres were tested with application of the chemotherapeutic agent oxaliplatin. Concentrations of 200 μM, 400 μM or 600 μM of oxaliplatin were used. Solutions were made by diluting a stock solution of 10 mM into warm SIF. After the fibres were fully characterised a single concentration of oxaliplatin was applied directly into the metal ring which sealed off the receptive field of the fibre. The solution was continuously gassed with oxygen. Application of oxaliplatin on the receptive field of the fibre lasted for 10, 15, 20 or 30 minutes. In some preliminary experiments different concentrations of oxaliplatin were applied for 5 minutes. However, receptive properties of fibres remained unchanged; therefore a minimum application period of 10 minutes was chosen. Fibres were tested with different combinations of oxaliplatin concentration and application times. A breakdown is provided in Table.7.2.1 (see below).
At the end of the designated application time, the oxaliplatin solution was sucked away from the receptive field and the metal ring was lifted to allow the flow of SIF. This usually lasted between 15-20 seconds. The metal ring was then re-placed and the SIF was quickly evacuated from within the ring. The thermocouple was kept in its original location. A cold stimulus was then applied to the receptive field of the unit which lasted for 60 seconds. A second cold stimulus was re-applied after a couple of minutes and in some recordings a third cold stimulus was also applied. A 30 s cold stimulus was used when testing mechanically insensitive cold fibres. In some recordings this was then

followed by the application of a noxious heat stimulus and a mechanical stimulus (constant force stimulation or mechanical ramp).
In fibres which displayed changes to their receptive properties after application of oxaliplatin, the duration of this action was not investigated. Therefore, it is not known whether there is complete washout of oxaplatin from the preparation and whether the fibres response profile returns to pre-oxaliplatin baseline activity after a longer duration wash-out. However, if an area of skin which had already been exposed to oxaliplatin was re-investigated, fibres from this area were always hyperexcitable (data not shown) and therefore this indicated that washout of the drug was poor. Therefore, the same skin area in subsequent recordings, of the same preparation was avoided for further re-testing to avoid re-exposure to noxious or chemical stimuli to avoid possible sensitisation or desensitisation of units.
For quantitative analysis all fibres were required to either be activated by adequate mechanical, thermal or electrical stimuli to determine the proportion of oxaliplatin sensitive fibres. This was done as a precaution to obtain a percentage that was not artificially lowered because of the fact that fibres were lost due to deteriorating recording conditions. All statistical analyses were carried out as described in Chapter 2.

Table.7.2.1. Dose of oxaliplatin applied on all fibre types

Fibre type	RA	SA	SA (c)	DH	AM	AMC	AMH	CM/ CMH	CMC/ CMCH	CLTM	CC
200 µM	7	5	1	2	1	0	1	1	0	0	0
400 µM	9	2	0	0	8	2	0	2	6	4	4
600 µM	5	3	1	1	2	1	1	1	1	0	3
Total tested	21	10	2	3	11	3	2	4	7	4	7

<u>Oxaliplatin application onto whole nerve trunk</u>

In a different set of experiments, oxaliplatin was directly applied onto the whole nerve trunk. Recordings were then carried out from fibres in the periphery, whose receptive fields did not overlap with the site of oxaliplatin application.

These experiments were carried out to investigate whether oxaliplatin application to the whole nerve would have any effect on activity evoked in the receptive terminals.

3 to 4 units were characterised in each recording. This was carried out by placing multiple thin filaments onto the recording electrode. Once fibres had been characterised using electrical, mechanical and thermal stimulation, the nerve trunk was isolated using a metal ring and 600 μM oxaliplatin was applied for 30 minutes. At the end of 30 minutes, the oxaliplatin was removed, and the ring was lifted to allow SIF to flow through. This lasted between 15-20 s and the ring was resituated around the whole nerve. A cold stimulus was then applied for 1 minute to see whether any spontaneous activity developed from the fibres in the periphery. If there was no activity, a cold stimulus was then applied directly to the individual receptive fields.

If there was no induced response, oxaliplatin (600 μM, 30 minutes) was applied directly to the receptive fields of the fibres. This was carried out to investigate whether the effect of oxaliplatin was specific to the receptive endings of the fibre. A cold stimulus was then re-applied to see whether a novel response to cold developed.

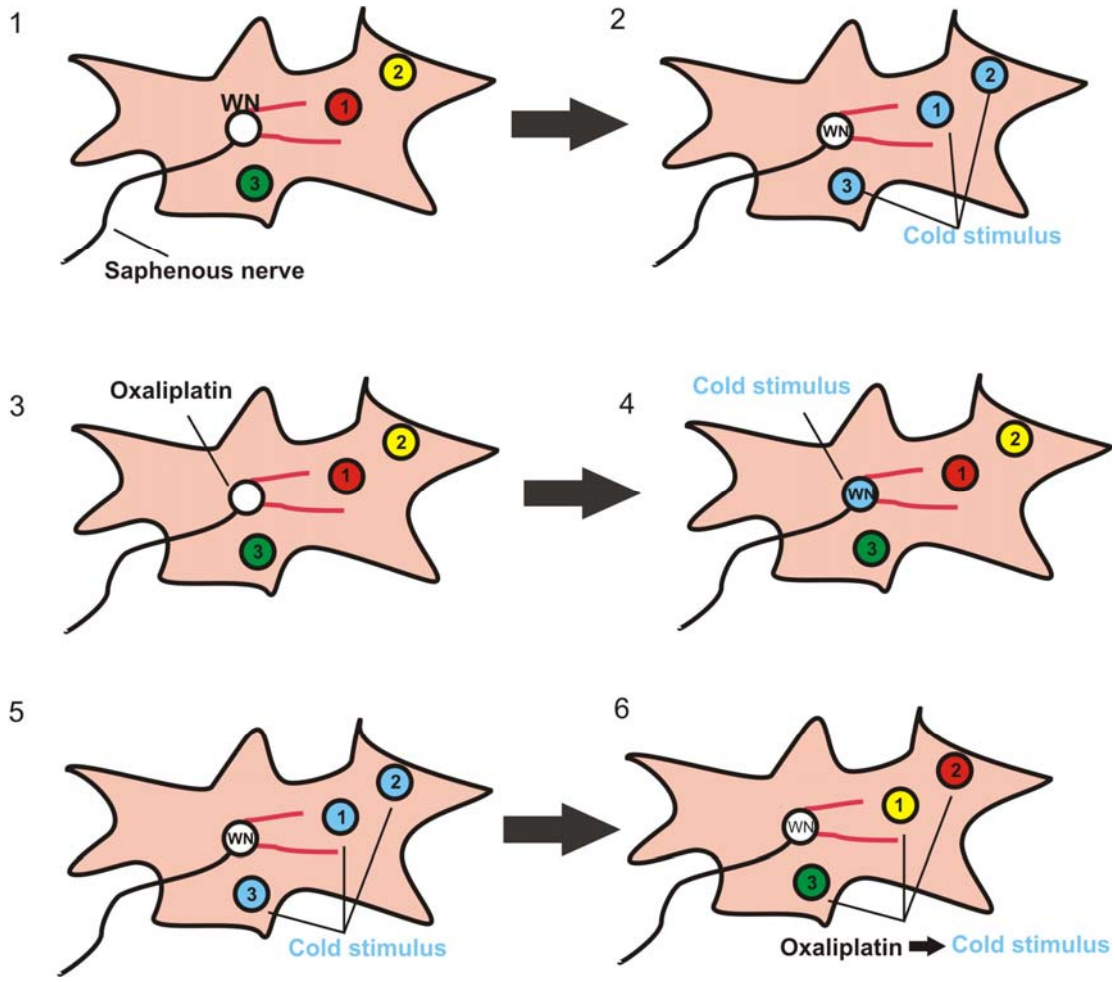

Fig.7.2.1. Schematic representation of an experiment investigating the effects of oxaliplatin onto the whole nerve
Areas numbered 1, 2 and 3 represent receptive fields of individually characterised units on the skin. WN represents the area of whole nerve on which oxaliplatin was applied.

7.3 Results

Single unit recordings were made from a total of 74 afferent fibres from 26 animals. Of those 33 were classified as Aβ fibres, 19 as Aδ fibres and 22 as C fibres.

The mean conduction velocity of the Aβ fibres was 17.2 ± 0.8 m/s; that of Aδ fibres was 5.3 ± 0.7 m/s; and that of C fibres was 0.43 ± 0.02 m/s. The general properties of all sensory fibres in the saphenous nerve are provided in Table.7.3.1. Fibres recorded in this study are not a representative sample, because some experiments focused on afferents that were sensitive to cold only.

In 3 experiments the effects of oxaliplatin application onto the whole nerve trunk were investigated.

TABLE 7.3.1. Conduction velocity, von Frey hair thresholds and cold sensitivity of myelinated Aβ, thinly myelinated Aδ and unmyelinated C fibres

Fibre Type	n	Conduction Velocity, m/s	von Frey threshold	Cold responsive before oxaliplatin	Novel/ increased cold response after oxaliplatin
RA	21	17.7 ± 1.1	2 (1, 4)	0/21	10/21
SA	10	14.9 ± 0.9	2.5 (0.3, 8)	0/10	6/10
SA (c)	2	22.6 ± 0.14	1, (1, 1)	2/2	0/2
DH	3	6.6 ± 1.5	0.3 (0.3,0.3)	0/3	1/3
AM	11	5.1 ± 0.9	22.4 (16, 32)	0/11	3/11
AMC	3	7.5 ± 0.02	32 (11.2, 32)	3/3	3/3
AMH	2	1.39 ± 0.04	19.2 (16, 22.4)	0/2	0/2
CM/CMH	4	0.48 ± 0.04	27.2 (19.2, 38.4)	0/4	0/4
CMC/CH	7	0.40 ± 0.03	32 (22.4, 32)	7/7	0/7
CLTM	4	0.44 ± 0.07	0.85 (0.7, 1)	3/4	0/4
CC	7	0.42 ± 0.05	-	7/7	0/7

All values are means ± SE; except for von Frey hair thresholds which are median and first (Q_1) and third (Q_3) quartiles. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; SA (c), cold sensitive slowly adapting afferent fibres; AM, high threshold mechano-sensitive A fibre; AMC, high threshold mechano-cold A fibre; AMH, high threshold mechano-heat A fibre; DH, D-hair receptors; CM, C mechano-sensitive nociceptor; CMC, C mechano-cold nociceptor, CMH, C mechano-heat sensitive nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM, C low-threshold mechano-sensitive fibre; CC, C mechano-insensitive cold fibre.

Aβ fibres

A summary of general properties of all the Aβ fibres is provided in Table.7.3.1. Table.7.3.2 (page 294) provides a quantitative summary of how the fibres responded to a cold stimulus before and after the application of oxaliplatin.

Rapidly adapting (RA) mechanoreceptors

A total of 21 RA fibres was recorded, none of which were initially spontaneously and none of which responded to a noxious cold or heat stimulus.
Oxaliplatin was applied onto the receptive fields of all the RA units. Units were never directly activated during the application of the drug (see Fig.7.3.2 for an example).
After application of oxaliplatin all RA fibres were re-tested for their response to a cold stimulus. Subsequently, a novel cold sensitivity developed in 10 out of 21 (47.6 %) fibres.
Fig.7.3.1. shows the proportion of RA units which became cold responsive after application of oxaliplatin ranging from 200-600 μM. In most cases all concentrations of oxaliplatin were able to induce a novel cold response in a proportion of fibres if applied for at least 15 minutes. In some preliminary recordings a shorter duration of application time, 5 minutes was used, but there were never any subsequent changes in receptive properties.
The average response threshold to cold after oxaliplatin was 13.2 ± 2.0 $^{\circ}$C ranging from 4.5-23.5 $^{\circ}$C (see Table7.3.2).
Most fibres (70 %) discharged in bursts of action potentials during the cold stimulus. An example of one such unit is illustrated in Fig.7.3.3. The majority of fibres, (60 %) responded during the first cold stimulus after oxaliplatin removal. However, 3 out of 10 (30 %) RA units responded during the second cold stimulus and 1 out of 10 (10 %) responded to the third cold stimulus.
Plotting the mean discharge rate to cold as a function of time revealed that after application of oxaliplatin, on average RA fibres discharged at high frequencies, in an irregular bursting manner (Fig.7.3.4A). Their mean maximal discharge rate was 22 impulses per second. There was a significant difference ($p<0.005$,

Wilcoxon matched pairs test) in the mean response to a cold stimulus before and after oxaliplatin, as illustrated in Fig.7.3.4B.
A sub-population of RA units (n=12) were re-tested for their response to a noxious heat stimulus. None became sensitive to heat.

Mechanical sensitivity of 12 RA units was re-investigated after exposure to oxaliplatin, using a constant force stimulation of 100 mN. 5 out of these 12 units (41.7 %) displayed a change in mechanical adaptation property and began responding throughout the constant force stimulation (CFS), often in bursts (Fig.7.3.5 and 6). Their firing pattern was studied by plotting the mean discharge rate as a function of stimulus force before and after oxaliplatin application (Fig.7.3.7A). This revealed that as well as a change in adaptation property, there was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the total mean number of impulses during the CFS after oxaliplatin application (Fig.7.3.7B).
Interestingly, only 3 out of these 5 units developed a novel cold sensitivity after exposure to oxaliplatin. Therefore fibres which became mechanically hypersensitive after oxaliplatin application did not always develop a novel cold sensitivity and vice versa.
The mean response of the remaining RA fibres (n=7) which did not display a change in adaptation properties after oxaliplatin application was plotted (Fig.7.3.8). Although after oxaliplatin application there was an increase in the total number of impulses during the CFS, this difference was not significant ($p>0.1$, Wilcoxon matched pairs test).

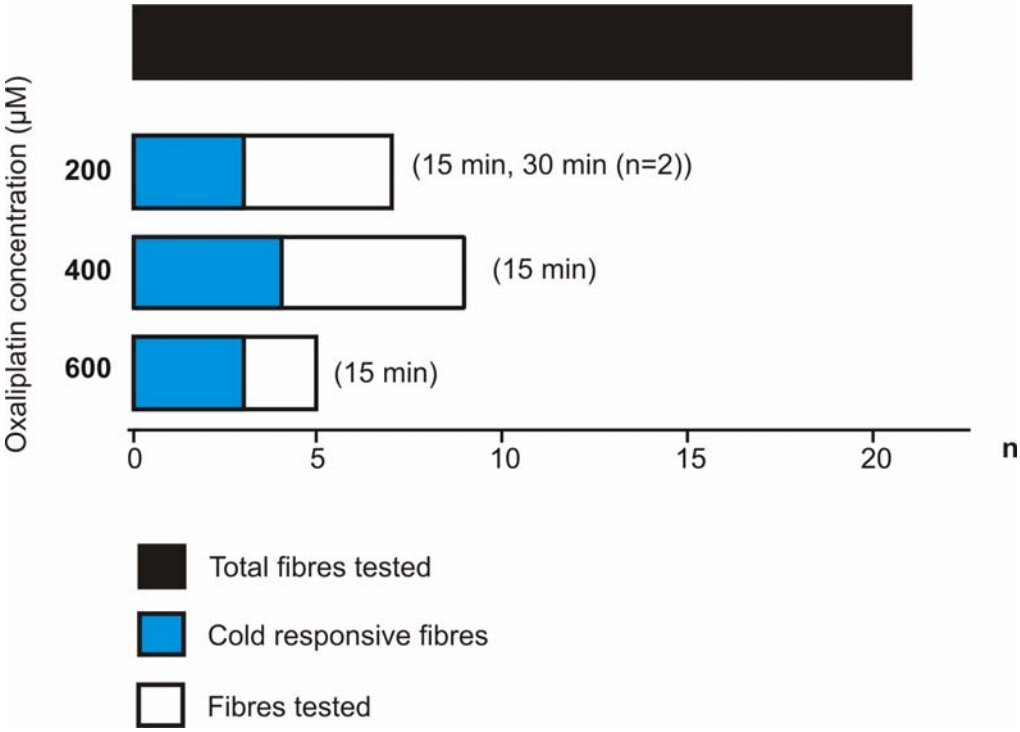

Fig.7.3.1. Proportion of rapidly adapting (RA) mechanoreceptors which displayed a novel cold response after oxaliplatin application to the receptive field

The proportion of induced cold sensitivity in the total number of fibres tested with oxaliplatin at different concentrations is shown.
For cold induced fibres, the time for which oxaliplatin was applied for is given in brackets.

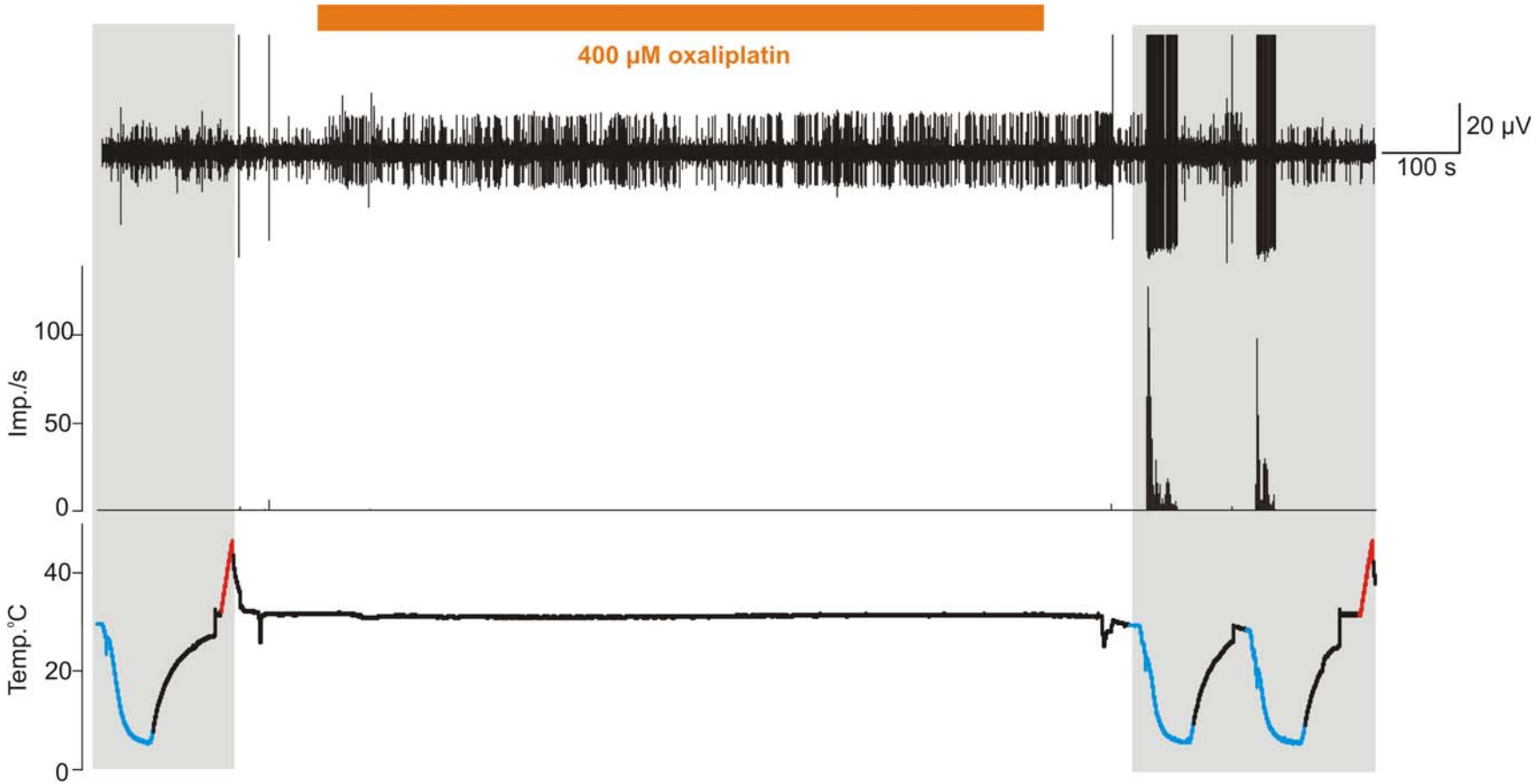

Fig.7.3.2. Response of a rapidly adapting (RA) Aβ mechanoreceptor to thermal stimuli before and after oxaliplatin application
There was no direct response from the fibre during the application period of the drug (15 minutes). After the drug had been removed, the receptive field was re-tested with thermal stimuli. The fibre then displayed a novel cold response, but remained insensitive to heat. A smaller, uncharacterised unit can be seen firing in the background. Areas highlighted in light grey are shown in more detail in Fig.7.3.3. The response of this fibre controlled mechanical stimuli before and after oxaliplatin application is shown in Fig.7.3.5.

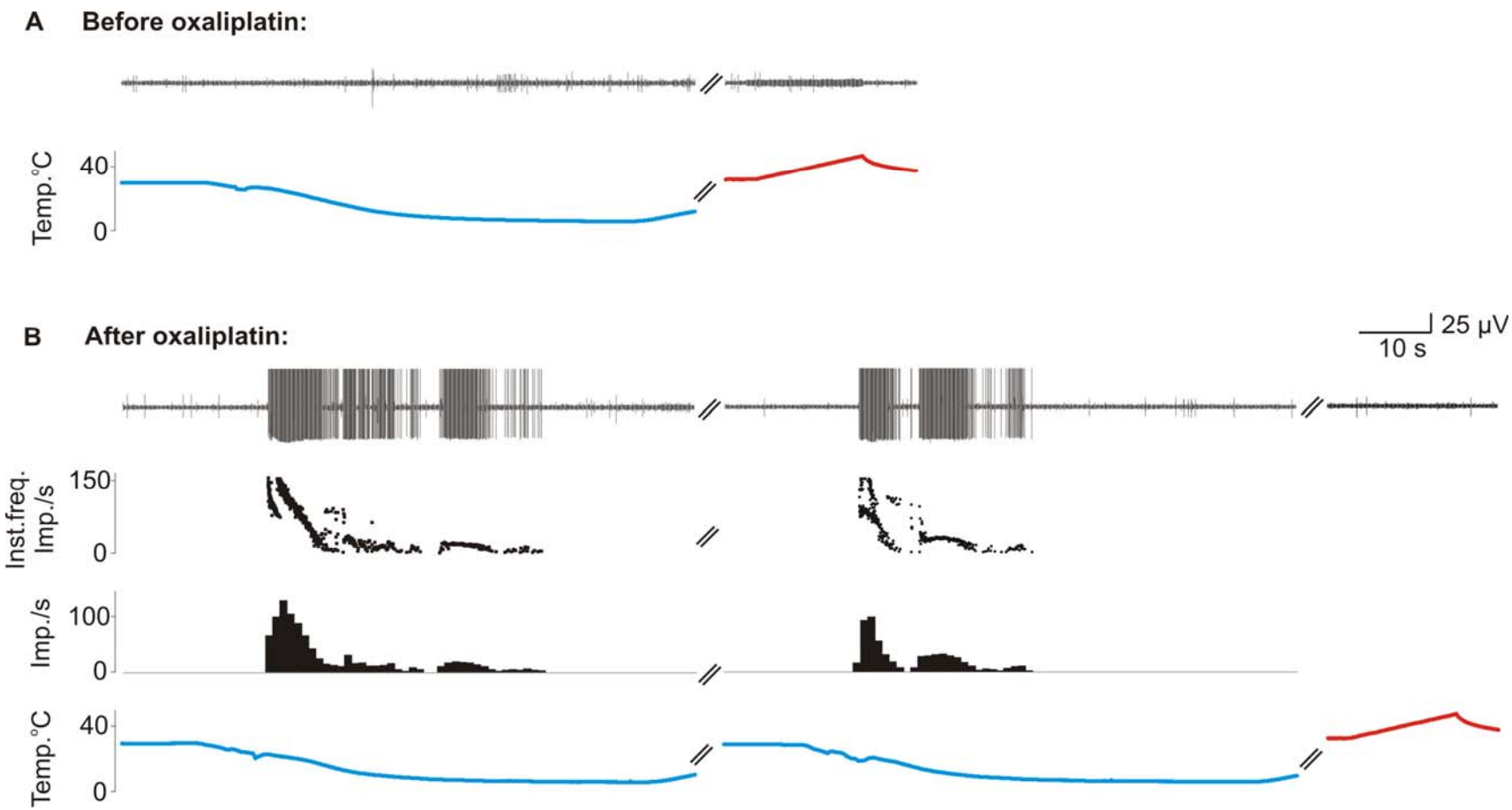

Fig.7.3.3. Response of a RA Aβ fibre to a cold and heat stimulus before and after application of 400 µM oxaliplatin for 15 minutes to its receptive field

(A) There was no response to either a noxious cold or heat stimulus before application of oxaliplatin. (B) Subsequently, the fibre responded to two repeated cold stimuli separated by 3 minutes, in a high frequency bursting manner. The fibre remained unresponsive to noxious heat.

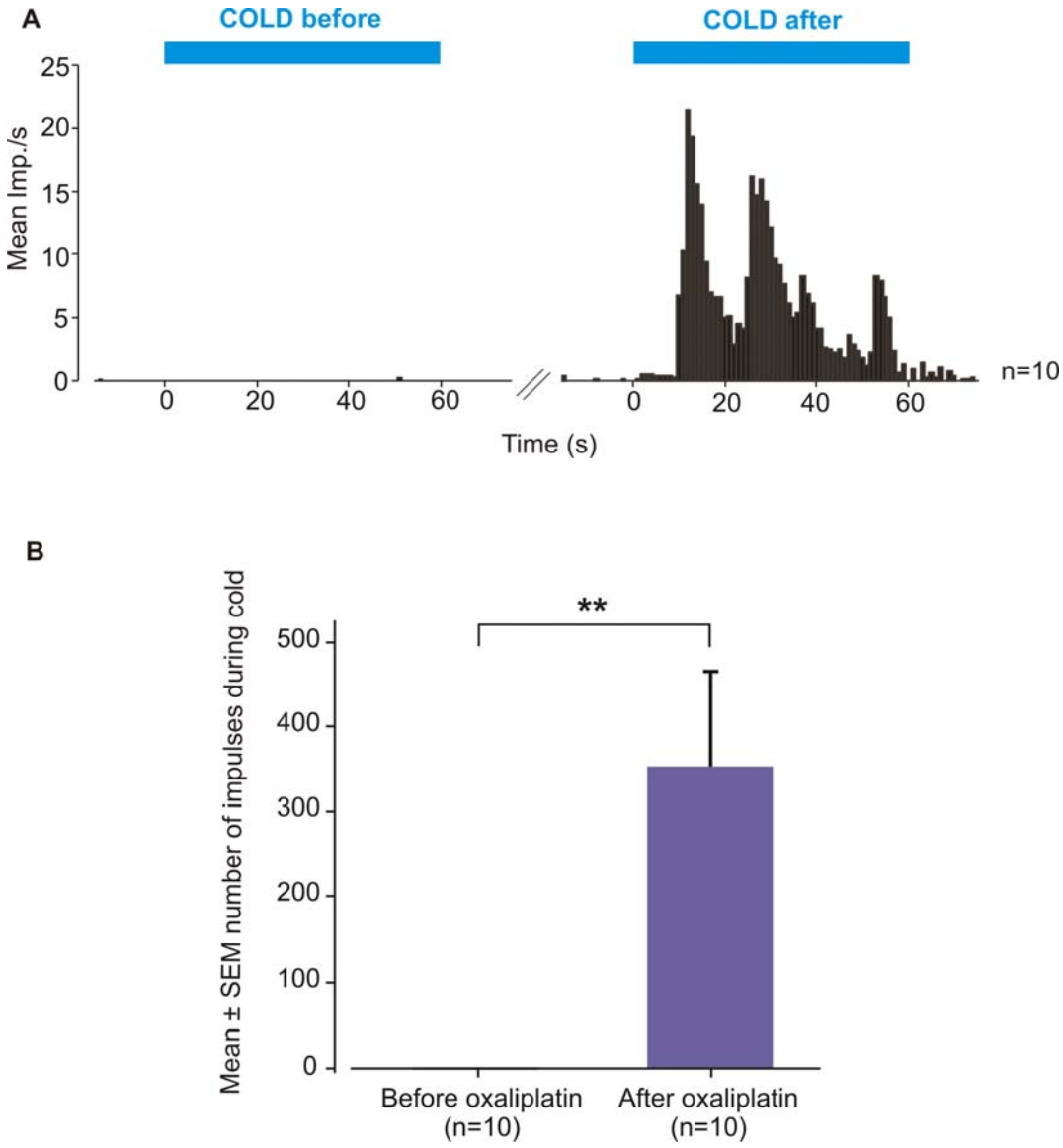

Fig.7.3.4. Mean response of RA fibres to a cold stimulus before and after application of oxaliplatin

(A) Units were not activated by cold stimuli before the application of oxaliplatin. Subsequently they responded to cold in irregular, high frequency bursting discharges. (B) There was a significant difference ($p<0.005$, Wilcoxon matched pairs test) in the mean number of impulses generated during a cold stimulus before and after oxaliplatin application.

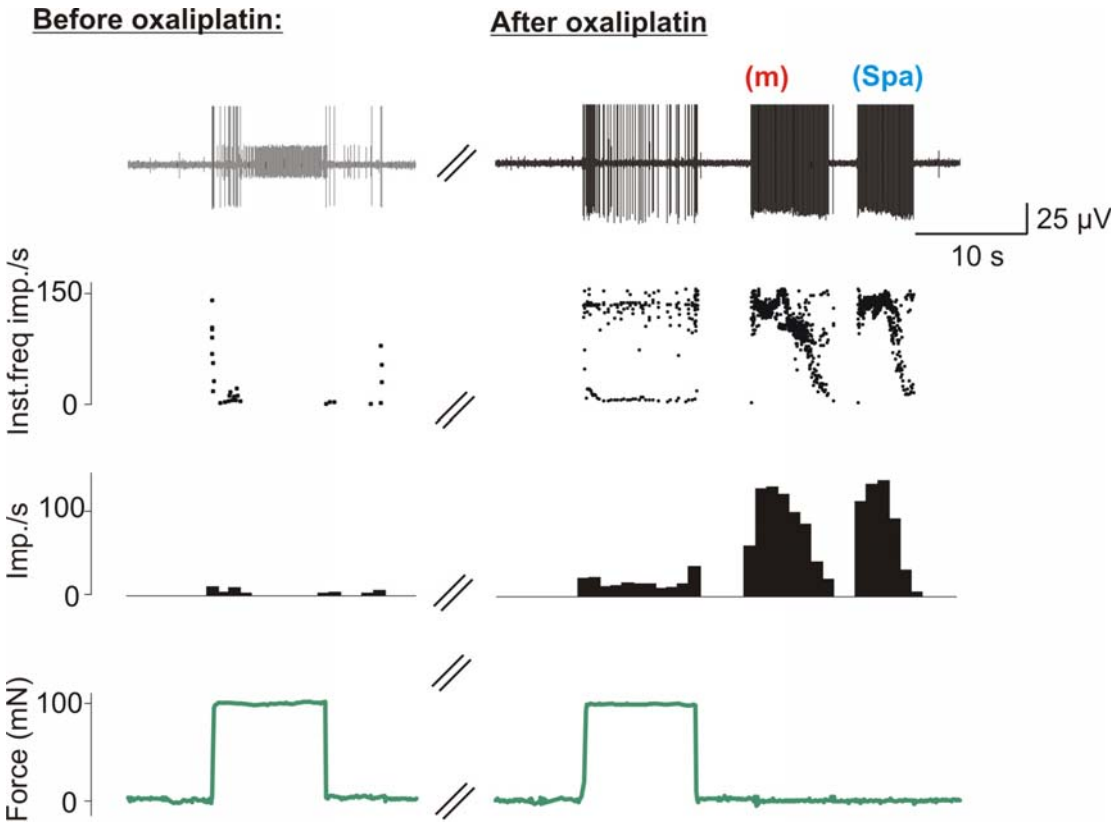

Fig.7.3.5. A rapidly adapting mechanoreceptor (RA) responding to a constant force stimulation of 100 mN before and after application of 400 µM oxaliplatin to the receptive field for 15 minutes
(A) The fibre adapted rapidly to the mechanical stimulus, responding at the beginning and end of the stimulus only. (B) Subsequently the unit fired continuously in bursts of action potentials during mechanical stimulation. A manual glass rod stimulus (m) was then applied to the receptive field, to which the fibre responded in high frequency bursts. This was shortly followed by spontaneous bursting activity from the fibre (Spa).

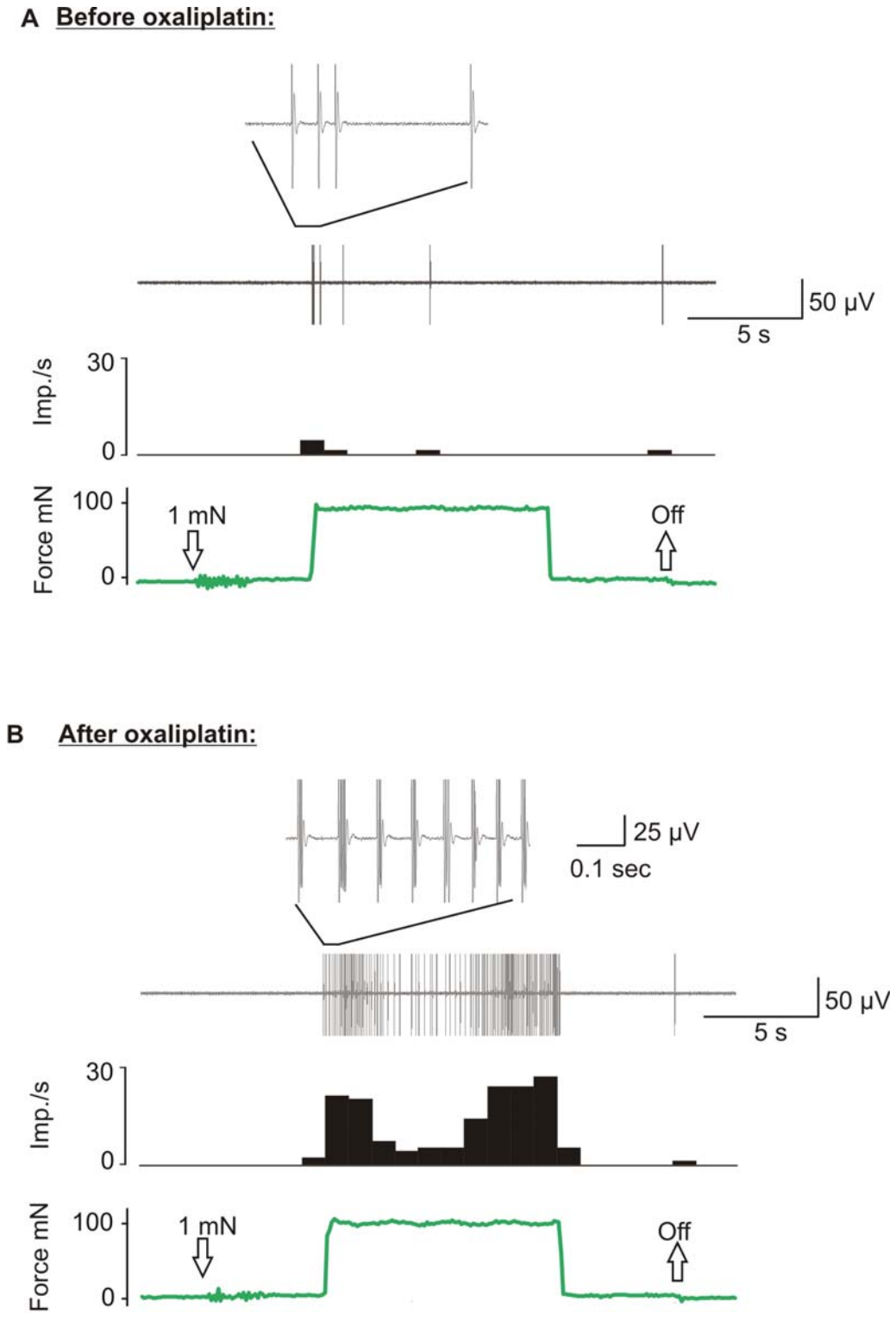

Fig.7.3.6. Example of the mechanical response of a RA mechanoreceptor before and after application of 400 µM oxaliplatin for 15 minutes to its receptive field

(A) The fibre displayed a rapidly adapting response to a 100 mN constant force stimulus. (B) After the application of oxaliplatin, the fibre responded throughout the stimulus, in high frequency bursts.

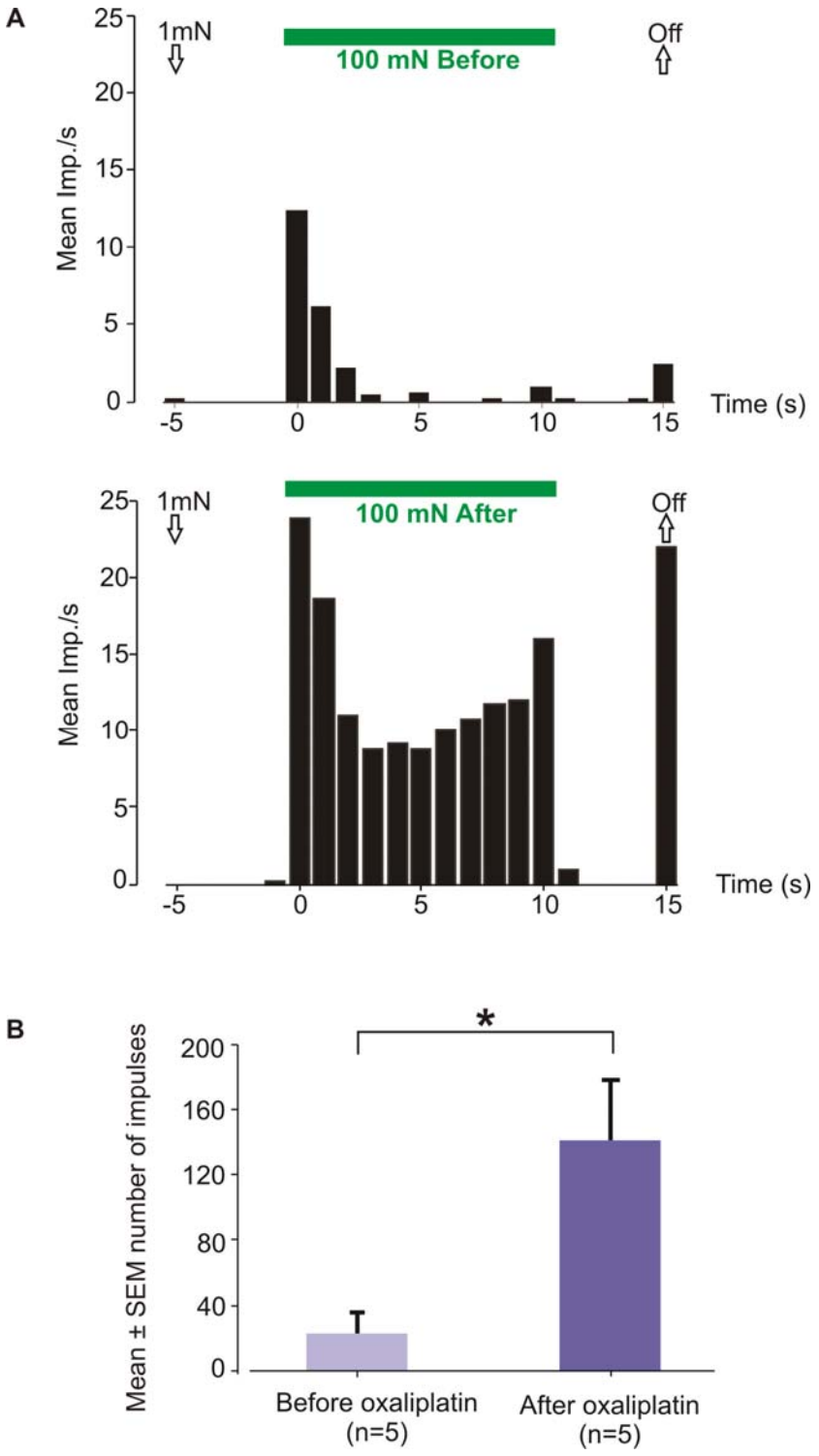

Fig.7.3.7 Mean response of rapidly adapting (RA) fibres to a 100 mN constant force stimulus before and after oxaliplatin application
(A) On average fibres (n=5) fired mostly at the beginning of the stimulus. However, after being exposed to oxaliplatin they discharged continuously during the mechanical stimulation. (B) There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean total number of impulses generated during the mechanical stimulation after oxaliplatin application compared to before.

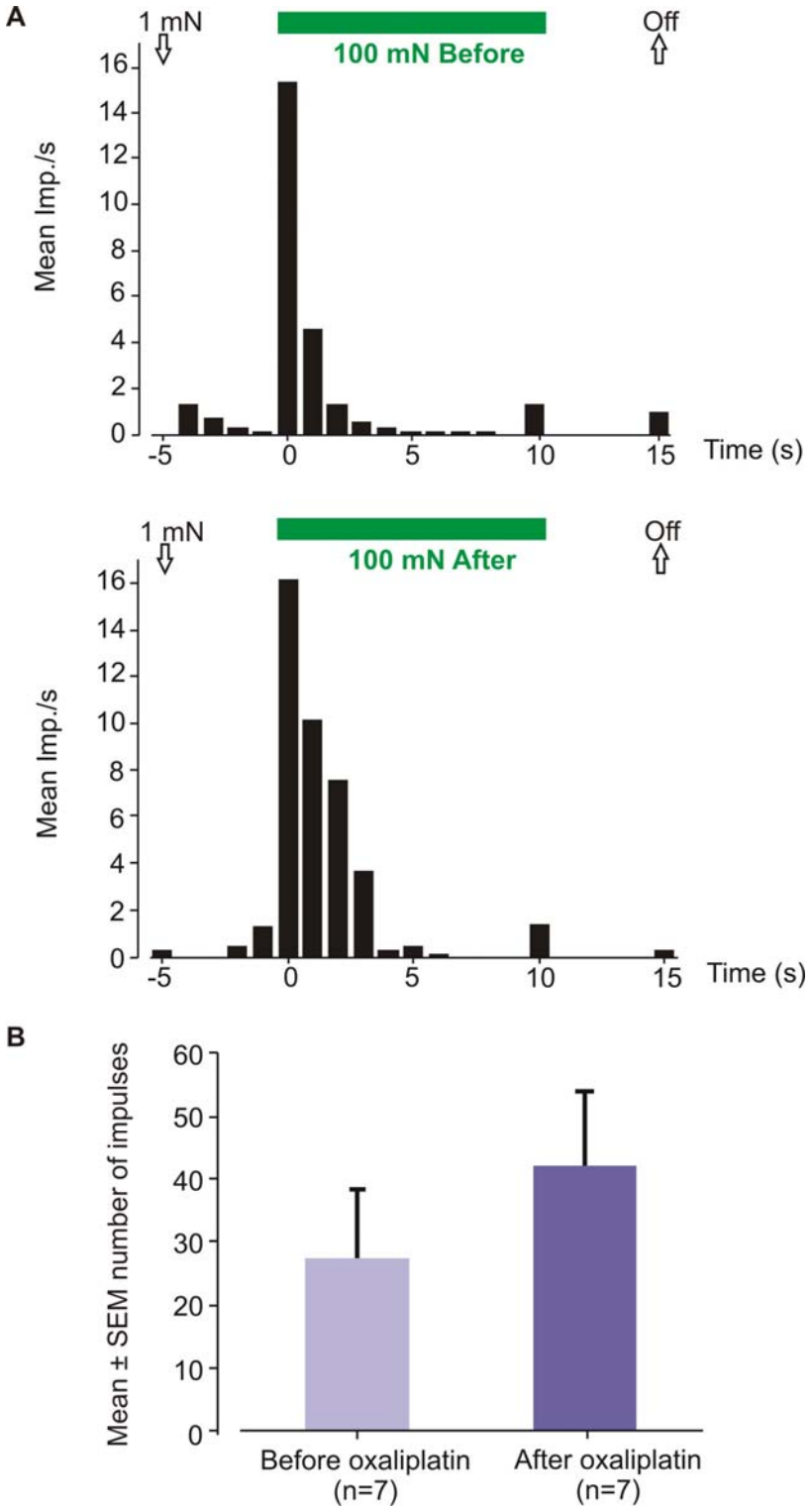

Fig.7.3.8. Mean rapidly adapting (RA) mechoreceptor response to constant force stimulation (CFS) of 100 mN before and after application of oxaliplatin

(A) Fibres (n=7) discharged mainly during the ramp phase of the stimulus, adapting rapidly to the CFS. Subsequently, the firing pattern remained unchanged, but the number of impulses during the initial ramp phase of the stimulus increased. (B) However, this difference was statistically insignificant (p>0.1, Wilcoxon matched pairs test).

Slowly adapting (SA) mechanoreceptors

A total of 12 SA fibres was recorded. None of the fibres displayed any initial spontaneous activity. Out of the 12 SA fibres, 2 (16.7 %) responded with a brief, non sustained excitation to a cold stimulus. An example of one such unit is shown in Fig.7.3.9. The average response threshold to cold was 29.7 (28.1-31.1 $^{\circ}$C, see Table.7.3.2, page 289). None were responsive to a noxious heat stimulus.

Oxaliplatin was applied on the receptive fields of all the SA fibres. Direct activation of these units during the application period was never observed. Subsequently, all SA fibres were re-tested for their response to a cold stimulus. 6 out of 12 (50 %) SA units displayed a novel response to a cold stimulus. This was not significantly different ($p=0.9$, χ^2 test) from the proportion of RA units which displayed a novel cold response after oxaliplatin treatment.

Fig.7.3.10 shows the proportion of SA units which became cold responsive after application of oxaliplatin at doses ranging from 200-600 µM.

None of the units which displayed this novel cold sensitivity were initially cold sensitive. The majority (66.7 %) of SA units discharged in bursts of action potentials during the cold stimulus. In contrast, some units displayed a regular, non-bursting firing pattern to the cold stimulus. An example of one such unit is illustrated in Fig.7.3.11. The average response threshold to cold was 13.7 ± 2.4 $^{\circ}$C ranging from 6.6-22.0 $^{\circ}$C, which was not significantly different from that of cold induced RA fibres ($p>0.1$, unpaired students t-test). Plotting the mean discharge to cold revealed that like the RA fibres, on average SA fibres also discharged in irregular bursts after oxaliplatin application (Fig.7.3.12A). The mean maximal discharge rate was 23 impulses per second. There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean response to a cold stimulus after oxaliplatin application, as illustrated in Fig.7.3.12B.

Similar to RA fibres, not all of the SA fibres responded to the first cold stimulus post oxaliplatin. 4 out of 6 (67 %) units responded to the first cold stimulus whereas 2 out of 6 (33 %) units responded to the second cold stimulus.

2 SA units were re-tested with a noxious heat stimulus. None became sensitive to heat.
2 SA units were re-tested with constant force stimulation of 100 mN after exposure to oxaliplatin. Their mean firing pattern remained unchanged (Fig.7.3.14) although there was a decrease in overall firing frequency after oxaliplatin application.
In contrast, it was found that the two SA fibres which were initially cold responsive, became less cold responsive after the application of oxaliplatin (see Fig.7.3.9 for an example). Fig.7.3.13 displays the averaged cold responses before and after oxaliplatin application for these two fibres.

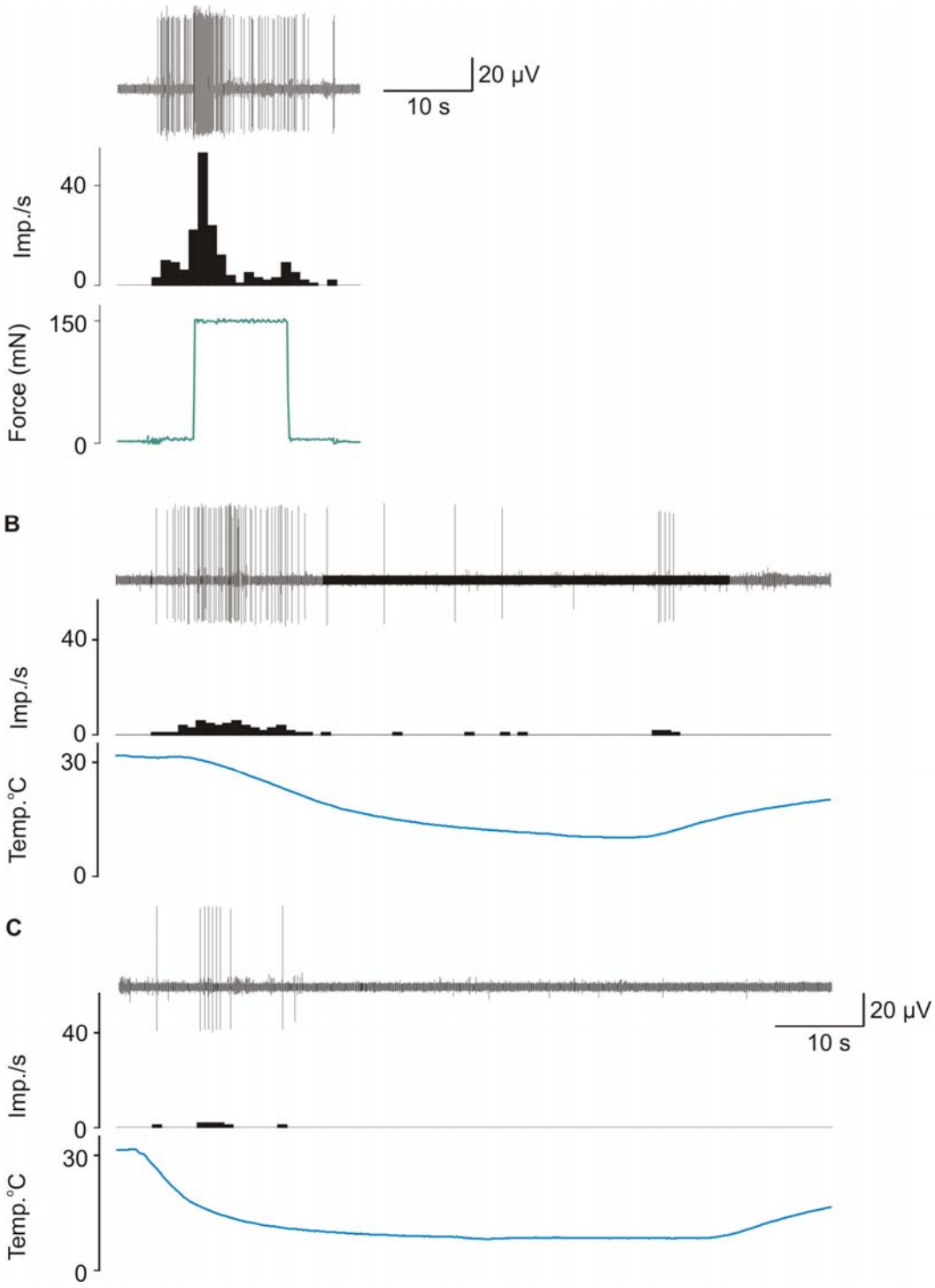

Fig.7.3.9. Example of a cold sensitive slowly adapting (SA) mechanoreceptor before and after application of oxaliplatin
(A) The fibre responded vigorously during the ramp phase of the constant force stimulus which was then followed by a lower frequency firing throughout. (B) The fibre then responded briefly to the cold stimulus before application of oxaliplatin. (C) After application of 600 µM oxaliplatin to the receptive field for 10 minutes, a cold stimulus was re-applied, to which the fibre's response had decreased.

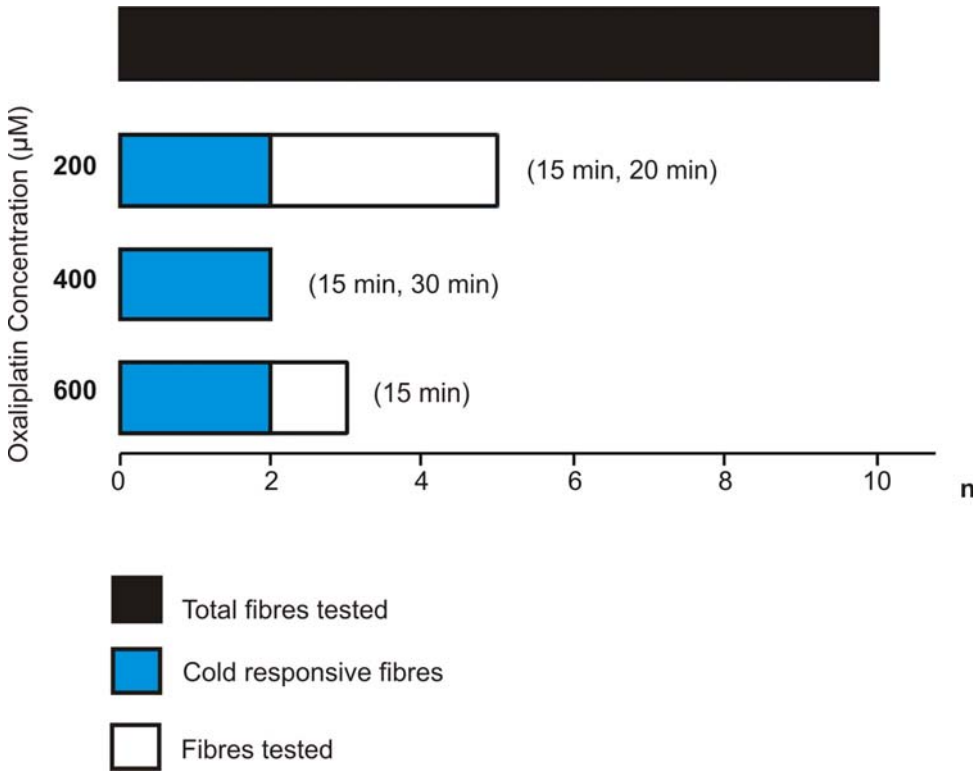

Fig.7.3.10. Proportion of SA fibres which displayed a novel cold response after oxaliplatin application
The proportion of induced cold sensitivity in the total number of fibres tested with oxaliplatin at different concentrations is shown.
For cold induced fibres, the time for which oxaliplatin was applied for is given in brackets.

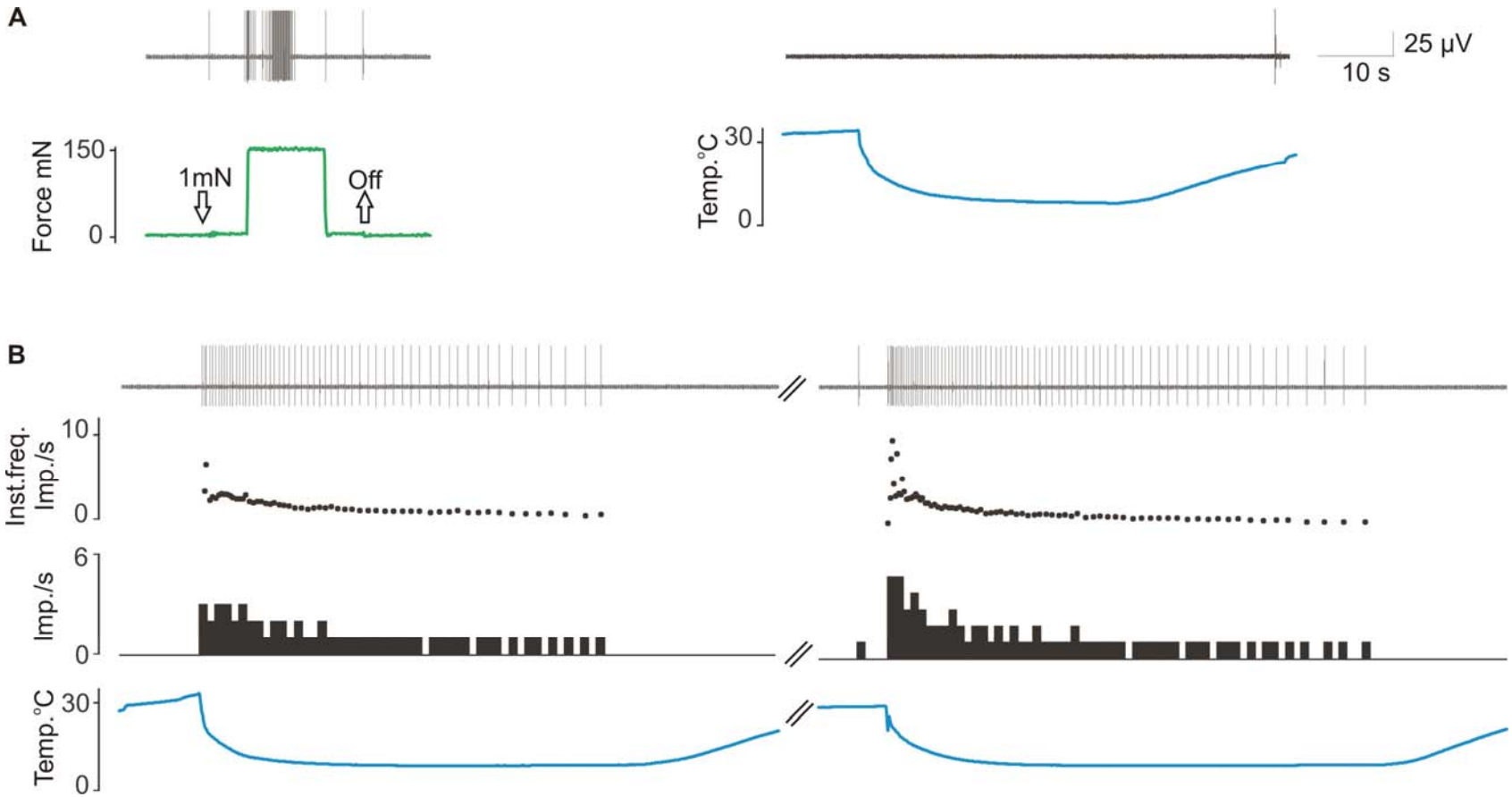

Fig.7.3.11. Example of a cold insensitive slowly adapting (SA) mechanoreceptor before and after application of oxaliplatin
(A) There was no response to a cold stimulus before application of the drug. (B) After the application of 600 µM oxaliplatin for 15 minutes, the unit responded to two cold stimuli which were separated by 5 minutes.

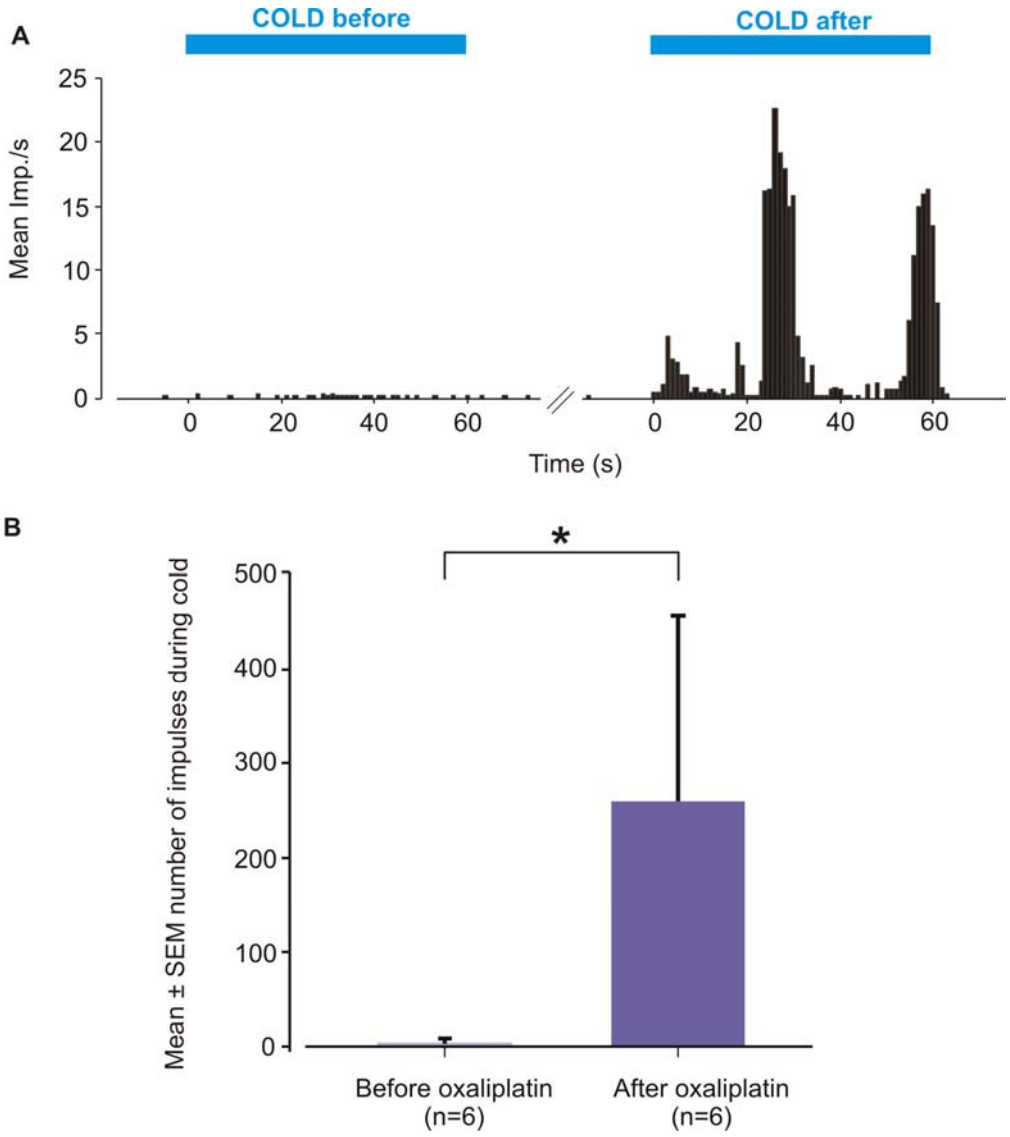

Fig.7.3.12. Mean response of SA fibres to a cold stimulus before and after oxaliplatin application

(A) Units were not activated by cold before the application of oxaliplatin. Note the low level of activity during cold before application of oxaliplatin is due to a fibre which became spontaneously active. Subsequently they responded to cold in irregular, high frequency bursting discharges. (B) There was a significant increase ($p<0.05$, Wilcoxon matched pairs test) in the mean number of impulses generated during a cold stimulus after oxaliplatin application.

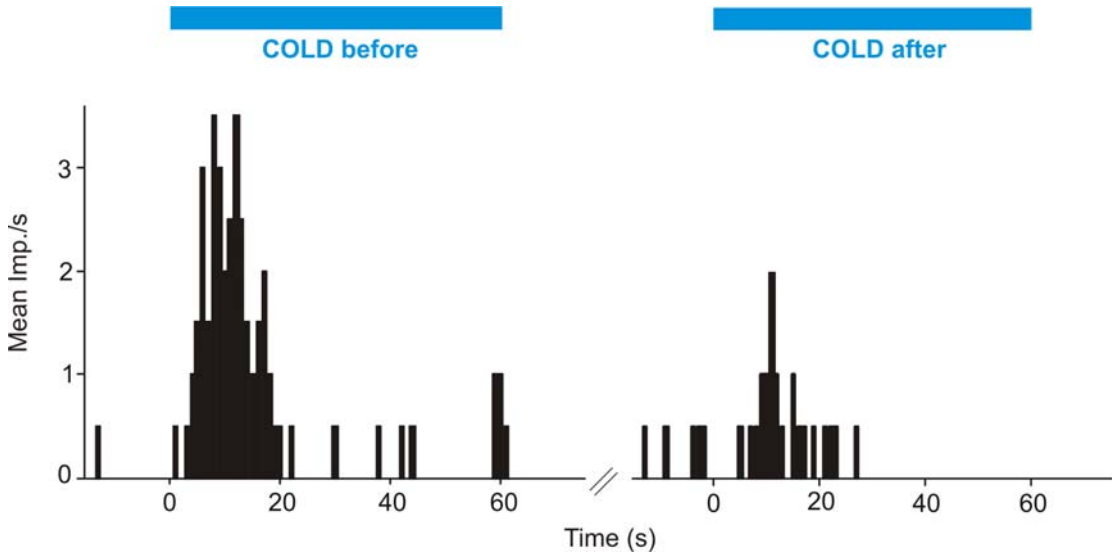

Fig.7.3.13. Mean response of cold sensitive mechanoreceptors (SA) to a cold stimulus before and after application of oxaliplatin (n=2)
Fibres responded typically during the ramp phase of the cold stimulus, reaching a peak discharge rate of 3 impulses per second. Subsequently the firing frequency decreased after application of oxaliplatin.

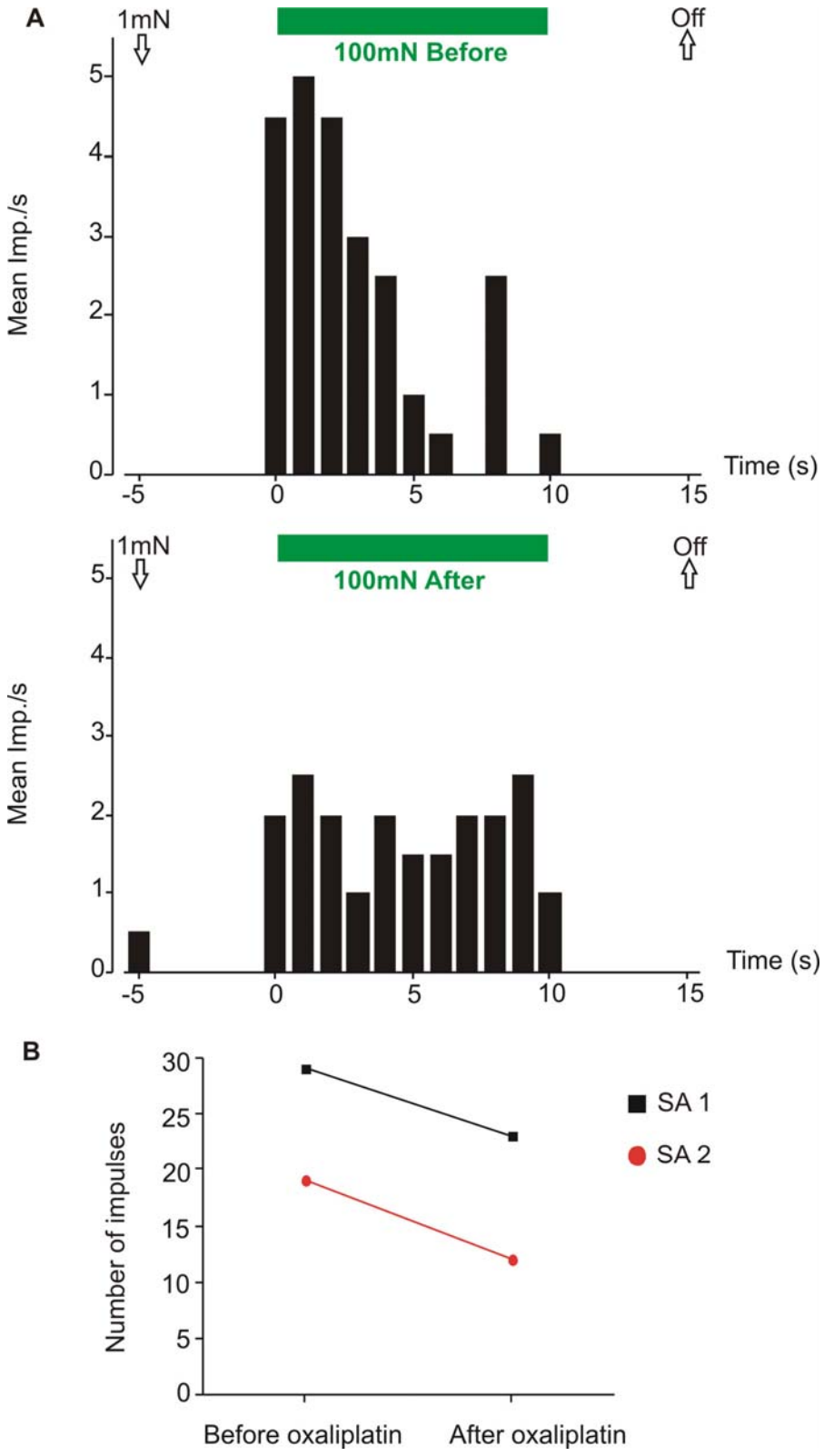

Fig.7.3.14. Mean slowly adapting (SA) mechanoreceptor response to constant force stimulation (CFS) of 100 mN before and after application of oxaliplatin

(A) Fibres (n=2) discharged mostly during the ramp phase of the stimulus followed by a lower level of firing throughout the CFS. Subsequently, the level of firing was lower. None of the units discharged in bursts. (B) The total number of impulses generated during the CFS after oxaliplatin was slightly lower for both fibres.

TABLE.7.3.2. Mean number of spikes to a cold stimulus and cold thresholds in myelinated Aβ fibres

Fibre Type	n	Number of spikes during cold stimulus before oxaliplatin	Cold Threshold, ˚C	∆, ˚C	Number of spikes during cold stimulus after oxaliplatin	Cold Threshold, ˚C after oxaliplatin	∆, ˚C
RA	10	0.2 ± 0.2 (2)	-	-	352 ± 112.7 (40-940)	13.2 ± 2.0 (4.5-23.5)	17.3 ±2.3
SA	6	4.8 ± 4.6 (0-28)*	-	-	260 ± 196.7 (22-1240)	13.7 ± 2.4 (6.6-22.0)	17.9 ±2.1
SA (c)	2	38 (10-66)	29.7 (31-28.1)	2.4 (0.3-4.4)	11.5 (9-14)	21.5 (16.5-26.5)	9.8 (4.5-15.4)

All values are means ± SE. RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; SA (c) slowly adapting afferent fibres which were previously cold sensitive , Δ relative change in temperature required to elicit a cold response from base line temperatures. * Includes one SA unit which displayed spontaneous activity before and during the cold stimulus.

Aδ fibres

A summary of general properties of all the Aδ fibres is provided in Table.7.3.1. Table.7.3.3 (page 301) provides a quantitative summary of how the fibres responded to a cold stimulus before and after the application of oxaliplatin.

D-hair (DH) fibres

A total of 3 DH fibres were recorded. None displayed any spontaneous activity initially. None responded to a cold or noxious heat stimulus. Oxaliplatin was applied to the receptive fields of all the DH units. None of the units were directly activated during application period. All the fibres were tested with a cold stimulus after oxaliplatin application.
1 out of 3 DH units displayed a novel response to cold stimuli after exposure to oxaliplatin (600 μM, for 15 minutes). During the passive re-warming phase the fibre began to discharge spontaneously in high frequency bursts (Fig.7.3.15). The response threshold to cold was 29.1 $^{\circ}$C. Response to noxious heat and mechanical adaptation were not re-tested in this unit.

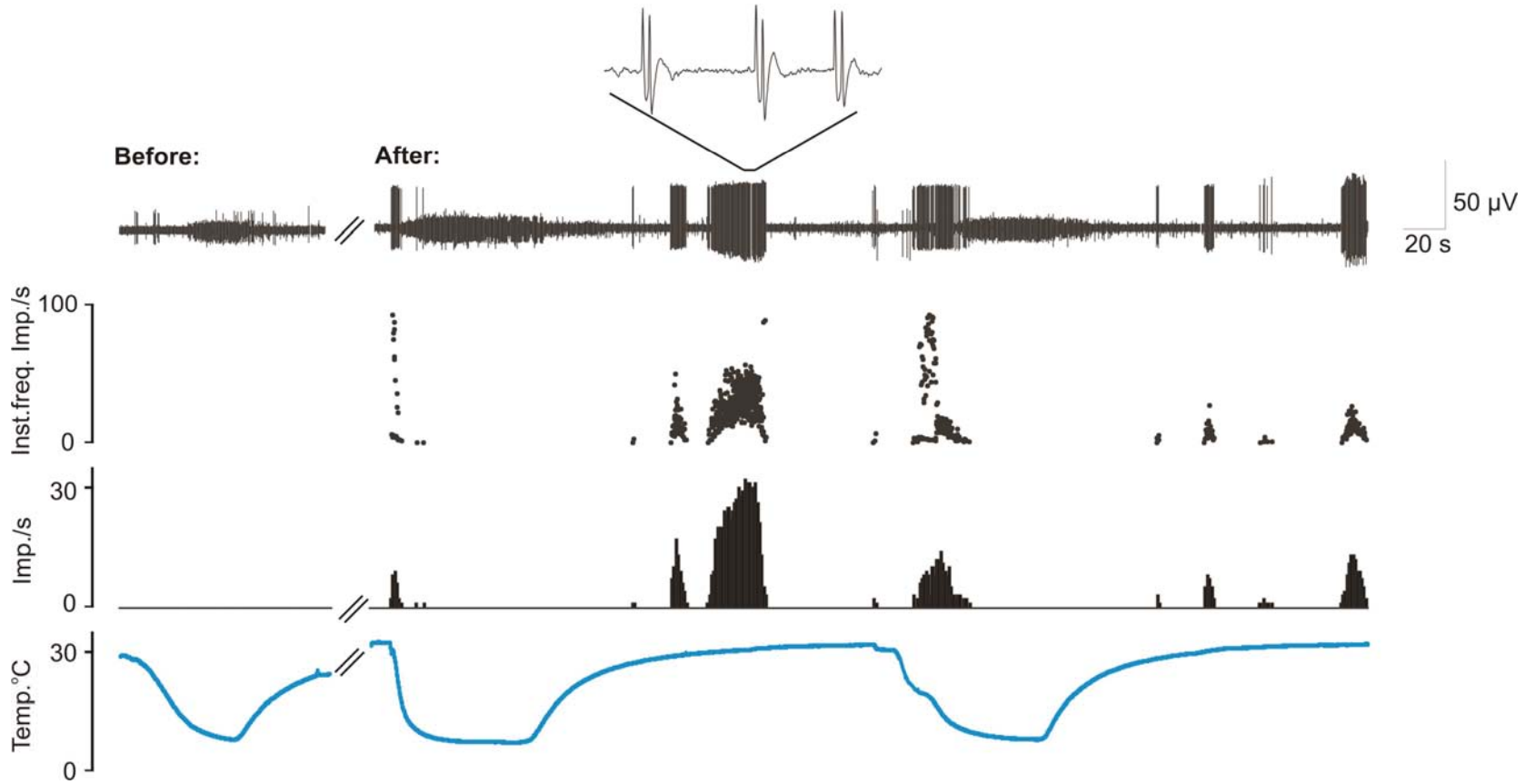

Fig.7.3.15. Example of a D-hair fibre before and after application of 600 µM oxaliplatin for 15 minutes
(A) The fibre responded typically during the onset of the mechanical stimulus. There was no response to a cold stimulus before application of oxaliplatin. (B) Subsequently the fibre discharged briefly in bursts during cold. This was then followed by a spontaneous, high frequency after discharge during the passive re-warming phase. The fibre exhibited the same response pattern to a repeated cold stimulus.

High threshold mechano-A (AM) nociceptors

A total of 16 AM fibres were recorded. None were initially spontaneously active.
Of the 16 AM units, 3 (18.8 %) responded to cold (AMC). The average response threshold to cold was 7.0 ± 1.9 °C, ranging from 3.5-10.2 °C. 2 AM units (12.5 %) were responsive to heat (AMH).

Oxaliplatin was applied to the receptive fields of all the AM units. None of the units were directly activated during application of oxaliplatin. All the fibres were re-tested with a cold stimulus afterwards.
In total 6 out of the 16 AM units (37.5 %) displayed either a novel response, or an increased response to a cold stimulus after oxaliplatin application. Overall, there was no significant difference ($p>0.3$, χ^2 test) in the proportion between Aβ and Aδ fibres which displayed a novel or increased cold response after the application of oxaliplatin.

3/11 cold-insensitive AM units displayed a novel cold response after oxaliplatin application, all firing in bursts during the cold stimulus. Fig.7.3.17 illustrates one such example. The proportion of AM units which became cold responsive after application of oxaliplatin ranging from 200-600 μM is shown in Fig.7.3.16.
The mean response to cold before and after oxaliplatin is represented in Fig. 7.3.18. After exposure to oxaliplatin, fibres discharged in irregular, high frequency bursts during the cold stimulus. Mean maximal responses tended to occur at very low temperatures.

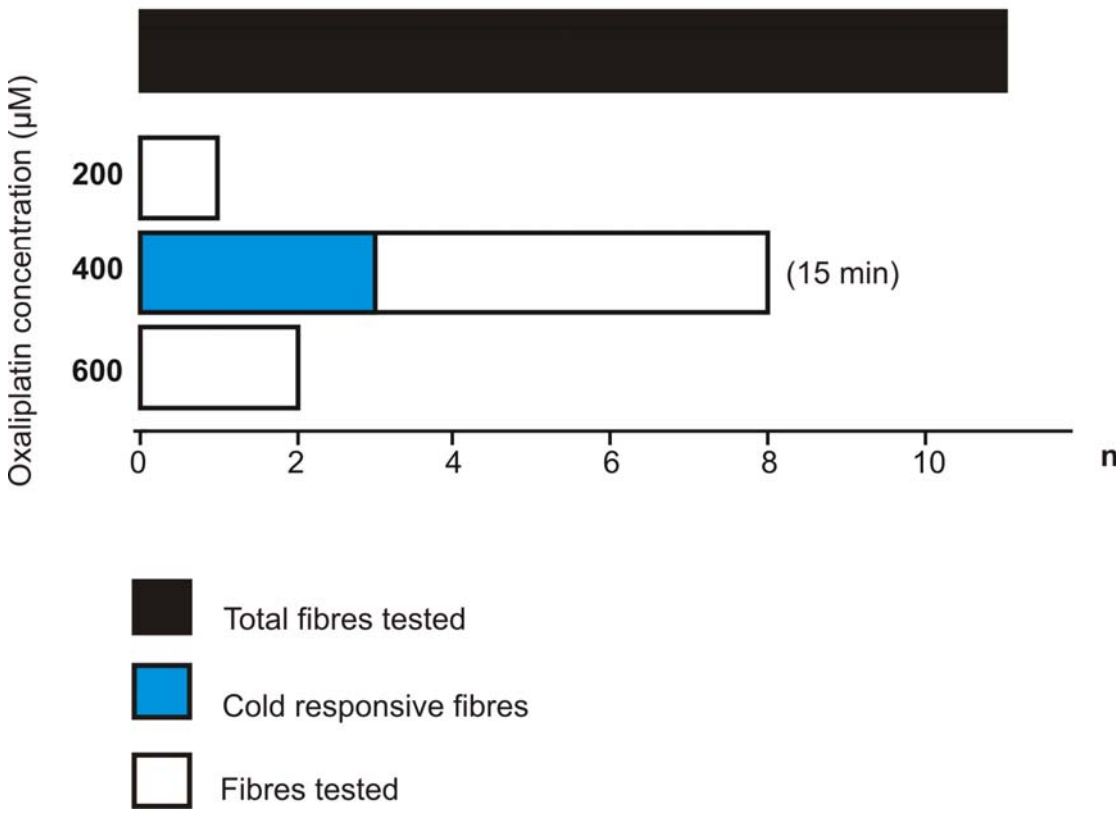

Fig.7.3.16. Proportion of AM fibres which displayed a novel cold response after oxaliplatin application
The proportion of induced cold sensitivity in the total number of fibres tested with oxaliplatin at different concentrations is shown.
For cold induced fibres, the time for which oxaliplatin was applied for is given in brackets.

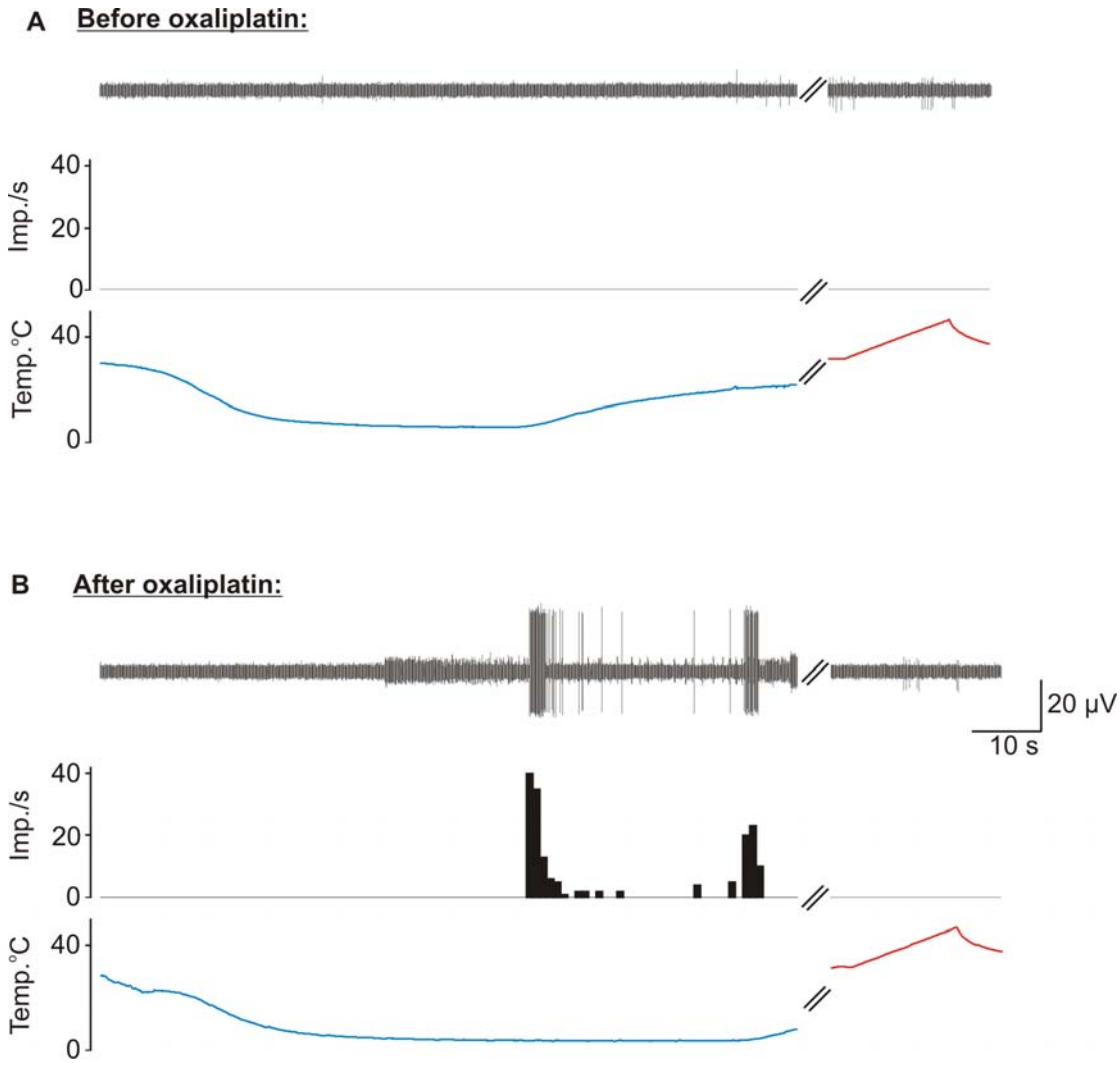

Fig.7.3.17. Example of a high threshold mechanically sensitive A fibre (AM) response to thermal stimulation before and after application of 400 uM oxaliplatin for 15 minutes

(A) The fibre did not respond to a noxious cold or heat stimulus before the application of oxaliplatin. (B) Subsequently, the fibre displayed a delayed response to a cold stimulus, but remained insensitive to noxious heat.

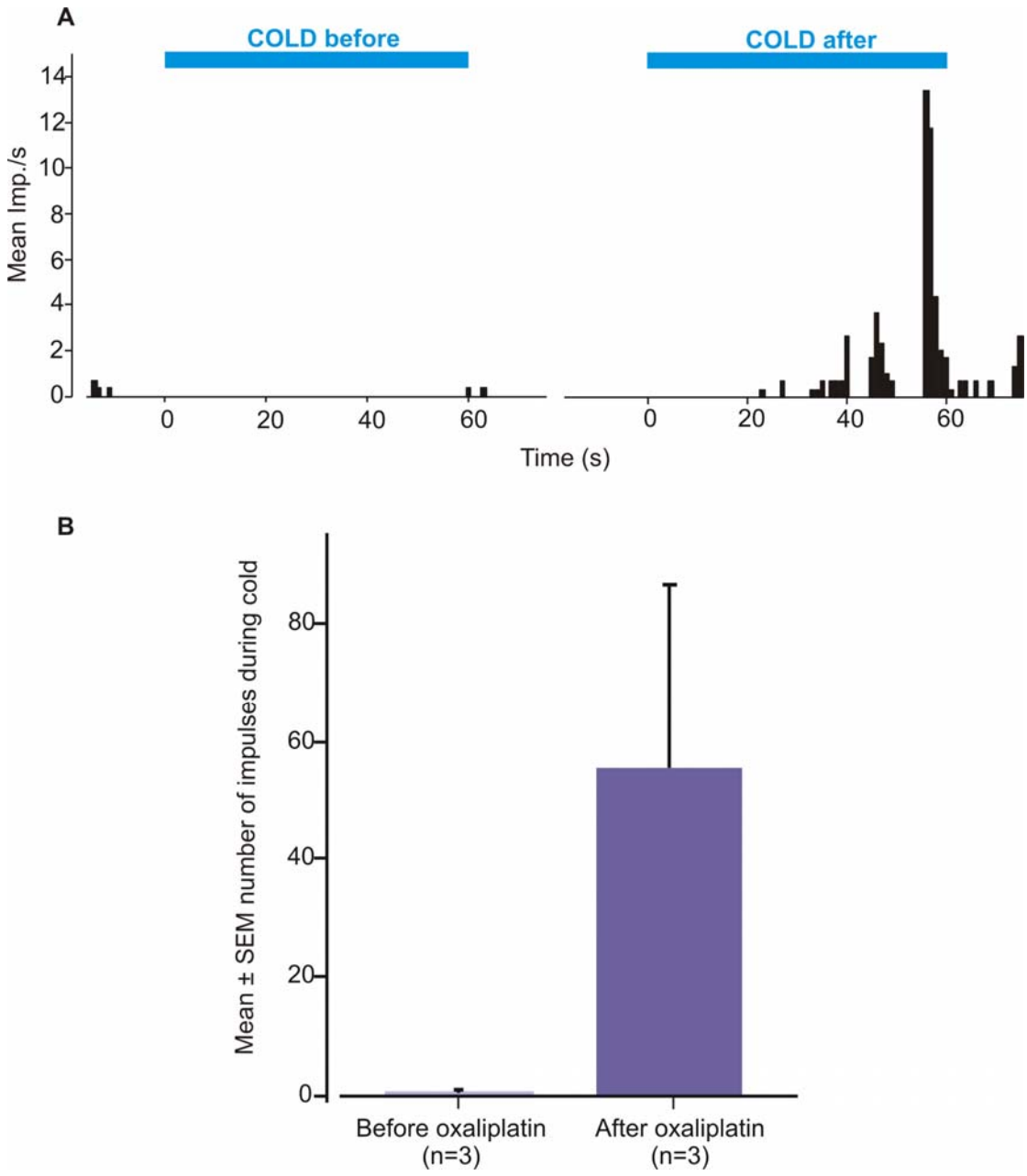

Fig.7.3.18. Mean response of AM fibres to a cold stimulus before and after oxaliplatin application (n=3)

(A) Units were not activated by a cold stimulus before the application of oxaliplatin. Subsequently they responded to cold in irregular, high frequency bursting discharges. (B) There was an increase in the mean total number of impulses during cold after oxaliplatin application.

All of the AMC units (n=3) displayed a marked increase in firing frequency to a cold response after application of oxaliplatin (see Fig.7.3.19 for which concentrations of oxaliplatin were used). 2 out of these 3 units started to respond in bursts of action potentials to the cold stimulus. An example is shown in Fig.7.3.20. Plotting the mean discharge rate to cold revealed that after application of oxaliplatin, AMC fibres displayed a different firing pattern (Fig.7.3.21). Whereas previously they would discharge at a regular interval in low frequencies, subsequent to oxaliplatin application, they discharged in an irregular manner at high frequencies, often in bursts. There was a clear increase in the mean response to cold after oxaliplatin application (Fig.7.3.21B), this difference was statistically insignificant ($p>0.1$, Wilcoxon matched pairs test) in the small sample investigated. Cold thresholds did not change significantly ($p>0.3$, paired students t-test) following application of oxaliplatin (see Table.7.3.3).

The AMH units (n=2) remained cold insensitive after application of oxaliplatin. Only 1 fibre was re-tested with a noxious heat stimulus. The fibres response threshold increased from 33.0 to 38.8 °C while there was a reduction in the total number of impulses during the stimulus from 42 to 12 (see Table.7.3.5).

In total, 13/16 AM units were re-tested for their response to noxious heat after application of oxaliplatin, of which none became heat sensitive.
10 out of the 16 units were re-tested mechanically using a ramp stimulus. 3/10 units discharged in bursts of action potentials after oxaliplatin application. Mean response of these fibres to a mechanical ramp before and after oxaliplatin is illustrated in Fig.7.3.22A. Statistically, there was no difference ($p>0.5$, Wilcoxon matched pairs test) in the mean total number of impulses generated during mechanical stimulation before and after oxaliplatin application (Fig.7.3.22B).

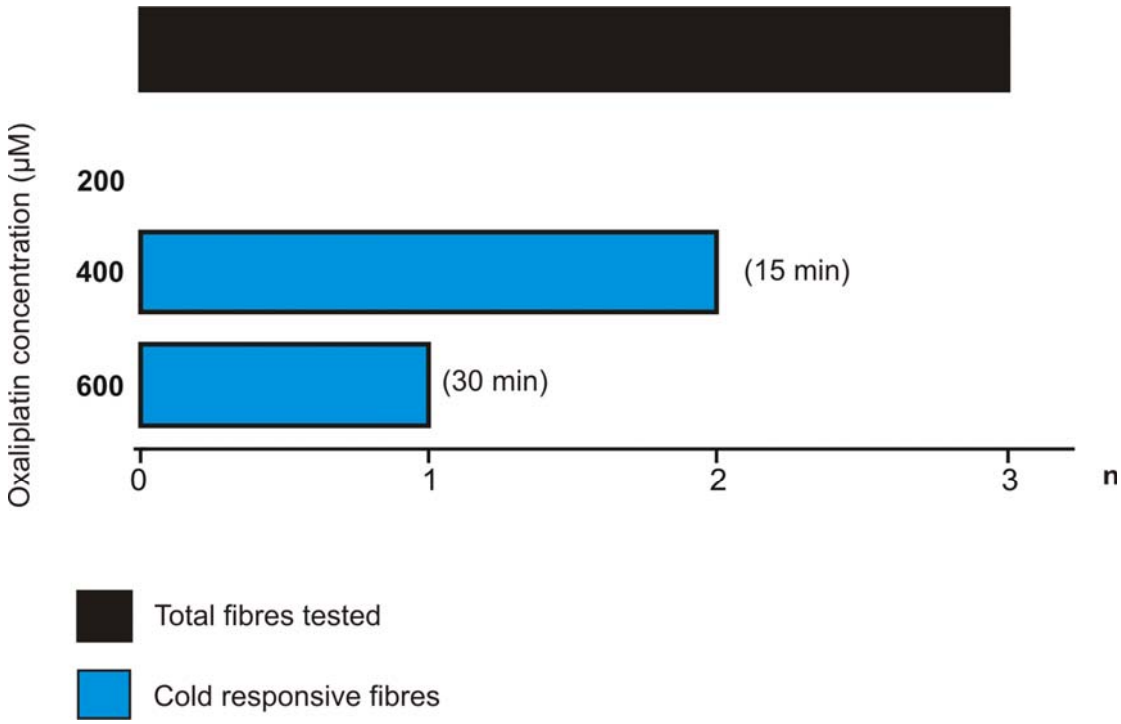

Fig.7.3.19. Proportion of AMC fibres which displayed an increased cold response after oxaliplatin application

The proportion of induced cold sensitivity in the total number of fibres tested with oxaliplatin at different concentrations is shown.
For cold induced fibres, the time for which oxaliplatin was applied for is given in brackets.

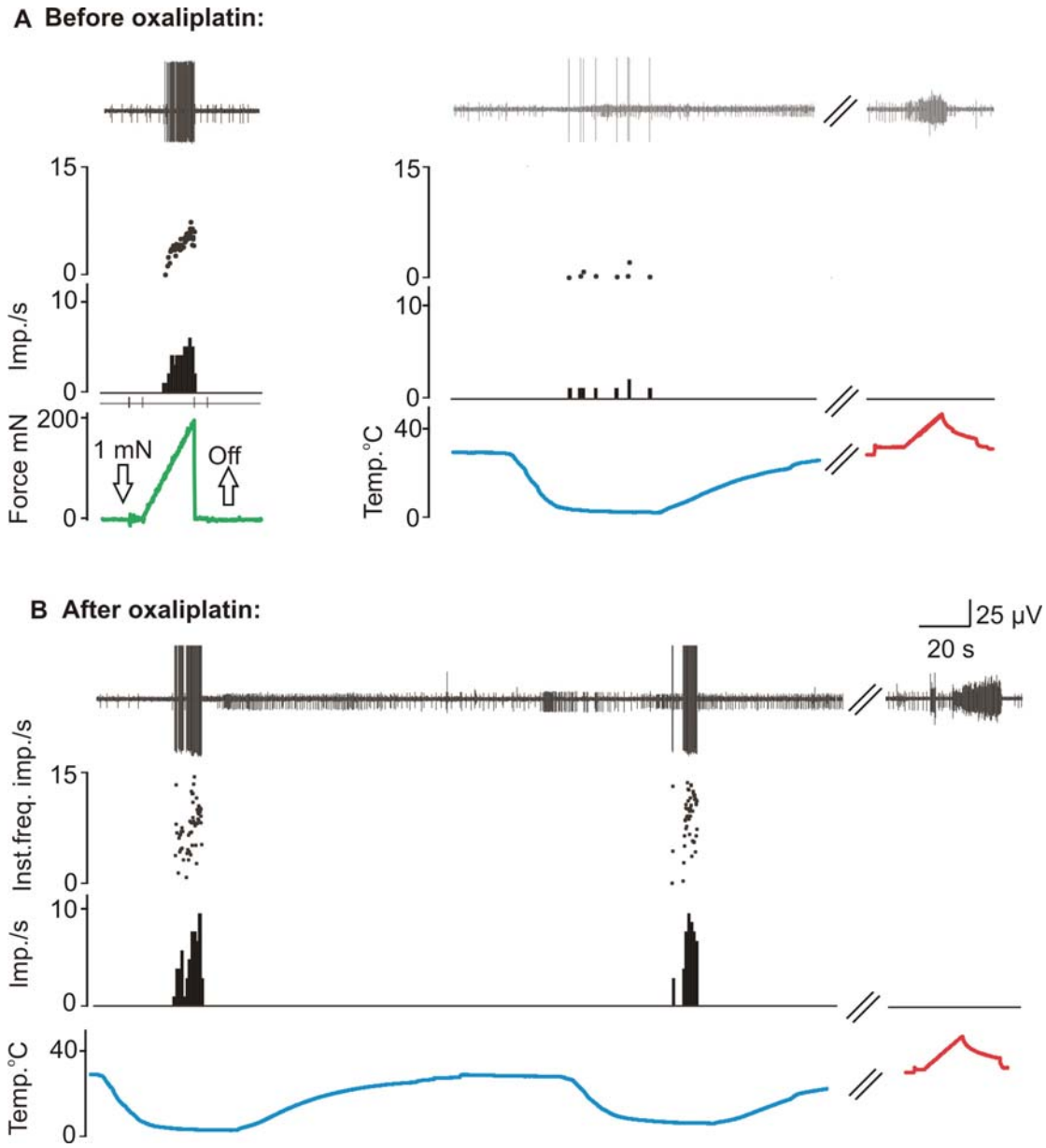

Fig.7.3.20. Example of an AMC fibre before and after oxaliplatin application

(A) The fibre displayed an increasing response to a mechanical ramp (0-200 mN). The fibre was cold sensitive, firing at a low frequency and never in bursts. (B) After the application of oxaliplatin (400 µM, 15 minutes) the fibre showed an increase in firing frequency and started to discharge in bursts of action potentials to a cold stimulus. The fibre remained insensitive to noxious heat.

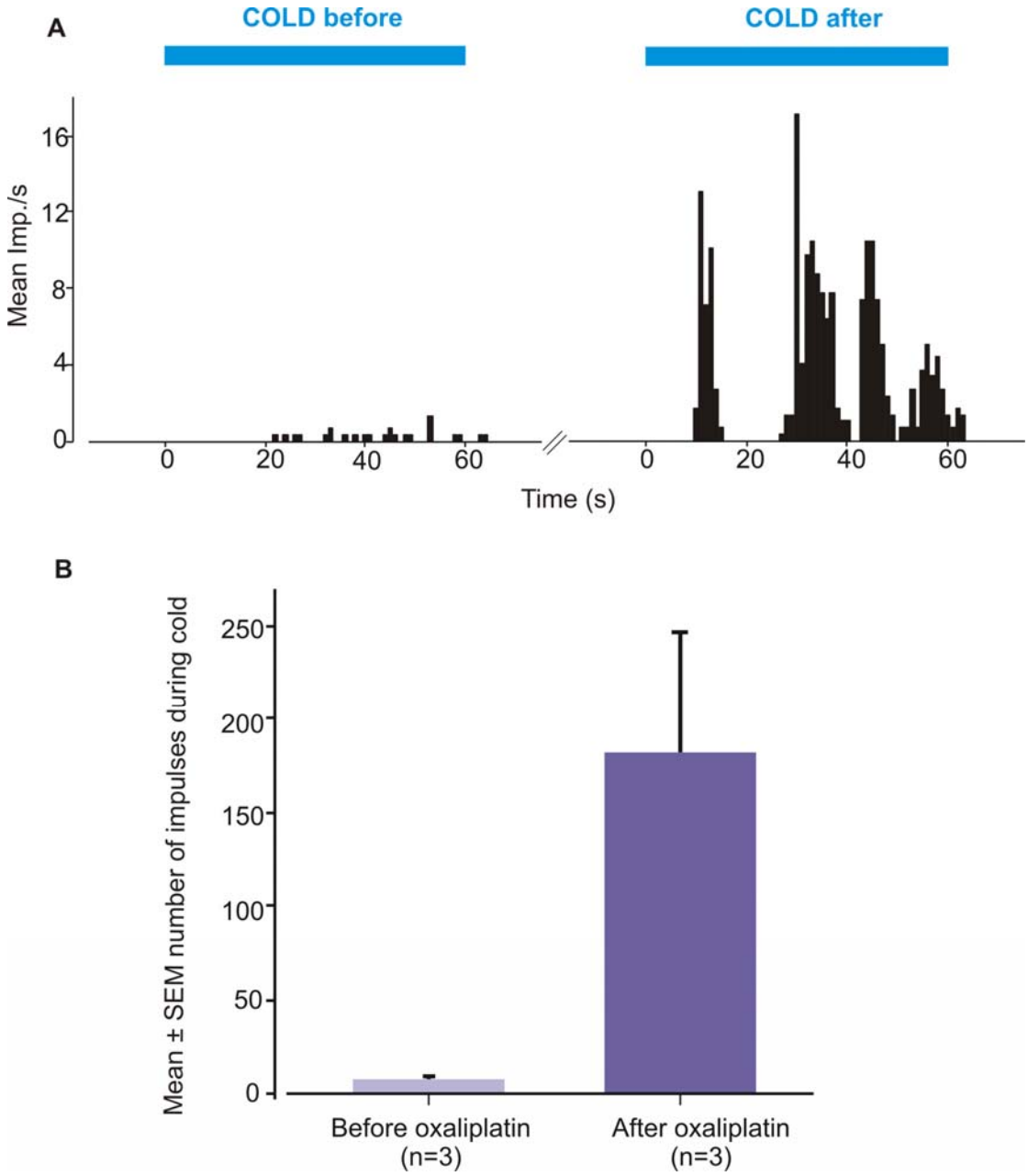

Fig.7.3.21. Mean response of AMC fibres to a cold stimulus before and after oxaliplatin application (n=3)
(A) Units were activated by a cold stimulus, firing in regular manner at low frequencies before the application of oxaliplatin. Subsequently they responded to cold in irregular, high frequency bursting discharges. (B) There was a clear increase in the mean total number of impulses during cold after oxaliplatin application, although this difference was statistically insignificant (p>0.1, Wilcoxon matched pairs test) in this small sample.

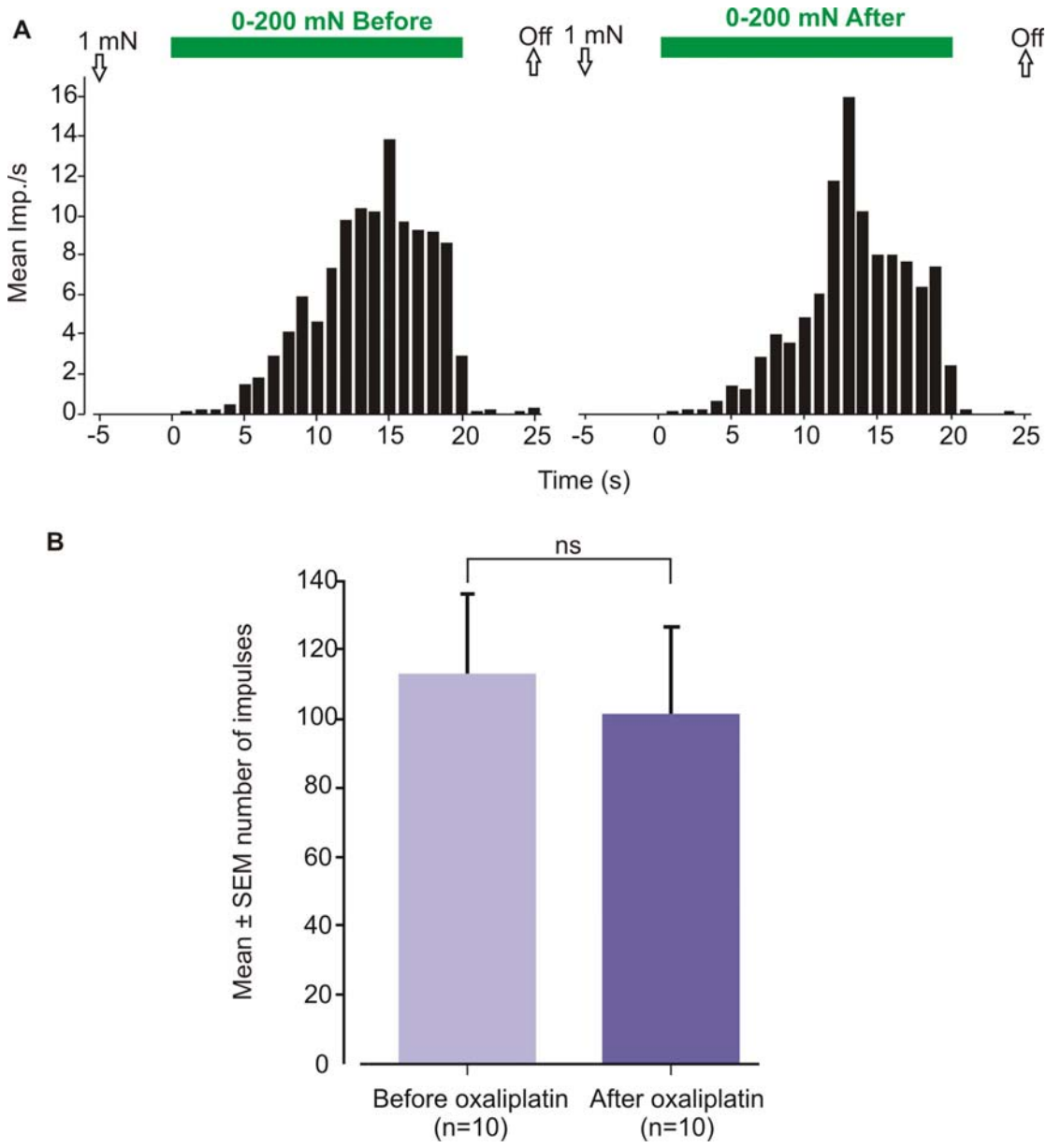

Fig.7.3.22. Mean response of AM fibres to a mechanical ramp (0-200 mN) before and after application of oxaliplatin

(A) There appeared to be no observable difference in the firing pattern or rate of discharge during a mechanical ramp between before and after application of oxaliplatin. (B) There was no significant difference (p>0.5, Wilcoxon paired test) in the mean response to a mechanical ramp stimulus after oxaliplatin application.

TABLE.7.3.3. Mean number of spikes to a cold stimulus and cold thresholds in thin myelinated Aδ fibres

Fibre Type	n	Number of spikes during cold stimulus before oxaliplatin	Cold threshold, ˚C	Δ, ˚C	Number of spikes during cold stimulus after oxaliplatin	Cold threshold, ˚C after oxaliplatin	Δ, ˚C
DH	1	0	-	-	30	29.1	3.4
AM	3	0.6 ± 0.6 (0-2)	-	-	55 ± 30 (21-117)	9.5 ± 4.8 (3.7-19)	21.5 ± 5.5
AMC	3	8 ± 1.7 (5-11)	7 ± 1.9	21.3 ± 2.3	182 ± 64 (60-279)	6.0 ± 1.1 (3.8-7.1)	23.2 ± 1.0

All values are means ± SE. AM, high threshold mechanosensitive A fibre; AMC, high threshold mechano-cold A fibre; DH, D-hair receptors; Δ, relative change in temperature required to elicit a cold response from base line temperatures.

C fibres

A total of 22 C fibres were recorded. Of these 15 were mechanically sensitive, while 7 units were mechanically insensitive cold fibres. None of the mechanically sensitive C fibres were initially spontaneously active. General properties of these fibres are provided in Table.7.3.1. Table.7.3.4 (page 315) provides a quantitative summary of how the fibres responded to a cold stimulus before and after the application of oxaliplatin.

Low threshold mechano-sensitive C fibres (C-LTM)

Of the four C-LTM units: 1 responded additionally to cold (CLTM-C), 1 responded to heat (CLTM-H) and 2 units responded to both cold and heat stimuli (CLTM-CH).

400 μM oxaliplatin was applied to the receptive fields of all fibres for 15 minutes. Subsequently all the units were then re-tested with a cold stimulus. In fibres which were previously cold sensitive, there appeared to be no obvious change in cold sensitivity after application of oxaliplatin, as illustrated by plotting the mean discharge rate to a cold stimulus before and after oxaliplatin application (Fig.7.3.23A). This was further supported by the fact that there was no significant difference ($p>0.5$, Wilcoxon matched pairs test) in the total number of action potentials produced during the cold stimulus before or after oxaliplatin exposure (Fig.7.3.23B). Cold thresholds also remained unchanged ($p>0.5$, paired students t-test). Novel heat sensitivity never developed in these fibres.

Novel cold sensitivity did not develop in the CLTM-H fibre (Fig.7.3.24). 2 out of 3 CLTM-H fibres were re-tested for their sensitivity to a noxious heat stimulus. The difference in the mean total number of spikes during a heat stimulus between before and after oxaliplatin was not different (see Table.7.3.5). Differences between heat thresholds were not statistically significant either ($p>0.5$, paired students t-test).

3 out of 4 units were re-tested with a mechanical ramp stimulus. Subsequent to oxaliplatin application, no differences in firing pattern or rate of discharge were observed (Fig.7.3.25A). Changes in the mean total number of impulses generated to the mechanical ramp stimulus (Fig.7.3.25B) were not significantly different ($p>0.2$, Wilcoxon matched pairs test). Interestingly, in contrast to A fibre mechanoreceptors, the C fibres never discharged in a bursting manner to either mechanical or thermal stimuli after being exposed to oxaliplatin.

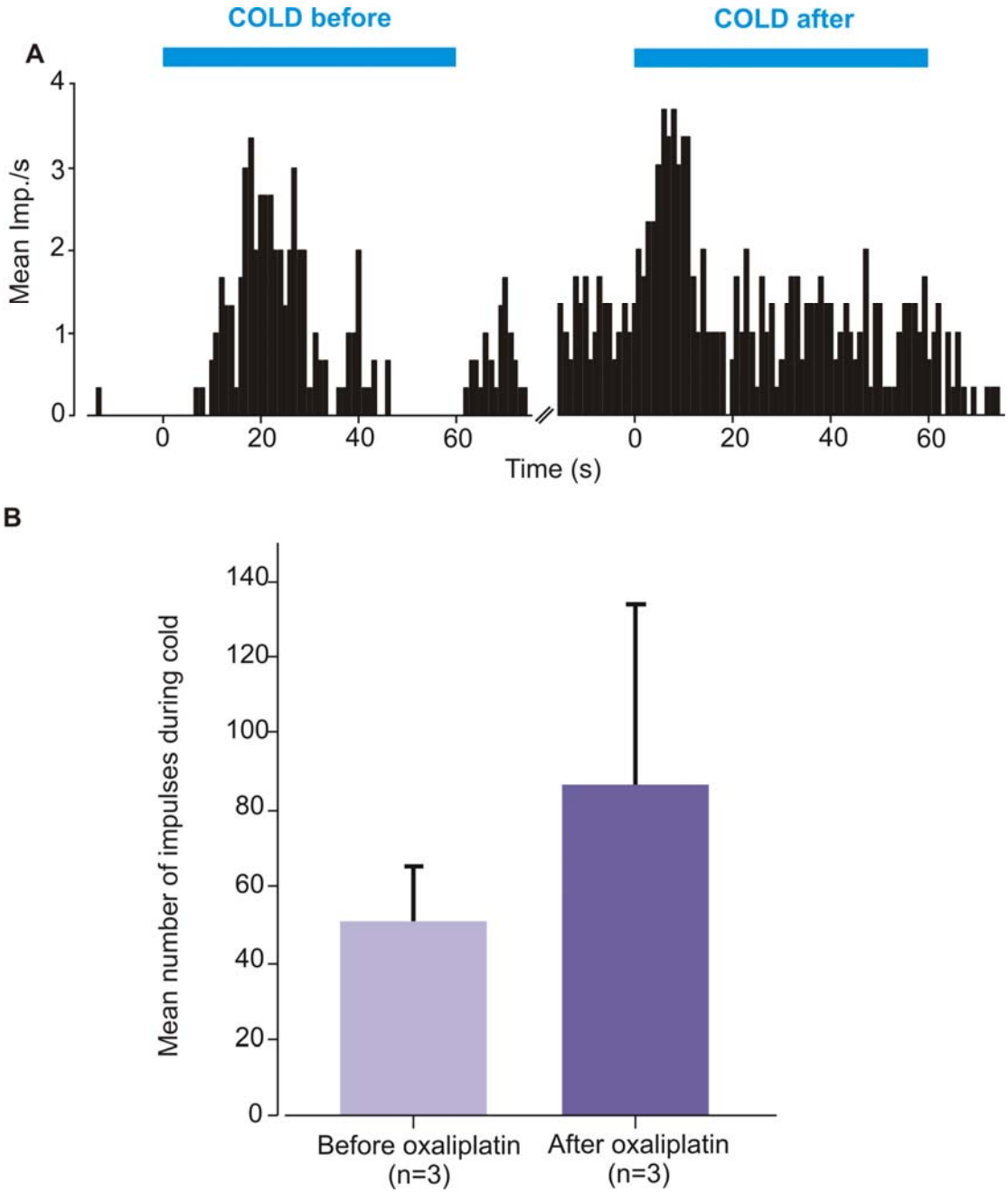

Fig.7.3.23. Mean response of CLTM-C fibres to a cold stimulus before and after application of oxaliplatin

(A) Fibres mainly responded during the dynamic phase of the cold ramp. This was then followed by a lower level of firing which was not sustained throughout the stimulus. There was also some activity during the re-warming phase. Subsequently, the firing pattern was similar except there was sustained firing throughout the cold stimulation. There was also some spontaneous activity before the application of cold. (B) Although on average there was more firing to cold after oxaliplatin application, this difference was not statistically significant ($p>0.5$, Wilcoxon matched pairs test).

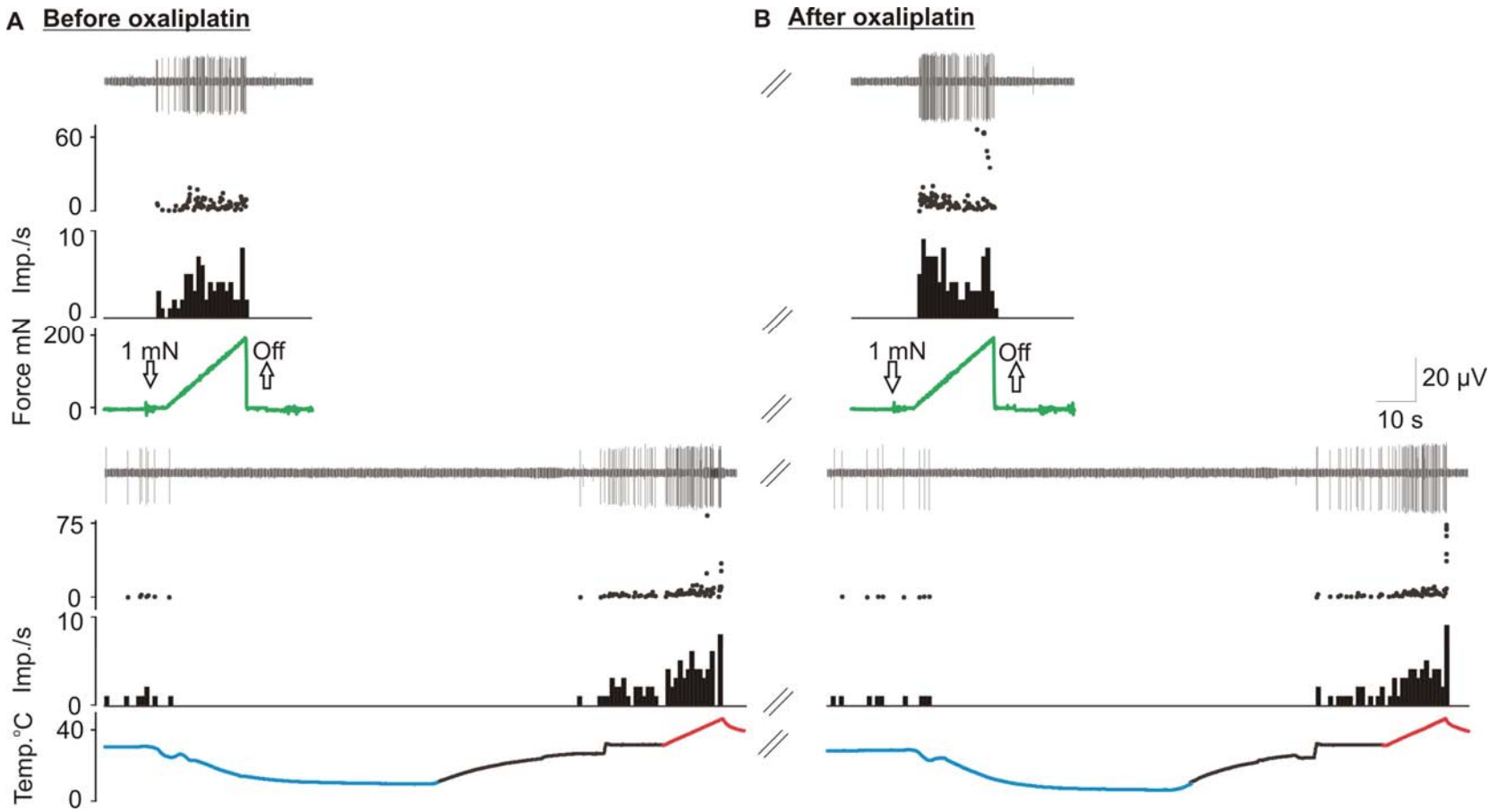

Fig.7.3.24. Example of a CLTM-H unit before and after application of 400 µM oxaliplatin for 15 minutes
(A) The fibre responded to the mechanical ramp and did not adapt to the force applied. There was some spontaneous activity which ceased upon application of a cold stimulus. This was then followed by a vigorous response to a noxious heat stimulus. (B) Subsequent to oxaliplatin application, the receptive properties of the fibre remained largely unchanged.

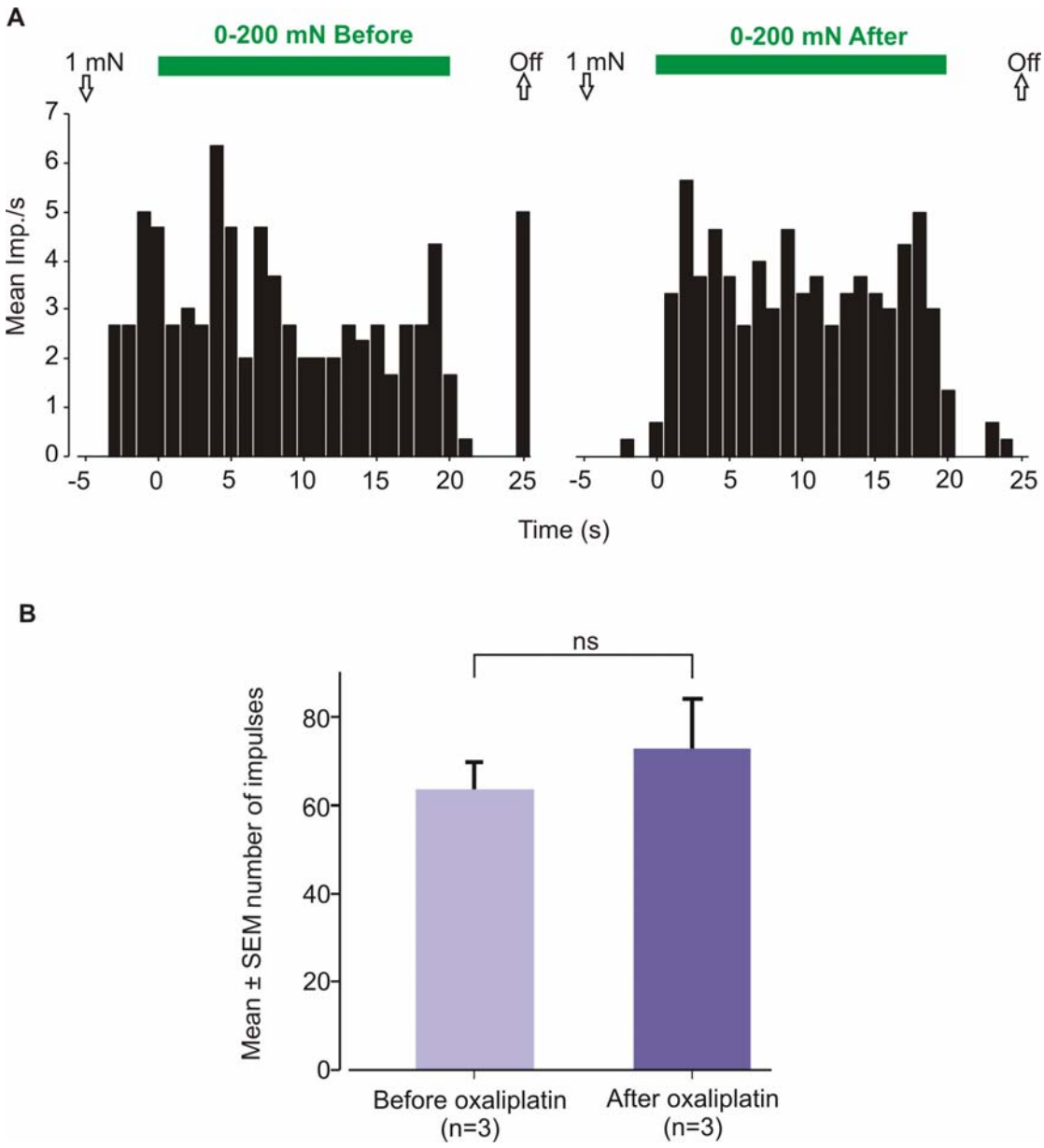

Fig.7.3.25. Mean response of CLTM fibres to a mechanical ramp (0-200 mN) before and after application of oxaliplatin

(A) There appeared to be no observable difference in the firing pattern or rate of discharge during a mechanical ramp between before and after application of oxaliplatin. (B) There was no significant difference (p>0.2, Wilcoxon pairs test) in the mean response to a mechanical ramp between before and after oxaliplatin application.

High threshold mechano-sensitive C fibres

A total of 11 C fibres were recorded. Of these 4 responded to a cold stimulus (CMC), 2 responded to heat (CMH), 3 responded to both cold and heat (CMCH) and 2 were thermally insensitive (CM).

Cold sensitive nociceptors (CMC and CMCH fibres)

Of the 11 C fibres, 7 (63.6 %) responded to cold (CMC/CMCH). The average response threshold to cold was 13.1 ± 3.0 °C, ranging from 4.0 to 24.9 °C (see Table.7.3.4).

Oxaliplatin was applied onto the receptive fields of all the fibres. None of the fibres were directly activated during oxaliplatin application. Fibres which became spontaneously active after initial mechanical and thermal testing did not show any changes in the rate of spontaneous activity during oxaliplatin application.
All units were then re-tested with a cold stimulus. None of the cold sensitive nociceptors displayed any significant change in their cold response after oxaliplatin application. An example of one such unit is illustrated in Fig.7.3.26. Plotting the mean response to a cold stimulus before and after oxaliplatin application revealed that, although there was no change in the mean peak discharge rate, there appeared to be a change in the firing pattern. On average, after oxaliplatin application, fibres were less responsive throughout the cold and only achieved the initial peak discharge rate at the very end of the cold stimulus (Fig.7.3.27A). However, there was no statistical significance ($p>0.2$, Wilcoxon matched pairs test) in the total number of impulses during cold before or after oxaliplatin application (Fig.7.3.27B). Differences between cold thresholds were also not statistically significant ($p>0.7$, paired students t-test, see also Table.7.3.4).

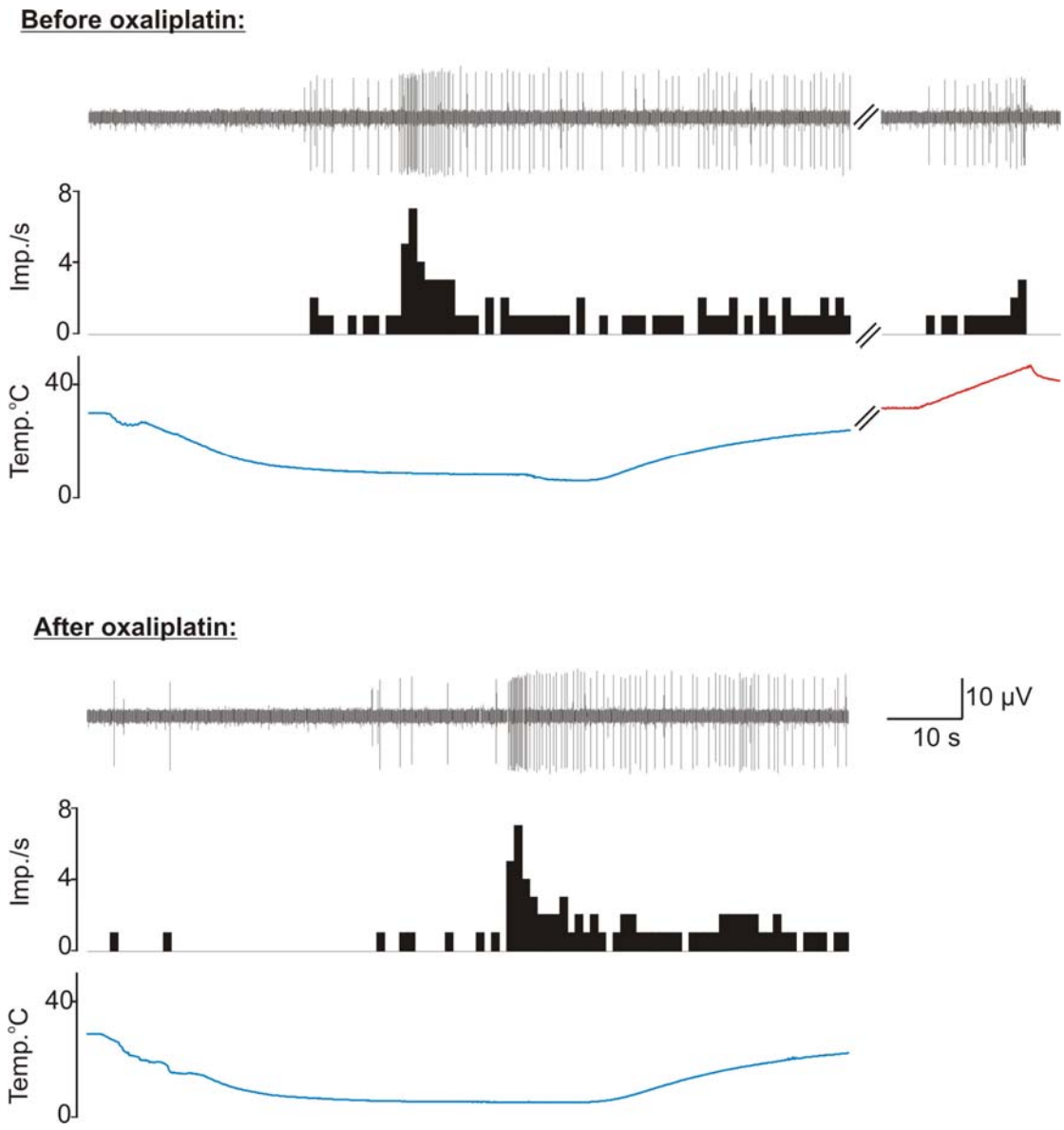

Fig.7.3.26. Example of a CMCH unit before and after application of 400 µM oxaliplatin for 15 minutes

The C fibre responded to both noxious cold and heat stimuli before the application of oxaliplatin. Subsequently, the fibre remained sensitive to cold, but responded in a more delayed manner. There was no difference in the peak discharge rate. The fibre never developed any bursting activity.

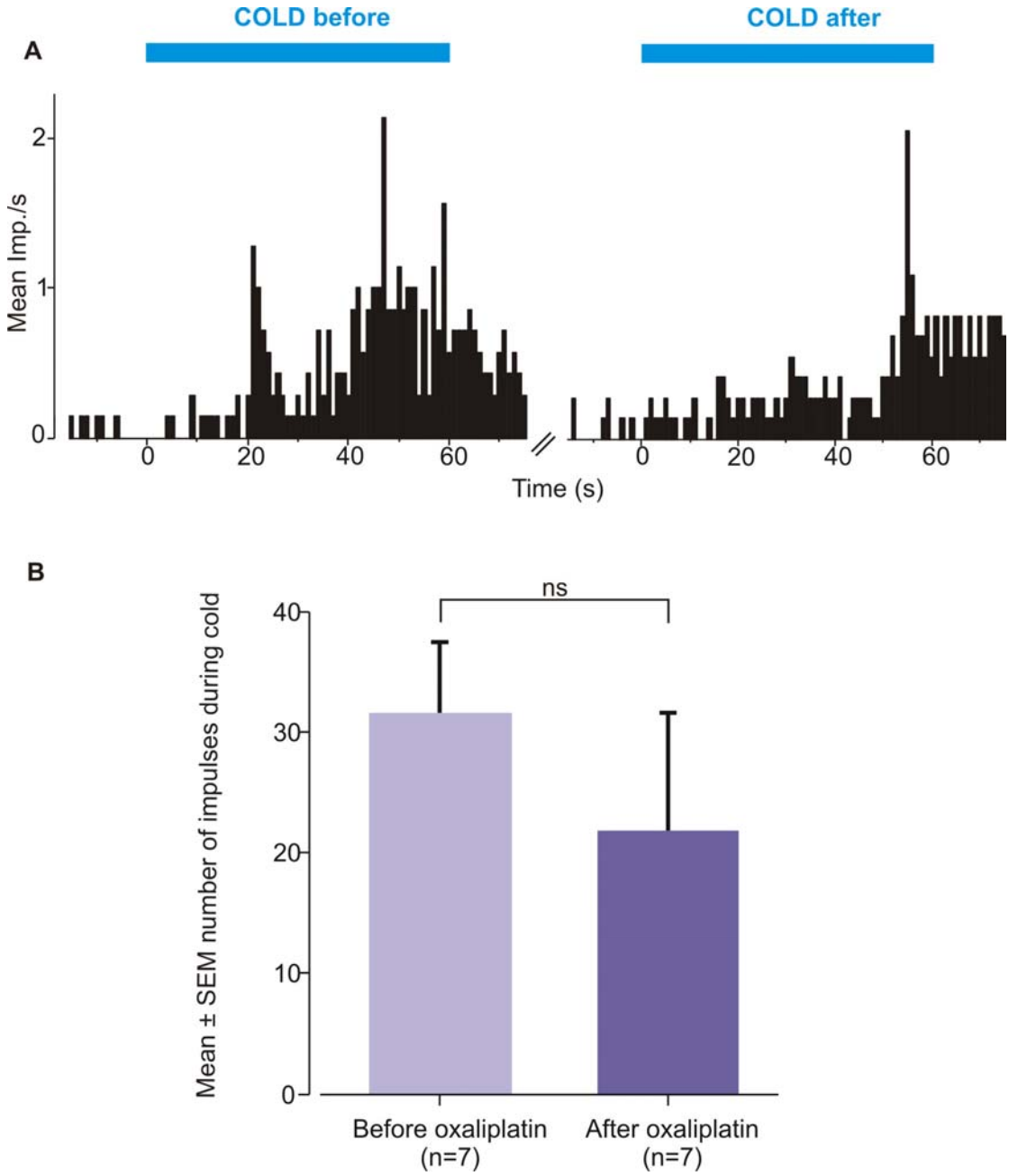

Fig.7.3.27. Mean response of cold sensitive nociceptors (CMC and CMCH fibres) to a cold stimulus before and after oxaliplatin application *(A) Units were activated by a cold stimulus, firing at low frequencies throughout. On average as the temperature decreased the mean firing rate increased. Subsequently, on average the firing rate during the cold stimulus decreased, although the mean peak discharge rate remained unchanged. (B) Although there was a decrease in the mean total number of impulses during cold after oxaliplatin application, this difference was statistically insignificant (p>0.2, Wilcoxon matched pairs test).*

Cold insensitive nociceptors (CM and CMH fibres)

None of the previously cold-insensitive units (CM (n=2) and CMH (n=2)) became cold responsive after application of oxaliplatin.

Heat sensitivity in C fibre nociceptors

Novel heat sensitivity never developed in previously heat insensitive nociceptors (CM and CMC) after application of oxaliplatin.
3 out of 5 CMH fibres were re-tested for their sensitivity to a noxious heat stimulus (Table.7.3.5). Difference in the total number of spikes during a heat stimulus before and after oxaliplatin was not significantly different ($p>0.2$, Wilcoxon matched pair test). Heat thresholds did not change significantly ($p>0.4$, paired students t-test) following application of oxaliplatin.

Mechanical sensitivity in C fibre nociceptors

No evident change was observed in 9 out of the 11 CM units which were re-tested mechanically. Plotting the mean discharge rate as a function of stimulus force revealed that there was no clear change in firing pattern or mean peak discharge rate (Fig.7.3.28A). There was no significant difference ($p>0.8$, Wilcoxon matched pairs test) in the mean total number of impulses generated during a mechanical ramp stimulus after application of oxaliplatin (Fig.7.3.28B). Nociceptors were never observed to fire in bursts after being exposed to oxaliplatin.

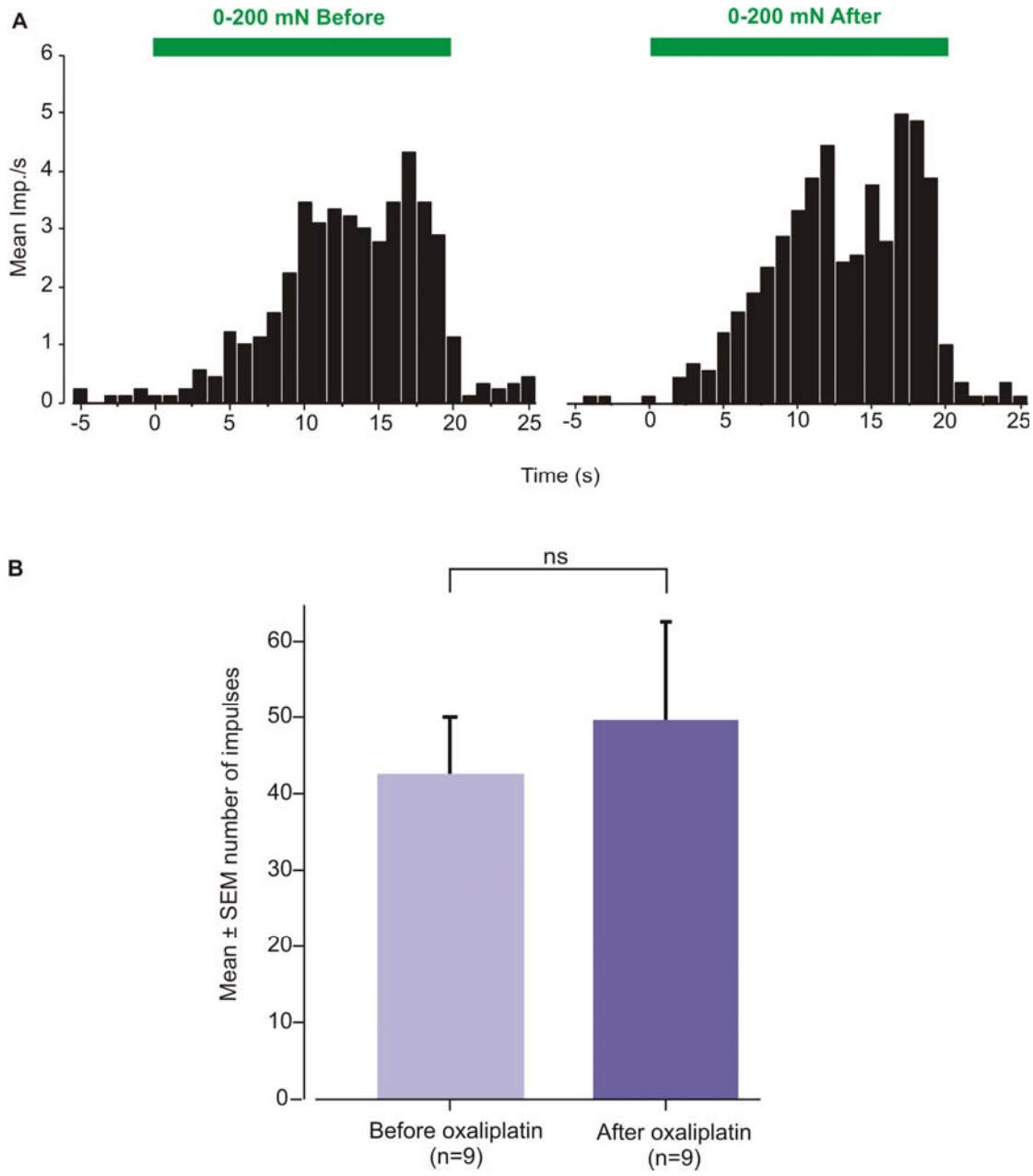

Fig.7.3.28. Mean response of CM fibres to a mechanical ramp (0-200mN) before and after application of oxaliplatin

(A) There appeared to be no observable difference in the firing pattern or rate of discharge during a mechanical ramp between before and after application of oxaliplatin. (B) There was no significant difference (p>0.8, Wilcoxon pairs test) in the mean response to a mechanical ramp after oxaliplatin application.

Cold sensitive mechanically insensitive C fibres (CC)

A total of 7 unmyelinated mechanically insensitive C fibres were recorded. 4 units displayed an ongoing discharge at bath temperatures of 32 °C, firing at low frequencies but not in bursts. The average response threshold to cold was 26.6 ± 2.3 °C, ranging from 13.0-30.5 °C (see Table.7.3.4).

Oxaliplatin was applied to the receptive fields of all the cold fibres. Fibres which displayed no ongoing activity were not activated during the application of oxaliplatin. There was no change in the rate of spontaneous activity in fibres which already displayed ongoing activity.

All units were re-tested with a cold stimulus after oxaliplatin application. An example of a cold fibre is given in Fig.7.3.29. There was no difference in the response pattern or mean peak discharge rate to cold after oxaliplatin application (Fig.7.3.30A). The difference in the mean total number of impulses generated during cold before and after oxaliplatin was also statistically insignificant ($p>0.4$, Wilcoxon matched pairs test, Fig.7.3.30B). However, the mean cold threshold was significantly higher ($p<0.05$, paired students t-test) after application of oxaliplatin.
All the cold fibres were re-tested with noxious heat after oxaliplatin application. None developed any novel heat sensitivity. All of the units remained mechanically insensitive after application of oxaliplatin and none displayed any bursting discharges.

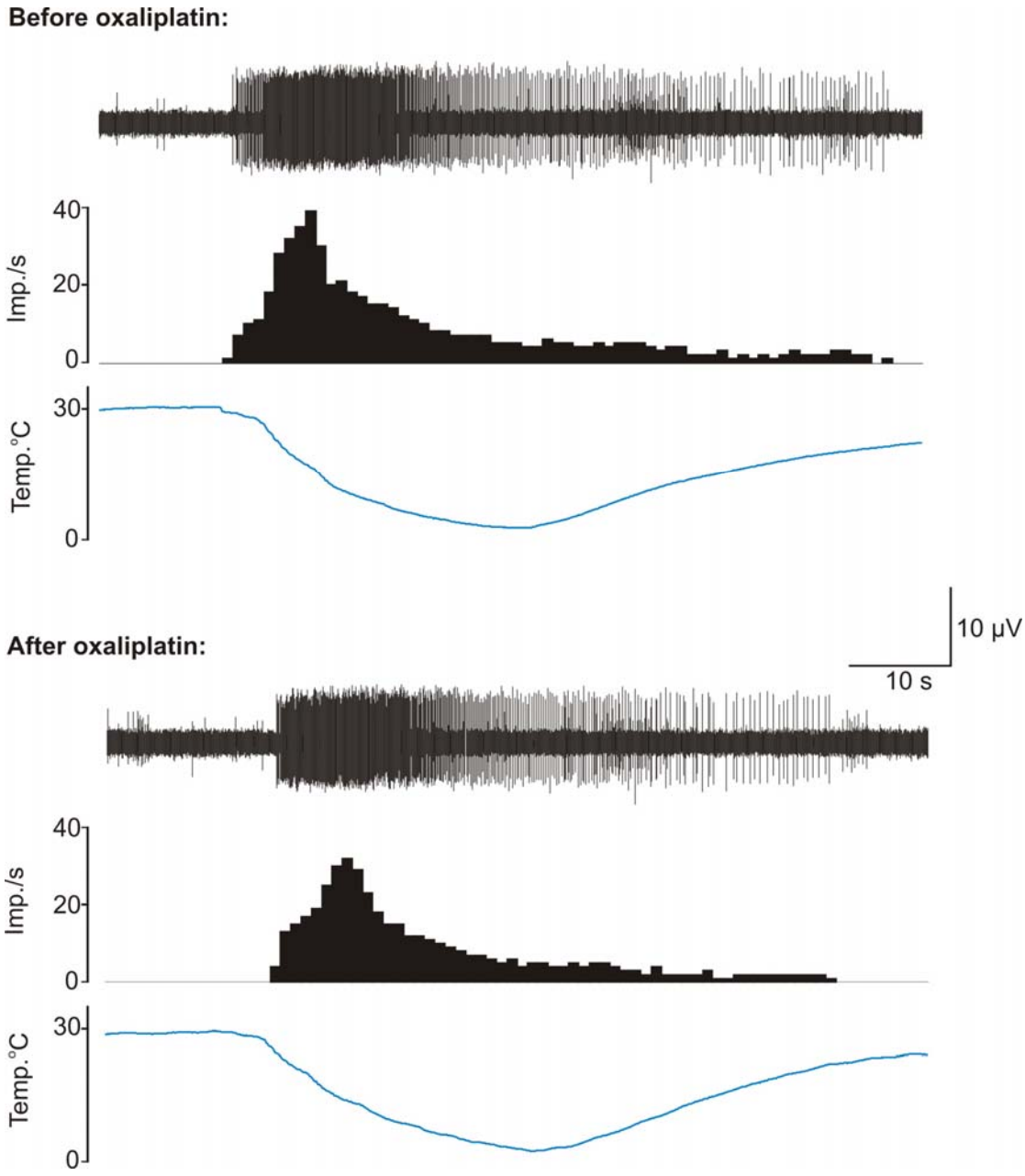

Fig.7.3.29. Example of a mechanically insensitive cold fibre (CC) responding to cooling before and after application of 400 µM oxaliplatin for 20 minutes

The fibre did not display any on going activity at bath temperatures. During cooling the fibre responded vigorously reaching a peak discharge rate of 38 impulses per second. Firing rate then quickly declined as the temperature got cooler. After application of oxaliplatin there appeared to be no significant change in the fibres response pattern to cold.

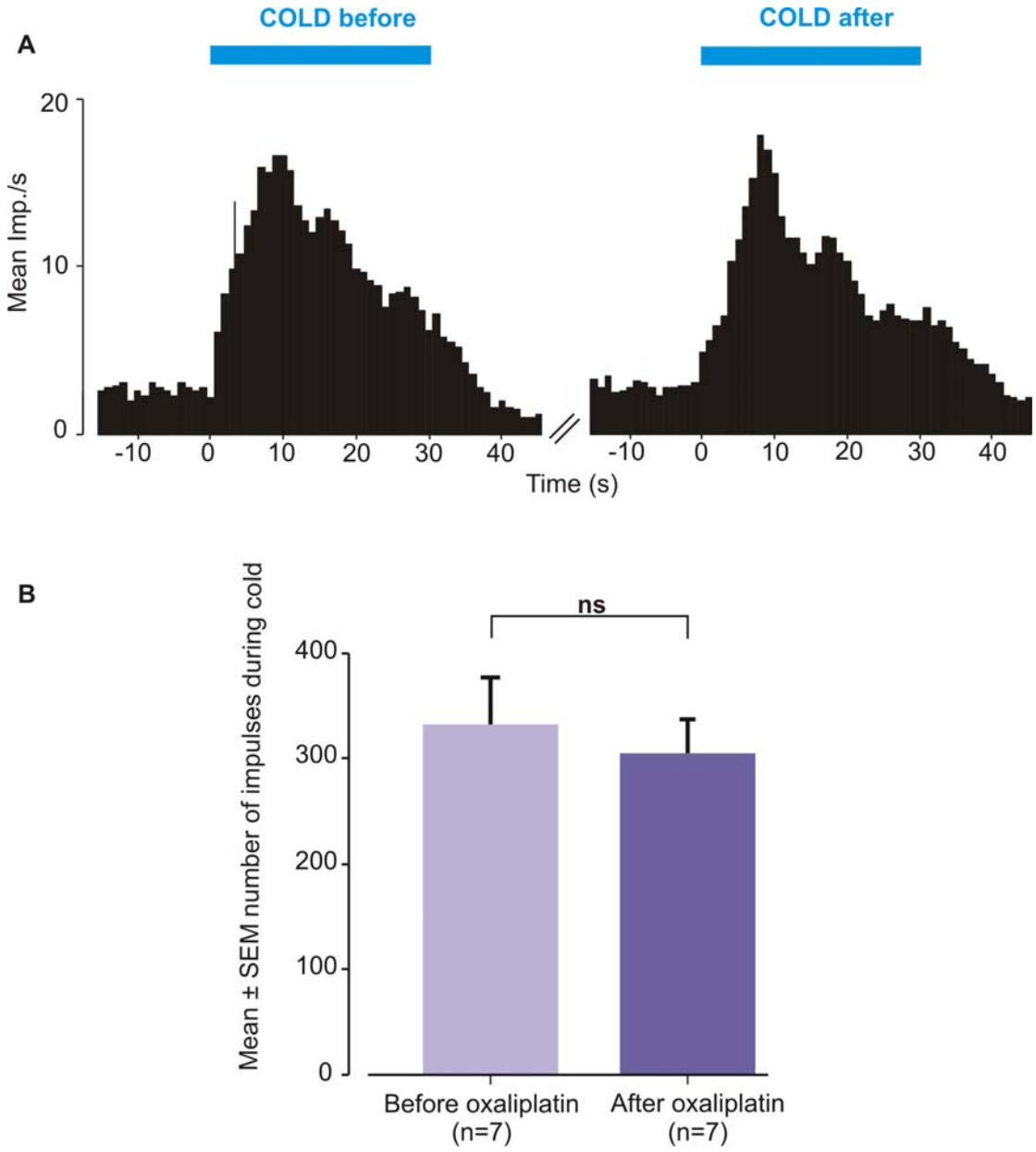

Fig.7.3.30. Mean response of mechanically insensitive cold fibres (CC) to a cold stimulus before and after oxaliplatin application
(A) Units were activated by a cold, firing at high frequencies during the ramp phase of the stimulus, followed by a lower level of discharge at colder temperatures. Subsequently there was no evident change in firing pattern to cold. (B) Although there was a slight decrease in the mean total number of impulses during cold after oxaliplatin application, this difference was statistically insignificant (p>0.4, Wilcoxon matched pairs test).

TABLE.7.3.4. Mean number of action potentials to a cold stimulus and cold thresholds in cold sensitive C fibres

Fibre Type	n	Number of spikes during cold stimulus before oxaliplatin	Cold threshold, ˚C	∆, ˚C	Number of spikes during cold stimulus after oxaliplatin	Cold threshold, ˚C after oxaliplatin	∆, ˚C
CMC/CMCH	7	31 ± 6 (15 - 55)	13.1 ±3.0 (4.0 - 24.9)	17.0 ± 3.0	21 ± 9 (0 - 73)	12.9 ±3.9 (4.0 - 31.2)	18.0 ± 3.8
CLTMC/CLTMCH	3	50 ± 14 (29 - 79)	21.5 ± 3.2 (15.6 - 26.5)	8.8 ± 3.0	87 ± 46 (32 - 180)	24.4 ± 2.4 (20.2 - 28.4)	6.3 ± 2.5
CC	7	333 ± 44 (160 - 479)	26.6 ± 2.3 (13.0 - 30.5)	4.0 ± 2.2	306 ± 31 (203 - 428)	23.3 ± 3.2 (6.0 – 29.0)	6.6 ± 3.2

All values are means ± SE. CMC, C mechano-cold nociceptor, CMHC, C mechano-heat-cold sensitive nociceptor; CLTM-C, C low-threshold mechanosensitive cold fibre; CLTM-CH, C low-threshold mechano-sensitive heat-cold fibre; CC, C mechano-insensitive cold fibre; Δ, relative change in temperature required to elicit a cold response from base line temperatures.

TABLE.7.3.5. Mean number of action potentials to a heat stimulus and heat thresholds in thin myelinated Aδ and C fibres

Fibre Type	n (before oxaliplatin)	Impulses during heat stimulus before oxaliplatin	Heat threshold, ˚C	n (after oxaliplatin)	Impulses during heat stimulus after oxaliplatin	Heat threshold, ˚C after oxaliplatin
AMH	2	22 ± 20 (2-42)	37.6 ± 5.2 (33.0-42.7)	1	12	38.8
CMH	5	30 ± 8 (9-54)	35.2 ± 0.2 (34.5-36)	3	12 ± 3 (5-18)	37.1 ± 1.9 (34.3-40.6)
CLTM-H	3	40 ± 8 (26-56)	38.3 ± 3.1	2	37 ± 16 (21-53)	36.2 ± 2.5 (33.7-38.6)

All values are means ± SE. AMH, high threshold mechano-heat sensitive A fibre; CMC, high threshold mechano-heat sensitive C fibre; CLTM-H, low threshold mechano-heat sensitive C fibre.

Oxaliplatin application to the whole nerve

In a different set of 3 experiments oxaliplatin was applied onto the trunk of the whole nerve (see Fig.7.2.1). Recordings were then carried out from fibres which were located in the periphery, away from the original oxaliplatin treated area.
These experiments were carried out to investigate potential sites of the nerve (axon or receptive field) that could be involved in the generation of oxaliplatin induced change of excitability.

In each experiment a multi-unit recording was carried out, in which 3 fibres were characterised. In total 7 RA, 1 SA and 1 AM fibre were recorded. All fibres were insensitive to cold and heat stimuli. 600 μM oxaliplatin was then applied onto the whole nerve for 30 minutes. A cold stimulus was then directly applied onto the whole nerve, where the oxaliplatin had been applied to see if any activity could be evoked from the units in the periphery. All the receptive fields in the periphery were then individually re-tested for their response to a cold stimulus. None of the units recorded from the periphery were initially cold sensitive. These fibres never became spontaneously active after a cold stimulus was applied onto the oxaliplatin treated area of the whole nerve. When a cold stimulus was applied directly onto their receptive fields, they remained unresponsive.
600 μM oxaliplatin was then re-applied onto the individual units in the periphery for 30 minutes. A cold stimulus was then re-applied directly onto the fibres and subsequently, 4 out of 9 (44 %) of fibres (3 RA and 1 AM unit) displayed a novel cold response. An example of one such multi-unit recording is shown in Fig.7.3.31.

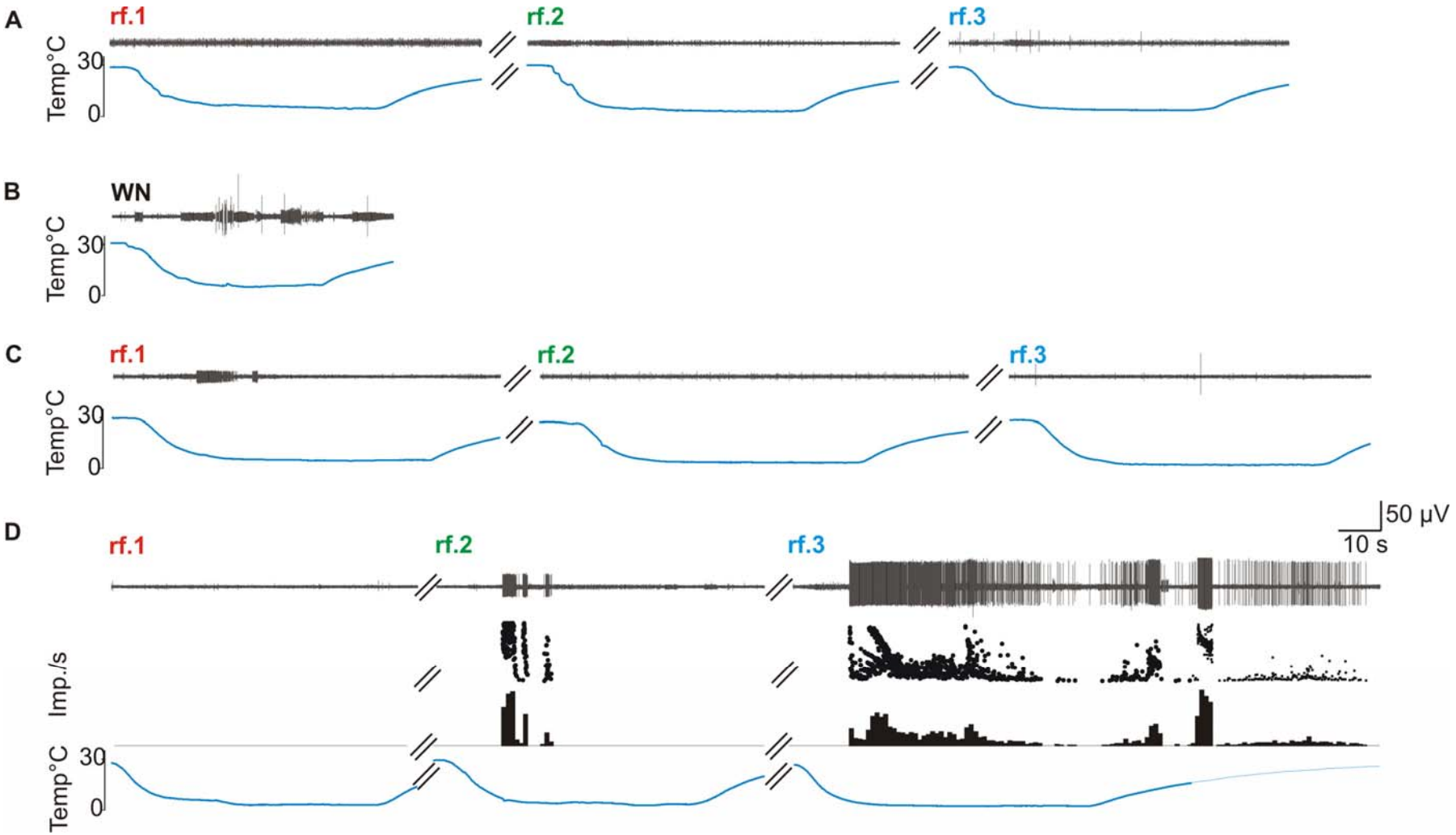
A
rf.1
rf.2
rf.3
Temp°C
30
0
B
WN
Temp°C
30
0
C
rf.1
rf.2
rf.3
Temp°C
30
0
D
50 µV
10 s
rf.1
rf.2
rf.3
Imp./s
Temp°C
30
0

Fig.7.3.31. Example of an experiment in which oxaliplatin (600 µM, 30 minutes) was applied onto the whole nerve trunk

(A) A cold stimulus was applied onto the receptive fields (rf) of 3 characterised units in the periphery (rf.1 and 3 were of RA fibres and rf.2 was of an AM fibre). None were initially cold sensitive. (B) Oxaliplatin was then applied to the whole nerve trunk. A cold stimulus was then applied directly to the whole nerve (WN). No activity was evoked from the periphery. (C) The fibres were then re-tested with a cold stimulus. All 3 units remained unresponsive. (D) Oxaliplatin was then applied directly to the receptive fields of the fibres. Subsequently cold stimuli were delivered as before, and an AM and RA unit displayed a novel cold response, firing in bursts. The RA unit also displayed a novel re-warming response.

7.4 Discussion

Until now, direct recordings from oxaliplatin treated primary afferent neurons innervating the rat skin in vitro have not been carried out.
This study shows for the first time, that oxaliplatin applied directly on the receptive fields induces a novel cold sensitivity in half of previously cold insensitive Aβ mechanoreceptors. Just over a third of Aδ nociceptors also displayed a novel or increased sensitivity to cold after oxaliplatin application. In contrast, receptive properties of C fibres remained unchanged. Responses to noxious heat also remained unchanged. An increase in the response to mechanical stimuli in a proportion of Aβ mechanoreceptors was found. In contrast there was no change in response to mechanical stimulation in Aδ and C fibres.
Application of oxaliplatin onto the whole nerve did not induce changes in receptive properties of fibres recorded from the periphery.

Almost half of the low threshold mechanically sensitive A fibres became cold responsive after oxaliplatin treatment

Thermal responses were absent among the majority of large myelinated Aβ afferents, with the exception of two slowly adapting (SA) fibres which responded during a cold stimulus. Thermal responses were also absent among D-hair (DH) receptors.

Just under half of the Aβ mechanoreceptors displayed a novel cold response after acute oxaliplatin application. This is the first time that the effects of cold temperature on oxaliplatin treated Aβ mechanoreceptors in the rat have been demonstrated in vitro. 1 out of 3 DH units also displayed a novel cold and re-warming response after oxaliplatin application.

Bath application of 250 μM oxaliplatin has been shown to increase the A fibre compound action potential amplitude of rat sural and vagal nerves in an isolated nerve preparation (Adelsberger et al., 2000). In the present study a

proportion of A fibres were also found to be sensitive to oxaliplatin, therefore consistent with the findings above.
High frequency busting discharges to cold and mechanical stimuli were common among Aβ units sensitive to oxaliplatin. Repetitive discharges after oxaliplatin have been reported previously. Using the mouse phrenic nerve hemi-diaphragm preparation, Webster et al (2000) reported the occurrence of abnormal trains of potentials which were evoked by a single stimulus in the presence of 500 μM oxaliplatin. In another investigation, sensory and motor nerve conduction studies on the median nerve were carried out in a group of patients receiving oxaliplatin (Besser et al., 2007). After oxaliplatin, single motor nerve stimuli induced repetitive muscle action potentials in 15 out of 20 patients. EMG of the thenar eminence revealed neuromyotonic discharges in 16 patients. No signs of hyperexcitability were observed before oxaliplatin. Similar findings have been reported in phase I trials (Lehky et al., 2004;Wilson et al., 2002). Within 24-48 hours after the first cycle of oxaliplatin infusion neuromyotonic discharges were observed in the muscles of patients.

Adelsberger et al (2000) describe the effects of oxaliplatin as being ‘delayed’ since no difference in the compound action potentials were observed at least 5-10 minutes after the application. Our findings support this, since no changes in receptive properties were observed in some preliminary recordings where an application time of 5 minutes was used. Therefore subsequently a minimum application time of 10 minutes was used. One possible explanation for this is this is a prolonged diffusion time of the drug through the connective tissue.

Development of a novel cold sensitivity in Aβ fibres which are not normally cold sensitive may account for the cold induced paraesthesias experienced by patients. Ling et al demonstrated the development of cold hypersensitivity in rats which were administered a single does of oxaliplatin (Ling et al., 2007). A significant reduction in tail withdrawal latency to cold stimuli was observed starting at 24 hours post injection. This finding was interpreted as a change in cold pain thresholds. However, our data have shown that oxaliplatin can induce a novel cold sensitivity in thermally insensitive Aβ mechanoreceptors,

suggesting that the cold hypersensitivity observed by Ling et al (2007) may also be attributed to the abnormal cold responses in these fibres.

An exciting finding in the present study is that cold responses in initially cold sensitive SA fibres were not enhanced by oxaliplatin. This may suggest that oxaliplatin does not affect the molecular mechanisms underlying this cold sensitivity.
Another observation is that only half of the RA and SA fibres became cold sensitive after oxaliplatin treatment. This suggests that maybe electrophysiological properties of SA and RA fibres are not homogeneous. Within each subpopulation, there may be differential expression of ion channels which result in only half of the fibres being sensitive to oxaliplatin.
Interestingly, there was no difference in the cold thresholds between RA and SA units. This could therefore represent a homogeneous population of fibres that express the same oxaliplatin sensitive cellular mechanism.

Some rapidly adapting, Aβ mechanoreceptors displayed a change in mechanical adaptation property after oxaliplatin treatment, but Aδ and C fibres displayed no changes

Almost half (41.7 %) of RA units which were re-tested with a mechanical constant force stimulus post oxaliplatin exposure displayed changes in their mechanical adaptation properties and began to discharge throughout the stimulation. There was also an increase in firing frequency, some fibres responding in bursts of action potentials.
In rats administered a single dose of oxaliplatin, Ling et al (2007) demonstrated the development of a mechanical hypersensitivity (Ling et al., 2007). A significant reduction in the paw withdrawal threshold using von Frey hairs was found. Based on their results the authors suggested that the mechanical hypersensitivity could involve small myelinated A fibres.
In the present study, there was no significant change in the mean firing pattern or rate of discharge in Aδ or C fibres to mechanical stimulation after oxaliplatin. However, mechanical von Frey hair thresholds were not re-tested, therefore a direct comparison between the two studies is difficult. Our results demonstrate

that a proportion of Aβ fibres clearly became mechanically hypersensitive and this could account for the mechanical hypersensitivity observed in the study by Ling et al (2007).

Over a third of Aδ nociceptors displayed either a novel or increased response to cold after oxaliplatin application

Thin myelinated Aδ nociceptors are thought to evoke a sharp, pricking pain in humans. These fibres have been shown to signal the first pain sensation evoked by a noxious heat and mechanical stimulation in humans, based on the reaction time to the first pain sensation. Blockade of these fibres has also been shown to reduce pain sensations (Meyer et al., 2005).

Painful or unpleasant sensations known as dysaesthesias induced by oxaliplatin in patients have been reported in many investigations (Binder et al., 2007;Cersosimo, 2005;Leonard et al., 2005).
Binder et al (2007) demonstrated the development of cold and mechanical hyperalgesia in patients who experienced pain post oxaliplatin infusion, by demonstrating a decrease in the cold and mechanical pain thresholds. Patients described the pain as ‘tingling’, ‘cold’, ‘freezing’, ‘pricking’, ‘heavy’ and ‘piercing’ (Binder et al., 2007). The authors concluded that the development of the neuropathy was due to the sensitisation of the nociceptive system, but could not speculate further on which fibre types were involved.
In the present study we have shown that novel cold sensitivity was induced in 3/16 high threshold mechanosensitive Aδ fibres. An increased response to cold was observed in all 3 high threshold mechano-cold sensitive Aδ fibres after oxaliplatin. These findings provide evidence for the involvement of Aδ nociceptors in oxaliplatin induced acute neuropathy. Since these fibres are known to normally elicit painful sensations, hypersensitivity to cold could account for the cold induced pain experienced by patients.
Interestingly, we found that although there was a significant increase in the cold response of AMC fibres after oxaliplatin, the cold thresholds did not differ significantly. Oxaliplatin may be acting by enhancing the pre-existing cold mechanism so that a stimulus of the same strength would result in an increased

response, but without changing the mechanism required for setting the threshold temperature required for channel activation. Alternatively oxaliplatin could be acting independently of the existing cold sensory mechanism, so that during a cold stimulus, both the pre-existing and the new cold mechanism are activated. But since there is no change in the cold threshold, this would imply that the new mechanism is not important in setting the threshold temperature. Only a proportion of Aδ nociceptors displayed sensitivity to oxaliplatin application. This suggests that like Aβ fibres, electrophysiological differences exist within Aδ nociceptors resulting in differential sensitivity to oxaliplatin.

C fibres did not display changes in cold sensitivity after oxaliplatin treatment

On the whole, there were no noticeable changes in the cold sensitivity of C fibres after acute oxaliplatin application. The effects of acute oxaliplatin induced neuropathy have been attributed to changes in C fibres (Mantyh, 2006) but our results would argue against this.

We found that C fibres which were not initially cold sensitive (CM, CMH and CLTM-H fibres) never developed a novel cold response. This is in contrast to the findings in Aβ and Aδ fibres. Bursting discharges never developed in any of the C fibres after oxaliplatin, unlike those observed in A fibres. It appears therefore that factors that are differentially expressed in small and large DRG neurons could be important in determining the ability of large DRG neurons to become sensitive to oxaliplatin. Our findings are consistent with Adelsberger et al who observed a very small difference in the amplitude of C fibre compound action potential after oxaliplatin compared to the larger effect observed in the A fibre compound action potential.

C fibres which were cold sensitive (CMC, CLTM-C, and CC) never displayed an increased response to cold after oxaliplatin. Interestingly, this would suggest differences in the cold sensory mechanisms between AMC and CMC fibres, since both groups of nociceptors responded differently to cold after oxaliplatin. An increase in the cold threshold of cold thermoreceptors (CC fibres) was observed after oxaliplatin. It is unclear whether this is a specific effect of oxaliplatin or a desensitisation to cold. Since there was no effect on the actual

response during a cold stimulus after oxaliplatin, it seems unlikely that the change in the cold threshold was due to oxaliplatin.

Molecular and cellular mechanisms underlying the effects of oxaliplatin: Possible involvement of sodium channels

Since axonal hyperexcitability and repetitive discharges are common features in oxaliplatin induced neuropathy, many studies have suggested that it could be acting directly on ion channels such as sodium and potassium channels. An increase in sodium channel activity or decrease in potassium channel activity could be a mechanism for generating neuronal hyperexcitability and repetitive discharges.

Studies by Adelsberger et al (2000) and Webster et al (2005) suggest that sodium and not potassium channels are involved in oxaliplatin induced neuropathy.
Adelsberger and colleagues also demonstrated that oxaliplatin caused an increase in the refractory period of the nerve. They found that the most prominent effect of oxaliplatin was to slow down the inactivation kinetics of sodium currents in a large proportion of DRG cells. A shift in the activation threshold to more hyperpolarising potentials was also shown in these cells. Several clinical studies have shown an increase in the duration of the relative refractory period (RRP) of both motor and sensory nerves in patients post oxaliplatin infusion (Kiernan and Krishnan, 2006;Krishnan et al., 2005;Krishnan et al., 2006). These results therefore suggest that oxaliplatin induced neuropathy is mediated via voltage gated Na^+ channels.

In the present study, acute oxaliplatin application resulted in changes to the receptive properties of a proportion of Aβ and Aδ fibres, but never in C fibres. This suggests that oxaliplatin is acting on a sub-type of sodium channel that is expressed primarily on myelinated A fibres and not on unmyelinated C fibres. Large sized DRG neurons (diameter >45 μm) have been shown to express high levels of the sodium channel subtype $Na_v1.1$. Both large and medium sized (25-45 μm) neurons have been shown to express $Na_v1.6$ and $Na_v1.7$ (Black et

al., 1996). $Na_v1.6$ has been found to be highly expressed at the nodes of Ranvier in peripheral myelinated axons (Caldwell et al., 2000). Oxaliplatin could therefore be directly interacting with sodium channels $Na_v1.1$, $Na_v1.6$ and $Na_v1.7$ present on the peripheral neurons. All of these sodium channels are TTX-sensitive. Small diameter neurons have been shown to express high levels of the TTX-R channels, $Na_v1.8$ and $Na_v1.9$ (Akopian et al., 1996;Black et al., 1996;Dib-Hajj et al., 1998;Sangameswaran et al., 1996). A lack of oxaliplatin sensitivity among C fibres in our study would therefore indicate that $Na_v1.8$ and $Na_v1.9$ are insensitive or at least not as sensitive to oxaliplatin compared to the above mentioned TTX-S sodium channels. This would be consistent with the findings by Krishnan et al (2005) who suggested that the effects of oxaliplatin are mediated via transient and not persistent sodium conductances. It would also be in agreement with Adelsberger et al (2000) who demonstrated that the repetitive firing evoked by oxaliplatin was abolished on pre-treating the nerve with TTX.

Properties of sodium currents in large and medium DRG neurons support their role in oxaliplatin induced neuropathy

Everill et al (2001) demonstrated that large (48-50 μm diameter) neurons from the adult rat DRG, which presumably give rise to myelinated fibres, expressed two different types of sodium currents. 71 % of neurons were found to express both the fast inactivating TTX-S and slowly inactivating TTX-R current (Everill et al., 2001). Interestingly, 29 % of large neurons expressed only the fast inactivating TTX-S current. In addition they showed that the TTX-S current in these large neurons displayed a faster recovery from inactivation than the TTX-S current in smaller (<30 μm diameter) neurons, presumably C fibres. Aβ neurons are therefore expected to sustain higher frequency firing compared to C fibres because they are able to recover from inactivation faster. Similar findings were also reported by Renganathan et al (2001). They found that 52 % of large (>40 μm diameter) cutaneous afferents from mice DRG expressed only the fast inactivating, TTX-S sodium currents, while 48 % expressed both fast and slow. They further went on to show that the slow inactivating, TTX-R sodium currents could not be detected in large fibres in mice lacking $Na_v1.8$ (Renganathan et al., 2000).

40 % of large (35-50 μm diameter) DRG neurons have been shown to exhibit a TTX-S 'resurgent' current (Cummins et al., 2005). Resurgent currents have been described as an unusual type of sodium current. Upon depolarisation, the current activates and inactivates normally, but during repolarisation, a surge of sodium current reactivates. This is unusual since normal sodium channels are expected to remain closed at this time as they recover from inactivation. This novel current is also correlated with rapid recovery from inactivation, both events promote the firing of a second action potential (Bean, 2005). Cummins et al (2005) found that resurgent currents are mediated by $Na_v1.6$ as these currents were not observed in DRG neurons from mice lacking $Na_v1.6$.

The above differences in the expression of various types of sodium currents in A fibres may explain why only approximately half of the A fibres (large and medium sized) became cold sensitive after oxaliplatin. If this is the case, then those fibres which express higher levels of TTX-S sodium currents (approximately half of large DRG neurons) in combination with resurgent currents would be more likely to be involved in oxaliplatin induced neuropathy, since these fibres would already be able to sustain a higher frequency firing.

Neurotoxins which effect sodium channel inactivation may provide an insight into the mechanisms involved in oxaliplatin induced neuropathy

Slowing of sodium channel inactivation has been suggested as a mechanism by which oxaliplatin may mediate its effect. The results in the present study are limited and cannot provide details into the underling molecular mechanisms involved. However, examining the actions of neurotoxins which have been shown to affect the inactivation of sodium channels may provide a further insight into the possible mechanisms by which oxaliplatin may function.

The action of robustoxin, purified from the venom of the male Sydney-funnel web spider, was investigated on TTX-S and TTX-R sodium currents in rat DRG neurons (Nicholson et al., 1998). Robustoxin was found to affect only TTX-S and not TTX-R sodium currents. The main action of robustoxin was a

concentration dependent slowing of TTX-S sodium current inactivation. Addition of TTX completely blocked the persistent current, indicating that the effect was mediated via TTX-S sodium channels. The toxin also significantly increased the rate at which channels recovered from inactivation, an action which would promote repetitive action potential firing.
Similar findings were discovered with α-pompilidotoxin (α-PMTX), which is isolated from the venom of the solitary wasp (Sahara et al., 2000). α-PMTX slowed down the inactivation process of TTX-S but not of TTX-R sodium channels in rat trigeminal neurons. Both robustoxin and α-PMTX bind to neurotoxin site 3 on sodium channels. Oxaliplatin may also be acting at the neurotoxin receptor site 3, or a similar site on the sodium channel to exert similar actions as the toxins mentioned above.

A striking finding is that the toxins mentioned above seem to have no effect on TTX-R sodium channels. Our results also suggest the same for oxaliplatin. This suggests that TTX-R sodium channels must be structurally different to TTX-S sodium channels, making them insensitive to these toxins and possibly also to oxaliplatin. Indeed this does appear to be the case.
Saab et al demonstrated the ability of the α-scorpion Leiurus quinquestriatus toxin (LQTX) to slow down the inactivation kinetics of the TTX-S sodium channel $Na_v1.4$, but not of the TTX-R sodium channel $Na_v1.8$ (Saab et al., 2002). This toxin has been previously shown to bind to the S3-S4 linker of domain four (D4S3-S4). Sequence analysis has revealed that the D4S3-S4 linker is longer in $Na_v1.8$ than in $Na_v1.4$ by four amino acids: Serine, Leucine, Glutamic acid, and Aspargine, a sequence named as ‘SLEN’. The authors hypothesised that this structural difference made $Na_v1.8$ resistant to LQTX. They further went on to show that this was the case by producing $Na_v1.4$-SLEN ($Na_v1.4$ construct carrying the SLEN sequence at the corresponding position in the D4S3-S4 linker), which was found to be resistant to LQTX.
Interestingly, the linker joining the S3 and S4 in domain 4 is also reported to be longer in the TTX-R channel $Na_v1.9$ (Dib-Hajj et al., 1998). The extra length of the linker region in both $Na_v1.8$ and $Na_v1.9$ may be responsible for making these TTX-R sodium channels resistant to the actions of certain toxins and possibly also to oxaliplatin.

The D4S4 voltage sensor is thought to play an important role in coupling activation to inactivation, and it has been proposed that movement of D4S4 facilitates the binding of the inactivation gate. Toxins which act on TTX-S sodium channels, may be binding to the D4S3-S4 linker, trapping the D4S4 voltage sensor is a specific conformation and thereby preventing the formation of the normal biding site for the inactivation gate. Oxaliplatin may also be acting in a similar fashion. However in TTX-R sodium channels, the SLEN sequence may prevent the toxin or oxaliplatin from binding to the D4S3-S4 linker and therefore allow the channel to inactivate normally in the presence of the toxin/oxaliplatin. Or binding of the toxin/oxaliplatin may occur, but the SLEN sequence may then interfere with the trapping of the D4S4 voltage sensor, allowing normal inactivation to occur.

Ciguatera is a form of marine poisoning caused by the ingestion of ciguatoxins that accumulate in certain tropical fish (Isbister and Kiernan, 2005). Ciguatoxins are lipophilic and act at the neurotoxin receptor site 5 on sodium channels (Pearn, 2001). Symptoms of acute ciguatera poisoning include paraesthesia in the extremities and dysesthesias in the tongue and throat in over 90 % of cases (Chateau-Degat et al., 2007;Pearn, 2001;Schnorf et al., 2002). Furthermore, hypersensitivity to cold, commonly reported as a cold induced painful burning sensation, is reported by 95 % of patients (Schnorf et al., 2002). One study also reported that at the onset of the poisoning, patients demonstrated a higher sensitivity to light touch in the hands compared to control subjects (Chateau-Degat et al., 2007).
Ciguatoxin-1 has been shown to cause mammalian TTX-S sodium channels to open closer to the cells resting membrane potential and TTX-R sodium channels to recover from inactivation more quickly (Strachan et al., 1999). Although ciguatera toxin appears to affect both TTX-S and TT-R sodium channels, the similarity in acute sensory symptoms induced by ciguatera poisoning and oxaliplatin indicates that oxaliplatin may also be acting via a neurotoxin receptor site on the sodium channel.

Mutations in the skeletal muscle sodium channel gene SCN4A, provides a further understanding of the possible mechanisms involved in cold induced oxaliplatin neuropathy

A slowing of sodium channel inactivation kinetics has been implicated in muscle disorders associated with sodium channel mutations. Mis-sense mutations of the skeletal muscle specific isoform $Na_v1.4$ have been established as a cause of several forms of myotonia, a neuromuscular disorder which results in muscle stiffness and/or weakness. One form of myotonia known as paramyotonia congenita (PC) is characterized by myotonia that appears during exercise (called paradoxical myotonia) and interestingly is exacerbated by exposure to cold, a feature also prominent in oxaliplatin induced neuropathy. The molecular mechanisms involved in PC may therefore be able to provide some indication of the possible mechanisms involved in oxaliplatin induced neuropathy, especially cold induced neuropathy.

Mohamaddi et al (2003) compared two sodium channel mutants R1448H and M1360V to wild type channels, expressed in HEK cells, at different temperatures (15, 25 and 35 °C). The R144H8 mutant is located in the S4 segment of domain IV (D4S4). The M1360V mutation is located in the segment S1 of domain IV (D4S1). Both mutations resulted in slower inactivation and faster recovery from inactivation compared to WT channels at all temperatures tested (Mohammadi et al., 2003). In addition they found that there was a shift of the steady-state activation curve to hyperpolarized potentials at lower temperatures for the mutant R1448H channel but not the mutant M1360V channel.

Bouhours et al reported of a novel mutation, T1313A, expressed on the DIII-DIV linker. They expressed the mutant in HEK cells and found that it slowed down fast inactivation and accelerated recovery (Bouhours et al., 2004). Channel inactivation slowed down even further at 11°C compared to 21°C. This suggested that cold induced myotonia resulted from the combined effect of the mutation and cooling.

The effect of cooling in oxaliplatin induced neuropathy may result from a similar mechanism, whereby oxaliplatin slows down channel inactivation and during cold inactivation slows down even further, which results in the generation of a persistent sodium current, resulting in action potentials. During cold, a shift in the activation curve to more hyperpolarising potentials would lower the activation threshold of the neurons, making them more excitable. Adelsberger and colleagues noticed both these changes in DRG sodium currents after oxaliplatin, a slowing of inactivation and a shift in the activation threshold to more hyperpolarising potentials. Slowing of inactivation kinetics was noted to be the most prominent finding at room temperature. At colder temperatures the shift in the activation threshold may be even greater. Repeating the experiment at different temperatures will confirm whether this is the case.

Oxaliplatin may not be affecting the cold sensitivity of the fibre itself, but cold temperatures may act by exacerbating the effect of oxaliplatin. This may explain why novel heat responses were never observed after oxaliplatin. Noxious heat may act by opposing the action of oxaliplatin, speeding up the rate at which inactivation occurs, thereby inhibiting the generation of persistent sodium current.

Our data would support the hypothesis that oxaliplatin does not affect the cold sensitive mechanisms of the neuron, primarily because of the differential sensitivity observed between AMC and CMC fibres. Cold responses were significantly enhanced after oxaliplatin in all the AMC fibres, but no such change was observed in the CMC and CC fibres. Results from chapter 4 suggest that all of these fibre types express the cold sensitive TRPM8 channel, which mediates their cold sensitivity. If oxaliplatin was acting through the TRPM8 receptor, enhancement in cold responses from AMC, CMC and CC fibres would be expected, but this was not found. It therefore suggests that oxaliplatin is acting independently of TRPM8, via a different ion channel or mechanism which is primarily expressed in A rather than C fibres. TTX-S sodium channels appear to be a strong candidate.

The importance of the TTX-R, $Na_v1.8$ in the transmission of cold has already been demonstrated. Cooling was shown to enhance the slow inactivation of TTX-S voltage gated sodium channels (Zimmermann et al., 2007). In contrast the inactivation properties of $Na_v1.8$ were found to be entirely cold resistant. They further went on to show that mice lacking $Na_v1.8$ displayed negligible responses to noxious cold and mechanical stimulation during cold, indicating that $Na_v1.8$ is required for the transmission of noxious cold and other painful stimuli during cold. Therefore, although $Na_v1.8$ may not act as a primary cold sensor itself, it is required for the transmission of signals during cold and noxious cold temperatures in unmyelinated fibres. This indicates the importance of sodium channels and how their differing properties can play an essential role in determining the transduction of a particular sensory modality.

Stronger activation by cold was required to induce a novel cold response in AM fibres compared to AMC fibres. Since AMC fibres express the TRPM8 receptor, during a cold stimulus, TRPM8 becomes activated which then possibly results in activation of sodium channels, including those which are affected by oxaliplatin. Since AM fibres do not have an existing cold sensory mechanism (such as TRPM8), stronger cooling may be required to bring them to firing threshold.

In Aβ and Aδ (DH and AM) fibres which became cold responsive after oxaliplatin, cold sensitivity was not always induced during the first cold stimulus post oxaliplatin. Mechanical probing of the receptive field with a glass rod was then carried out to ensure that the fibre was still responsive. It was observed, that a cold stimulus subsequent to this would nearly always result in a novel cold response. This suggests that these fibres required activation, before the effect of oxaliplatin could be observed.

Direct activation of the fibre during oxaliplatin application was never observed. This suggests that oxaliplatin was not acting as a direct channel opener. This was also observed by Webster et al (2005), who found that during the incubation period, no effect of oxaliplatin was observed, but a single stimulus after resulted in a huge production of miniature end plate potentials. This

indicated that the effects of oxaliplatin were not seen until nerve stimulation occurred, a finding similar to ours.

However, in the present study, some fibres did not require prior mechanical stimulation to induce a cold response. In these fibres it is likely that oxaliplatin had lowered the activation threshold, so that fibres were closer to their firing thresholds. In addition to this, cooling has been shown to inhibit a potassium current which is active at the resting membrane potential in all DRG cells (Reid and Flonta, 2001). Therefore during a cold stimulus, these fibres would have reached their firing thresholds.

Application of oxaliplatin directly onto the whole nerve did not result in changes in receptive properties of fibres in the periphery. There are two possible explanations for this observation.
Firstly, it may suggest that the whole nerve does not express the oxaliplatin sensitive mechanisms required to induce an effect in the periphery. An alternative explanation could be that oxaliplatin was unable to diffuse through the connective tissue around the whole nerve and therefore no effects in the periphery were seen.

Conclusions

This is the first study which has demonstrated that oxaliplatin applied directly on the receptive fields induces a novel cold sensitivity in a proportion of previously cold insensitive Aβ mechanoreceptors and both low and high threshold Aδ fibres. An increase in mechanical sensitivity was also observed a in proportion of Aβ mechanoreceptors. Interestingly, the receptive properties of C fibres remained largely unchanged after application of oxaliplatin. A summary diagram representing the proportion of fibres which displayed a novel or increased sensitivity to cold after application of oxaliplatin to their receptive fields is shown in Fig.7.4.1 below.

Although the present data do not provide any direct evidence for the mechanisms involved in oxaliplatin induced neuropathy, the fact that only the

receptive properties of myelinated fibres were affected suggests that oxaliplatin may be affecting sodium channels specifically expressed in myelinated neurons.

To test for isoform specific effects of oxaliplatin, the different isoforms of sodium channels could be expressed into a heterologous expression system and the kinetics of the currents could be tested after superfusion of oxaliplatin. The effects of oxaliplatin could be investigated using the skin-nerve preparation from mice lacking TTX-S sodium channel isoforms.

Patch clamp recordings from DRG cells of both large and small diameter neurons could be carried out, as described by Adelsberger et al (2000) in combination with application of cold stimuli.

Finally, carrying out quantitative sensory testing in patients treated with oxaliplatin will give a further insight into which sensory modalities are affected by oxaliplatin. Mechanical, heat and cold perception should be tested to assess the role of specific fibre types within as well as outside areas of sensory disturbance in patients with and without pain following both acute and chronic oxaliplatin treatment.

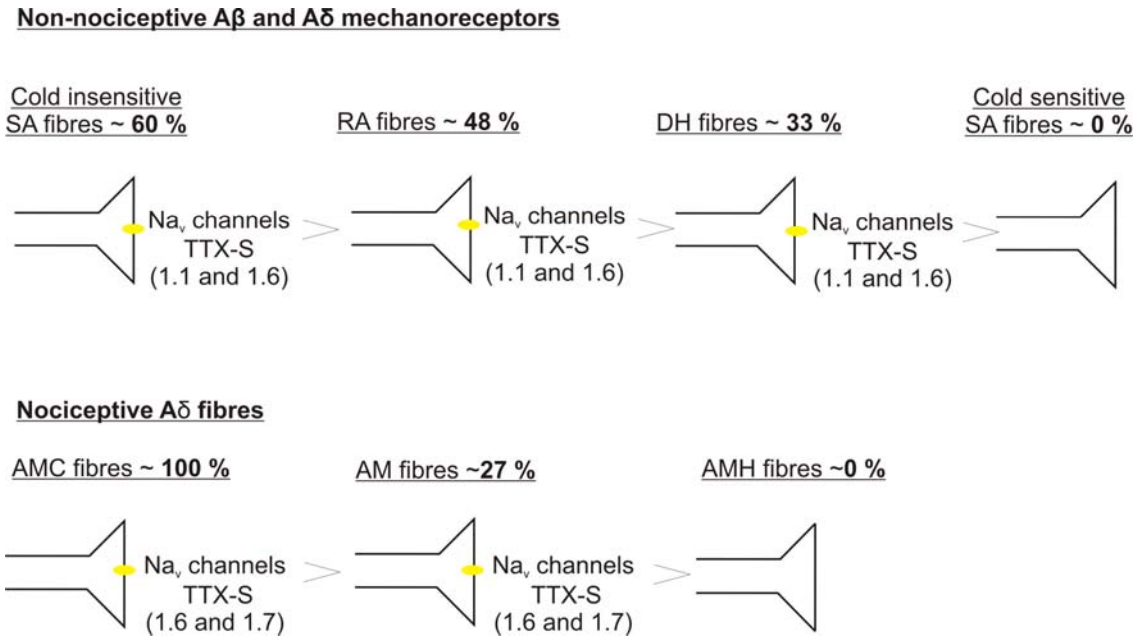

Fig.7.4.1 Schematic representation of sodium channel subunit expression on the peripheral terminals of fibres proposed to be involved in mediating oxaliplatin induced cold sensitivity

The proportion of fibres which displayed either a novel or increased cold sensitivity after application of oxalipltin to their receptive fields is shown as a percentage (%) next to the name of each fibre type. Within each fibre group, the sub-population of fibres which displayed the highest sensitivity to oxaliplatin is shown first and those which displayed the least sensitivity are shown last. For example, within the non-nociceptive Aβ and Aδ mechanoreceptors, 60 % of cold insensitive SA fibres displayed a novel cold response after application of oxaliplatin, compared to 0 % of cold sensitive SA fibres. Therefore cold insensitive SA fibres are shown first and cold sensitive SA fibres are shown last.

Chapter 8

8. General Discussion

In this thesis, single unit recordings using the in vitro skin nerve preparation were carried out to investigate the mechanisms involved in the transduction of thermal stimuli in primary afferent sensory neurons innervating the hind leg in rodents. The studies focused particularly on cold sensitivity. The results of these investigations have provided a novel insight into the functional expression pattern of TRP channels on the receptive nerve endings of fibres found in this preparation. Important data have also emerged regarding the effects of oxaliplatin on sensory nerve fibres. These results may explain for the first time the cause of the acute sensory neuropathy experienced by over 90 % of patients, where symptoms are aggravated by cold temperatures. Studies have also revealed an important role of voltage gated potassium channels in mediating cold sensitivity in sensory neurons. Finally, for the first time, electrophysiological analysis has been carried out on mice lacking the heat activated TRPV2 receptor. There has been extensive publication of findings from animals lacking TRPV1 (Caterina et al., 2000b) TRPV3 (Moqrich et al., 2005), TRPV4 (Chung et al., 2004;Mizuno et al., 2003;Suzuki et al., 2003), TRPM8 (Bautista et al., 2007;Colburn et al., 2007;Dhaka et al., 2007) and TRPA1 (Bautista et al., 2006;Kwan et al., 2006;McNamara et al., 2007). This is therefore one of the last of the knockout animals of a TRP channel which has been strongly implicated in nociception and therefore these data have been eagerly awaited for.

8.1 Summary of findings

A summary of all the results in this thesis is provided below in Tables 8.1 and 8.2. Fig.8.1 is a summary diagram, illustrating the expression pattern of various ion channels involved in mediating or regulating sensitivity to cold and heat on the receptive endings of primary afferent neurons. The summary tables and diagram will be referred to in the following text.

Table.8.1 Overall results for non-nociceptive fibres

Fibre type	Fibre sub-type	Cold sensitive (%)	Perceived sensation upon cooling	TRPM8 expression: menthol sensitivity (%)	TRPA1 expression: MO sensitivity (%)	TRPV1 expression: CAP sensitivity (%)	Increase/novel cold sensitivity after oxaliplatin (%)	Increase/novel cold sensitivity after K_v channel blockade (%)
Aβ: mechanoreceptors	RA	✘	-	✘	✘	✘	✓ **(47)**	✓ **(40)**
	SA	✘	-	✘	✘	✘	✓ **(60)**	✓ **(88)**
	SA (c)	✓ **(20)**	unknown	✘	✘	✘	✘	✓ **(75)**
Aδ: low threshold mechanoreceptor	DH	✘	-	✘	✘	✘	✓ **(33)**	✓ **(25)**
C: non-nociceptors	CLTM	✘	-	✘	✘	✘	✘	✘
	CLTM-C	✓ **(50)**	unknown	✘	✘	✘	✘	✓ **(33)**
	CC	✓	non-painful cool	✓ **(67)**	✘	✘	✘	✘

RA, rapidly adapting afferent fibres; SA, slowly adapting afferent fibres; SA (c), cold sensitive slowly adapting afferent fibres; DH, D-hair receptors; CLTM, C low-threshold mechano-sensitive fibres; CLTM-C, C low-threshold mechano-cold sensitive fibres; CC, C mechano-insensitive cold fibres.
The percentage of cold sensitivity within a sub-population of fibres is given in the third column. For example 20 % of Aβ slowly adapting mechanoreceptors are cold sensitive. These results are taken from Chapter 4, where a large sample of fibres was studied from the rat hairy skin. Results for TRP channel sensitivity is also taken from Chapter 4. Results for oxaliplatin sensitivity are taken from Chapter 7. Results for potassium channel blockade studies are taken from Chapter 6. The results have been formulated by pooling data for both 4-AP and TEA.

Table.8.2 Overall results for nociceptive fibres

Fibre type	Fibre sub-type	Cold sensitive (%)	Perceived sensation upon cooling	TRPM8 expression: menthol sensitivity (%)	TRPA1 expression: MO sensitivity (%)	TRPV1 expression: CAP sensitivity (%)	Increased/novel cold sensitivity after oxaliplatin (%)	Increased/novel cold sensitivity after K_v channel blockade (%)
Aδ: high threshold mechanoreceptor	AM	✘	-	✘	✘	✘	✓ (27)	✓ (8)
	AMC	✓ (20)	cold pain	✓ (83)	✓ (60)	✘	✓ (100)	✓ (100)
	AMH		-	✘	✓ (33)	✓ (100)	✘	✘
C: nociceptors	CM	✘	-	✘	✘	✘	✘	✘
	CMC	✓ (40)	burning pain	✓ (81)	✓ (29)	✘	✘	✓ (47)
	CMH	✘		✘	✓ (29)	✓ (65)	✘	✓ (36)

AM, high threshold mechano-sensitive A fibres; AMC, high threshold mechano-cold A fibres; AMH, high threshold mechano-heat A fibres; CM, C mechano-sensitive nociceptors; CMC, C mechano-cold and C mechano-cold and heat nociceptors , CMH, C mechano-heat sensitive nociceptors. The percentage of cold sensitivity within a sub-population of fibres is given in the third column. For example 40 % of C-fibre nocicpetors are cold sensitive.

8.2 The majority of Aβ mechanoreceptors are insensitive to temperature and TRP channel agonists, but develop cold sensitivity after potassium channel blockade or application of oxaliplatin

The majority of rapidly and slowly adapting mechanoreceptors were not sensitive to cold or heat stimuli applied to their receptive fields. Consistent with this, these fibres were insensitive to application of TRP channel agonists to their receptive fields, suggesting a lack of expression of these channels (see Chapter 4).

However, as previously reported (see chapter 4) a small proportion of slowly adapting fibres displayed a brief response during cooling. Until now the molecular mechanism behind this brief response to cooling has not been investigated. These fibres were insensitive during menthol application which suggested that TRPM8 was not the cold transducer on these afferents. Results from chapter 6 revealed that after application of 4-AP, 75 % of these fibres displayed a sustained response to cooling. This suggested that a 4-AP sensitive K^+ current was acting to reduce the cold responsiveness of these fibres. Since very few cold sensitive SA fibres were encountered in the present studies, future experiments could focus on recording from a larger sample of these fibres.

47 % of RA fibres and 60 % SA fibres displayed a novel response to cold after application of oxaliplatin. This has never been reported before and the abnormal development of cold sensitivity in these fibres may account for the hypersensitivity to cold experienced by patients receiving oxaliplatin therapy. 40 % of RA and 88 % of SA fibres displayed a novel sensitivity to cold after application of 4-AP/TEA. The molecular mechanism by which oxaliplatin exerts its effects is still not fully understood. Studies have suggested the involvement of voltage gated Na^+ or K^+ channels (see Chapter 7). It is interesting that application of both oxaliplatin and voltage gated potassium channel blockers act to induce novel cold responses in RA and SA fibres and this may suggest that oxaliplatin acts by blocking voltage gated K^+ channels. However, this seems very unlikely. The cold responses of RA and SA fibres after application of oxaliplatin were very different when compared to after application of 4-AP/TEA. Overall, the response to a cold stimulus after

application of oxaliplatin was greater in RA and SA fibres than after 4-AP/TEA application. Fibres discharged at very high frequencies in an irregular bursting manner (see chapter 7.3), whereas after application 4-AP/TEA fibres displayed a much lower level of discharge and in a more regular manner (see chapter 6.3). This suggests that oxaliplatin does not act by blocking voltage gated potassium channels and as discussed in chapter 7.4 is likely to be acting via TTX-S sodium channels.

8.3 Thermally insensitive A and C fibre nociceptors are insensitive to TRP channel agonists

Thermally insensitive AM and CM fibres did not respond during TRP channel agonist application, suggesting that these fibres do not express TRPV1, TRPA1 or TRPM8. However, 27 % and 8 % of AM fibres displayed a novel cold response after application of oxaliplatin and 4-AP/TEA, respectively. The receptive properties of CM fibres remained unchanged. Therefore, the proportion of thermally insensitive A and C fibre nociceptors which become cold sensitive after oxaliplatin or blockade of voltage gated potassium channels is much lower than that of Aβ mechanoreceptors. AM and CM fibres are less sensitive to the actions of oxaliplatin and broad spectrum potassium channel blockers suggesting that they express lower levels or different ion channels and receptors on their receptive fields compared to Aβ mechanoreceptors.

8.4 Thermally sensitive A fibre nociceptors respond to TRP channel agonists

The majority of thermally sensitive A fibre nociceptors responded directly to application of TRP channel agonists to their receptive fields. 83 % of cold sensitive AMC fibres responded during application of menthol, in a dose dependent manner, providing evidence for the expression of TRPM8 on these fibres. 60 % of these fibres also responded to mustard oil, indicating the expression of TRPA1 also. All heat sensitive, AMH fibres responded to capsaicin, suggesting that TRPV1 contributes to heat transduction in these fibres. A third of these fibres also responded to mustard oil. Overall the findings indicate that TRPA1 is expressed on both heat and cold sensitive A fibre nociceptors, TRPM8 is expressed on cold sensitive nociceptors and TRPV1 on heat sensitive nociceptors.

All AMC fibres displayed a greater response during cooling after application of oxaliplatin and 4-AP/TEA. This is in contrast to AMH fibres which remained cold insensitive. This suggests that both oxaliplatin and potassium channel blockers are capable of modulating the cold responses in these afferents, but not necessarily always able to induce a novel cold sensitivity. The present data suggest that TRPM8 is required for the transduction of cold in AMC fibres, but sodium and potassium conductances are also involved in shaping the final response to cold. An increase in cold sensitivity in AMC fibres would also explain the acute painful and distressing sensations experienced by patients administered oxaliplatin, since these fibres would now respond in a greatly exaggerated manner in response to cold.

8.5 C fibre nociceptors are sensitive to TRP channel agonists and respond to broad spectrum potassium channel blockers, but not to oxaliplatin

81 % of cold sensitive C fibre nociceptors (CMC and CMCH) responded to application of menthol to their receptive fields. Only 29 % of these fibres responded to mustard oil suggesting that TRPM8 and not TRPA1 is the transducer of noxious cold on these afferents. A larger proportion of heat sensitive C fibre nociceptors (CMH) responded directly to application of capsaicin, indicating that the heat responses in these fibres are mediated by TRPV1. Almost one third of CMH fibres also responded to mustard oil, indicating that TRPA1 is expressed in both cold and heat sensitive C fibre nociceptors. The poor correlation between TRPA1 expression and cold sensitivity in nociceptive A and C fibres shown in this study indicates that TRPA1 is unlikely to play a significant role is detecting noxious cold. The role of TRPA1 in sensory nerve fibres is discussed in more detail in Chapter 4.4.
Almost half of CMC fibres displayed an increase in cold response after blockade of K^+ channels and this indicates that potassium conductances are involved in regulating the response to cold stimuli in these fibres. Unlike AMC fibres, however, not all CMC fibres displayed an increase in cold sensitivity after application of 4-AP/TEA. This is likely to reflect the differential expression of potassium channel subunits on different types of C fibres; C fibres are neurochemically diverse (Stucky and Lewin, 1999) and a differential expression of ion channels has been found between IB4 positive and IB4 negative neurons (Stucky and Lewin, 1999;Vydyanathan et al., 2005) (see chapter 6.4). A third of CMH fibres displayed a novel cold sensitivity after blockade of potassium channels and this indicates that there is also a functional diversity amongst the functional importance of potassium conductances in these neurons, which normally suppresses their cold sensitivity.
Strikingly, receptive properties of C fibre nociceptors remained unchanged after application of oxaliplatin. This is in contrast to the findings in Aβ and Aδ fibres and therefore suggests that a difference between myelinated and unmyelinated fibres may explain the differential sensitivity to oxaliplatin in

these fibres. On this basis, the results from the present study are consistent with the notion that oxaliplatin is acting via TTX-S but not TTX-R sodium channels.

8.6 Thermally sensitive low threshold mechano-C fibres are not sensitive to TRP channel agonists, but respond to broad spectrum potassium channel blockers

Half of the mechanically low threshold C (CLTM) fibres responded to cooling of their receptive fields in the rat hairy skin (chapter 4.3). However, these fibres did not respond to menthol applied to their receptive fields indicating that TRPM8 is not the cold transducer in these fibres. Half of CLTM fibres also respond to heating and these did not respond to capsaicin. This suggests that other mechanisms must be involved in thermal transduction, apart from heat and cold activated TRP channels.
A third of these fibres displayed an increase in cold response after application of 4-AP/TEA and this suggests that potassium conductances are likely to be involved in mediating their response to cold. However, this was observed in one of a small sample of 3 fibres and therefore a larger sample of CLTM-C fibres needs to be recorded in future experiments. The receptive properties of CLTM fibres remained unchanged after application of oxaliplatin, again supporting the notion that oxaliplatin is acting via sodium channels that are differentially expressed amongst C- and A-fibres.

8.7 The majority of cold thermoreceptors are sensitive to low concentrations of menthol, but are not affected by potassium channel blockers or oxaliplatin

Almost 70 % of mechanically insensitive cold fibres were activated during application of menthol in micromolar concentrations. This finding has been reported before (see chapter 4.4) and indicates that TRPM8 is the cold transducer on these afferents and plays a role in signalling non-painful cooling. However, the present study found that application of the higher concentration of menthol in fact had the opposite effect and reduced the

responses evoked from these afferents. Therefore, the results propose that the “cooling” affect of menthol is mediated by the activation of cold specific fibres and the “burning” quality of menthol is mediated by the activation of Aδ and C cold nociceptors that respond to the higher concentrations. These mechanically insensitive, cold sensitive fibres did not respond to mustard oil or capsaicin, indicating a lack of TRPA1 and TRPV1 expression. Blockade of voltage gated potassium channels did not alter the cold responses of these fibres and therefore suggests that potassium conductances are not involved in modulating the responses to cold in these fibres. The lack of sensitivity to oxaliplatin suggests that these fibres are also not involved in the acute neuropathy.

8.8 Mice lacking TRPV2 have normal heat sensitive nociceptors.

Finally, electrophysiological analysis of the receptive properties of afferents innervating the hairy and glabrous skin of mice lacking the TRPV2 receptor reveal that C fibre nociceptors retain sensitivity to heat. Very few heat sensitive Aδ nociceptors were recorded and therefore the significance of TRPV2 as a heat transducer on these afferents is still not fully understood. However, all the AMH fibres reported in chapter 4 were shown to be activated by capsaicin and therefore indicate that TRPV1 plays a role in the heat transduction of these afferents. It is important to note that a mechanical stimulus was used as a search strategy and therefore mechanically insensitive afferents were very rarely recorded. When fibres were stimulated with a noxious heat stimulus reaching 50 °C, mechanically insensitive, heat sensitive C fibres were recorded from TRPV2 wild-type mice. These fibres were not found when the standard heat stimulus, which reached 47 °C was used and indicates that other heat sensitive afferents exist in this preparation which were not investigated. TRPV2 may be expressed on these afferents and therefore future experiments should use an electrical search strategy to avoid sampling bias.

Final conclusions

The studies described in this thesis were carried out with the aim of achieving a better understanding of how primary afferent sensory neurons in the rodent are able to signal thermal stimuli, in particular cold.

Firstly, the high correlation of cold sensitivity among Aδ nociceptors, C fibre nociceptors and thermoreceptors with menthol sensitivity indicates that TRPM8 is the primary cold transducer in these afferents and is involved in the signalling of painful and non-painful cool stimuli. These findings are consistent with recent in vivo data that showed that mice lacking the TRPM8 receptor have severe behavioural deficits in response to cold stimuli (see chapter 4.4). TRPA1 has been proposed as a noxious cold sensor, but the low correlation between cold and mustard oil responses in Aδ and C fibres in this study would suggest that the role of TRPA1 is not specific for the detection of noxious cold. However, since mustard oil sensitivity was observed in both heat and cold sensitive nociceptors, it supports the role of TRPA1 in detecting noxious stimuli. Capsaicin sensitivity was found in heat sensitive A and C fibre nociceptors which is consistent with its role as a noxious heat detector. TRPV2 does not appear to be important in the transduction of noxious heat, at least not in mechanically sensitive A and C fibre nociceptors. Its role in mechanically insensitive afferents remains to be investigated.

Blockade of voltage gated potassium channels was shown to previously induce a novel cold sensitivity in a proportion of cold insensitive DRG neurons (see chapter 6.1 and 6.4). The results presented here are in accordance with these previous findings and furthermore have revealed the identity of the sensory neurons that display a novel response to cold; thermally insensitive Aβ mechanoreceptors, D-hair fibres, AM and CMH fibres. In addition to these findings, blockade of potassium channels was found to increase the cold responses of already cold sensitive A and C fibre nociceptors, suggesting that the presence of potassium conductances act to limit the response to cold in these groups of afferents.

Direct application of the chemotherapeutic agent oxaliplatin on characterised sensory afferents has revealed that a proportion of low threshold Aβ mechanoreceptors and Aδ D-hair and nociceptors develop a novel sensitivity

to cold stimuli. If the molecular target of oxaliplatin are indeed TTX-S sodium channels, it suggests that altering sodium channel properties can affect the cold sensitivity of a neuron. The importance of the TTX-R sodium channel $Na_v1.8$ in the transmission of cold in unmyelinated nociceptive neurons has already been demonstrated (Abrahamsen et al., 2008;Zimmermann et al., 2007) and could suggest that TTX-R and TTX-S sodium channels contribute in different ways to the cold perception. Although $Na_v1.8$ may not act as a primary cold sensor itself, it is required for the transmission of signals during cold temperatures. All of the data put together therefore indicate that a cohort of ion channels and receptors are required for the transmission of cold signals.

The data presented in this thesis indicate that cold depolarises sensory nerve fibres by activating an excitatory ‘cold receptor’, TRPM8, and by modulating a combination of ionic conductances that together shape the final response to a cold stimulus.

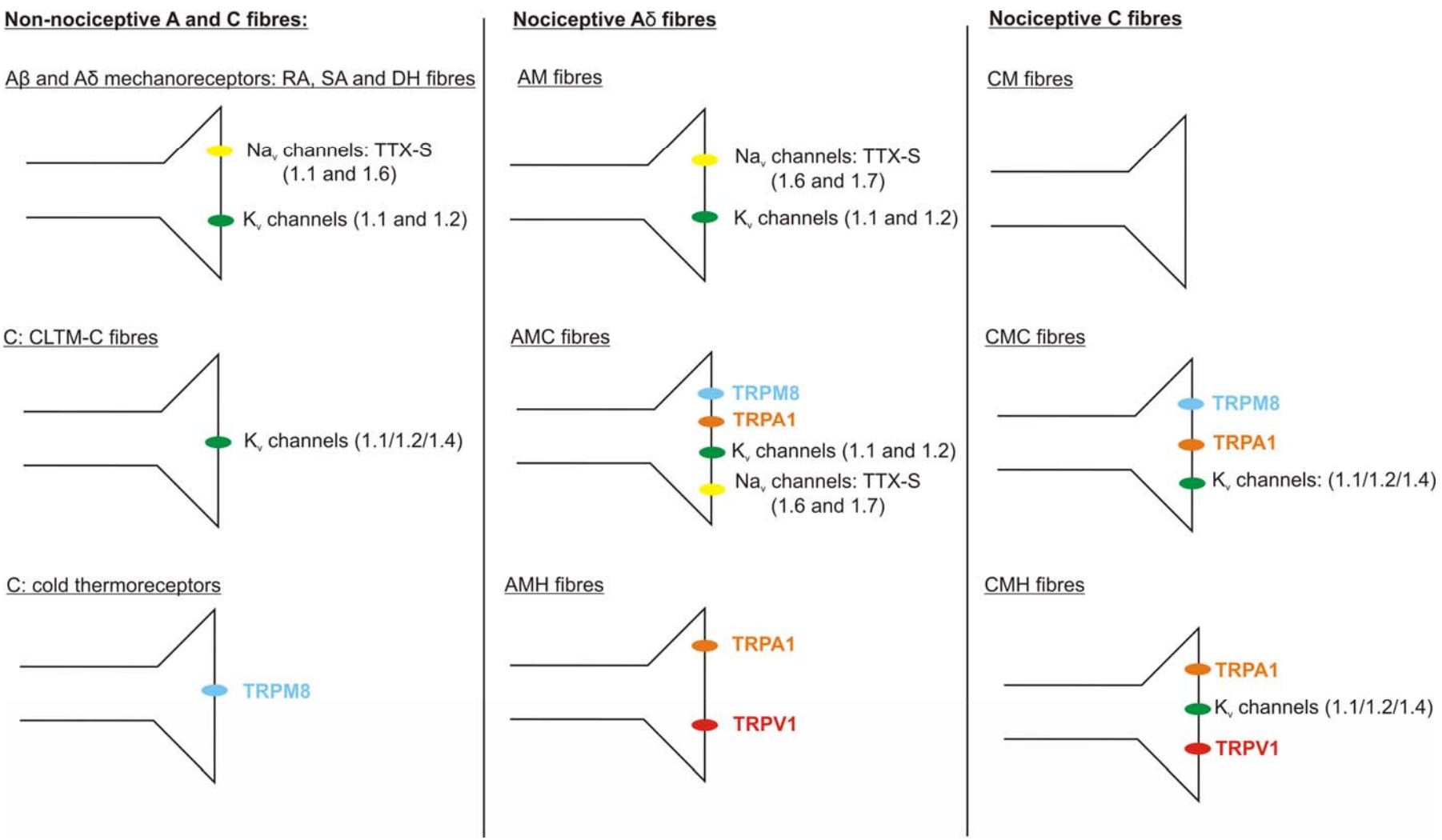
Non-nociceptive A and C fibres:
Aβ and Aδ mechanoreceptors: RA, SA and DH fibres
Na$_v$ channels: TTX-S (1.1 and 1.6)
K$_v$ channels (1.1 and 1.2)
C: CLTM-C fibres
K$_v$ channels (1.1/1.2/1.4)
C: cold thermoreceptors
TRPM8
Nociceptive Aδ fibres
AM fibres
Na$_v$ channels: TTX-S (1.6 and 1.7)
K$_v$ channels (1.1 and 1.2)
AMC fibres
TRPM8
TRPA1
K$_v$ channels (1.1 and 1.2)
Na$_v$ channels: TTX-S (1.6 and 1.7)
AMH fibres
TRPA1
TRPV1
Nociceptive C fibres
CM fibres
CMC fibres
TRPM8
TRPA1
K$_v$ channels: (1.1/1.2/1.4)
CMH fibres
TRPA1
K$_v$ channels (1.1/1.2/1.4)
TRPV1

Fig.8.1

Fig.8.1 A schematic diagram representing the expression pattern of different ion channels and receptors on the peripheral nerve endings of different fibre types, which are proposed to be involved in thermal transduction

Based on the results from investigations carried out in chapters 4, 6 and 7 the expression pattern of TRP channels, voltage gated potassium and sodium channels on the different classes of fibres studied are proposed. The basis of the differential expression of ion channel isoforms are discussed in more detail in the relative discussion chapters.

Reference List

1. Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321:702-705.

2. Adelsberger H, Quasthoff S, Grosskreutz J, Lepier A, Eckel F, Lersch C (2000) The chemotherapeutic oxaliplatin alters voltage-gated Na+ channel kinetics on rat sensory neurons. European Journal of Pharmacology 406:25-32.

3. Adriaensen H, Gybels J, Handwerker HO, Van Hees J (1983) Response properties of thin myelinated (A-delta) fibers in human skin nerves. J Neurophysiol 49:111-122.

4. Adrian ED (1926) The impulses produced by sensory nerve endings: Part I. J Physiol (Lond) 61:49-72.

5. Adrian ED, Zotterman Y (1926) The impulses produced by sensory nerve-endings: Part II. The response of a Single End-Organ. J Physiol (Lond) 61:151-171.

6. Ahluwalia J, Rang H, Nagy I (2002) The putative role of vanilloid receptor-like protein-1 in mediating high threshold noxious heat-sensitivity in rat cultured primary sensory neurons. Eur J Neurosci 16:1483-1489.

7. Airaksinen MS, Koltzenburg M, Lewin GR, Masu Y, Helbig C, Wolf E, Brem G, Toyka KV, Thoenen H, Meyer M (1996) Specific Subtypes of Cutaneous Mechanoreceptors Require Neurotrophin-3 Following Peripheral Target Innervation. Neuron 16:287-295.

8. Akins PT, McCleskey EW (1993) Characterization of potassium currents in adult rat sensory neurons and modulation by opioids and cyclic AMP. Neuroscience 56:759-769.

9. Akopian AN, Sivilotti L, Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. Nature 379:257-262.

10. Alrutz S (1897) On the temperature-Senses. Mind 6:445-448.

11. Anand P (2003) Capsaicin and menthol in the treatment of itch and pain: recently cloned receptors provide the key. Gut 52:1233-1235.

12. Ashcroft F (2000) Voltage gated potassium channels. In: Ion channels and disease San Diego, CA, USA: Academic Press.

13. Askwith CC, Benson CJ, Welsh MJ, Snyder PM (2001) DEG/ENaC ion channels involved in sensory transduction are modulated by cold temperature. Proc Natl Acad Sci U S A 98:6459-6463.

14. Atherton DD, Taherzadeh O, Elliot D, Anand P (2008) Age-Dependent Development of Chronic Neuropathic Pain, Allodynia and Sensory Recovery after Upper Limb Nerve Injury in Children. J Hand Surg Eur Vol 33:186-191.

15. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, Patapoutian A (2004) Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin. Neuron 41:849-857.

16. Banik RK, Brennan TJ (2008) Sensitization of primary afferents to mechanical and heat stimuli after incision in a novel in vitro mouse glabrous skin-nerve preparation. Pain.

17. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, Julius D (2006) TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. Cell 124:1269-1282.

18. Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. Nature in press.

19. Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Hogestatt ED, Julius D, Jordt SE, Zygmunt PM (2005) Pungent products from garlic activate the sensory ion channel TRPA1. PNAS 102:12248-12252.

20. Bean BP (2005) The Molecular Machinery of Resurgent Sodium Current Revealed. Neuron 45:185-187.

21. Beck PW, Handwerker HO, Zimmermann M (1974) Nervous outflow from the cat's foot during noxious radiant heat stimulation. Brain Research 67:373-386.

22. Benham CD, Gunthorpe MJ, Davis JB (2003) TRPV channels as temperature sensors. Cell Calcium 33:479-487.

23. Berglund B, Harju EL, Kosek E, Lindblom U (2002) Quantitative and qualitative perceptual analysis of cold dysesthesia and hyperalgesia in fibromyalgia. Pain 96:177-187.

24. Besser R, Luttgen N, Frieling T (2007) The spectrum of oxaliplatin-induced hyperexcitability of the peripheral nerves. Clinical Neurophysiology 118:e16.

25. Bessou P, Perl ER (1969) Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. J Neurophysiol 32:1025-1043.

26. Bester H, Chapman V, Besson JM, Bernard JF (2000) Physiological Properties of the Lamina I Spinoparabrachial Neurons in the Rat. J Neurophysiol 83:2239-2259.

27. Bever CT (1995) 4-Aminopyridine: Use in Multiple Sclerosis. CNS Drug Reviews 1:261-279.

28. Binder A, Stengel M, Maag R, Wasner G, Schoch R, Moosig F, Schommer B, Baron R (2007) Pain in oxaliplatin-induced neuropathy - Sensitisation in the peripheral and central nociceptive system. European Journal of Cancer 43:2658-2663.

29. Bini G, Cruccu G, Hagbarth KE, Schady W, Torebjork E (1984) Analgesic effect of vibration and cooling on pain induced by intraneural electrical stimulation. Pain 18:239-248.

30. Binzen U, Greffrath W, Hennessy S, Bausen M, Saaler-Reinhardt S, Treede RD (2006) Co-expression of the voltage-gated potassium channel Kv1.4 with transient receptor potential channels (TRPV1 and TRPV2) and the cannabinoid receptor CB1 in rat dorsal root ganglion neurons. Neuroscience 142:527-539.

31. Birinyi-Strachan LC, Gunning SJ, Lewis RJ, Nicholson GM (2005) Block of voltage-gated potassium channels by Pacific ciguatoxin-1 contributes to increased neuronal excitability in rat sensory neurons. Toxicology and Applied Pharmacology 204:175-186.

32. Black JA, Dib-Hajj S, McNabola K, Jeste S, Rizzo MA, Kocsis JD, Waxman SG (1996) Spinal sensory neurons express multiple sodium channel alpha-subunit mRNAs. Brain Res Mol Brain Res 43:117-131.

33. Blieberg H, Kemeny N, Rougier P, Wilke H (2002) Bimonthly leucovorin-5-fluorouracil with oxaliplatin or irinotecan: The FOLOX or FILFIRI regimens. In: Colorectal Cancer, a clinical guide to therapy pp 563-568. Informa health care.

34. Bostock H, Sears TA, Sherratt RM (1981) The effects of 4-aminopyridine and tetraethylammonium ions on normal and demyelinated mammalian nerve fibres. J Physiol 313:301-315.

35. Bouhours M, Sternberg D, Davoine CS, Ferrer X, Willer JC, Fontaine B, Tabti N (2004) Functional characterization and cold sensitivity of T1313A, a new mutation of the skeletal muscle sodium channel causing paramyotonia congenita in humans. J Physiol 554:635-647.

36. Burgess PR, Perl ER (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J Physiol 190:541-562.

37. Burgess PR, Petit D, Warren RM (1968) Receptor types in cat hairy skin supplied by myelinated fibers. J Neurophysiol 31:833-848.

38. Cabanes C, Viana F, Belmonte C (2003) Differential thermosensitivity of sensory neurons in the guinea pig trigeminal ganglion. J Neurophysiol 90:2219-2231.

39. Cain DM, Khasabov SG, Simone DA (2001) Response Properties of Mechanoreceptors and Nociceptors in Mouse Glabrous Skin: An In Vivo Study. J Neurophysiol 85:1561-1574.

40. Caldwell JH, Schaller KL, Lasher RS, Peles E, Levinson SR (2000) Sodium channel Nav1.6 is localized at nodes of Ranvier, dendrites, and synapses. PNAS 97:5616-5620.

41. Campbell JN, LaMotte RH (1983) Latency to detection of first pain. Brain Research 266:203-208.

42. Campbell JN, Meyer RA (1983) Sensitization of unmyelinated nociceptive afferents in monkey varies with skin type. J Neurophysiol 49:98-110.

43. Campbell JN, Meyer RA, LaMotte RH (1979) Sensitization of myelinated nociceptive afferents that innervate monkey hand. J Neurophysiol 42:1669-1679.

44. Campero M, Serra J, Bostock H, Ochoa JL (2001) Slowly conducting afferents activated by innocuous low temperature in human skin. J Physiol (Lond) 535:855-865.

45. Campero M, Serra J, Ochoa JL (1996) C-polymodal nociceptors activated by noxious low temperature in human skin. J Physiol 497 (Pt 2):565-572.

46. Carroll P, Lewin GR, Koltzenburg M, Toyka KV, Thoenen H (1998) A role for BDNF in mechanosensation. Nat Neurosci 1:42-46.

47. Caspani O, Zurborg S, Heppenstall PA (2007) 343 The contribution of TRPA1 and TRPM8 to cold allodynia and neuropathic pain. European Journal of Pain 11:152.

48. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000a) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306-313.

49. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398:436-441.

50. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816-824.

51. Cavaletti G, Tredici G, Petruccioli M, Donde E, Tredici P, Marmiroli P, Minoia C, Gatti A, Bayssas M, Griffon Etienne G (2001) Effects of different schedules of oxaliplatin treatment on the peripheral nervous system and the auditory pathway in the rat. Journal of the Peripheral Nervous System 6:44.

52. Cersosimo RJ (2005) Oxaliplatin-Associated Neuropathy: A Review. Ann Pharmacother 39:128-135.

53. Chambers MR, Andres KH, Duering M, Iggo A (1972) The structure and function of the slowly adapting type II mechanoreceptor in hairy skin. Q J Exp Physiol Cogn Med Sci 57:417-445.

54. Chateau-Degat ML, Beuter A, Vauterin G, Nguyen NL, Chinain M, Darius T, Legrand AM, Chansin R, Dewailly E (2007) Neurologic signs of ciguatera disease: evidence of their persistence. Am J Trop Med Hyg 77:1170-1175.

55. Chen CC, Rainville P, Bushnell MC (1996) Noxious and innocuous cold discrimination in humans: evidence for separate afferent channels. Pain 68:33-43.

56. Chi XX, Nicol GD (2007) Manipulation of the potassium channel Kv1.1 and its effect on neuronal excitability in rat sensory neurons. J Neurophysiol 98:2683-2692.

57. Chien LY, Cheng JK, Chu D, Cheng CF, Tsaur ML (2007) Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. J Neurosci 27:9855-9865.

58. Chung MK, Lee H, Mizuno A, Suzuki M, Caterina MJ (2004) TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. J Biol Chem 279:21569-21575.

59. Colburn RW, Lubin ML, Stone DJ, Jr., Wang Y, Lawrence D, D'Andrea MR, Brandt MR, Liu Y, Flores CM, Qin N (2007) Attenuated cold sensitivity in TRPM8 null mice. Neuron 54:379-386.

60. Craig AD, Bushnell MC (1994) The thermal grill illusion: unmasking the burn of cold pain. Science 265:252-255.

61. Cummins TR, Dib-Hajj SD, Herzog RI, Waxman SG (2005) Nav1.6 channels generate resurgent sodium currents in spinal sensory neurons. FEBS Lett 579:2166-2170.

62. Dallenbach K M (1939) Pain: History and Present Status. The American Journal of Psychology 52:331-347.

63. Darian-Smith I, Johnson KO, Dykes R (1973) "Cold" fiber population innervating palmar and digital skin of the monkey: responses to cooling pulses. J Neurophysiol 36:325-346.

64. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405:183-187.

65. Davis KD (1998) Cold-induced pain and prickle in the glabrous and hairy skin. Pain 75:47-57.

66. Davis KD, Lozano AM, Manduch M, Tasker RR, Kiss ZH, Dostrovsky JO (1999) Thalamic Relay Site for Cold Perception in Humans. J Neurophysiol 81:1970-1973.

67. Davis KD, Meyer RA, Campbell JN (1993) Chemosensitivity and sensitization of nociceptive afferents that innervate the hairy skin of monkey. J Neurophysiol 69:1071-1081.

68. Davis KD, Pope GE (2002) Noxious cold evokes multiple sensations with distinct time courses. Pain 98:179-185.

69. de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, Boni C, Cortes-Funes H, Cervantes A, Freyer G, Papamichael D, Le Bail N, Louvet C, Hendler D, de Braud F, Wilson C, Morvan F, Bonetti A (2000) Leucovorin and Fluorouracil with or without Oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol 18:2938-2947.

70. Dhaka A, Murray AN, Mathur J, Earley TJ, Petrus MJ, Patapoutian A (2007) TRPM8 is required for cold sensation in mice. Neuron 54:371-378.

71. Dib-Hajj SD, Tyrrell L, Black JA, Waxman SG (1998) NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. PNAS 95:8963-8968.

72. Donaldson H H (1885) On the temperature sense. Mind 10:399-416.

73. Dostrovsky JO, Craig AD (1996) Cooling-specific spinothalamic neurons in the monkey. J Neurophysiol 76:3656-3665.

74. Dubner R, Sumino R, Wood WI (1975) A peripheral "cold" fiber population responsive to innocuous and noxious thermal stimuli applied to monkey's face. J Neurophysiol 38:1373-1389.

75. Dubreuil AS, Boukhaddaoui H, Desmadryl G, Martinez-Salgado C, Moshourab R, Lewin GR, Carroll P, Valmier J, Scamps F (2004) Role of T-Type Calcium Current in Identified D-Hair Mechanoreceptor Neurons Studied In Vitro. J Neurosci 24:8480-8484.

76. Eckel F, Schmelz R, Adelsberger H, Erdmann J, Quasthoff S, Lersch C (2002) Prevention of oxaliplatin-induced neuropathy by carbamazepine: A pilot study. Dtsch Med Wochenschr78-82.

77. Everill B, Kocsis JD (2000) Nerve growth factor maintains potassium conductance after nerve injury in adult cutaneous afferent dorsal root ganglion neurons. Neuroscience 100:417-422.

78. Everill B, Kocsis JD (1999) Reduction in Potassium Currents in Identified Cutaneous Afferent Dorsal Root Ganglion Neurons After Axotomy. J Neurophysiol 82:700-708.

79. Everill B, Cummins TR, Waxman SG, Kocsis JD (2001) Sodium currents of large (Abeta-type) adult cutaneous afferent dorsal root ganglion neurons display rapid recovery from inactivation before and after axotomy. Neuroscience 106:161-169.

80. Extra JM, Espie M, Calvo F, Ferme C, Mignot L, Marty M (1990) Phase I study of oxaliplatin in patients with advanced cancer. Cancer Chemother Pharmacol 25:299-303.

81. Fedulova SA, Vasilyev DV, Veselovsky NS (1998) Voltage-operated potassium currents in the somatic membrane of rat dorsal root ganglion neurons: ontogenetic aspects. Neuroscience 85:497-508.

82. Finger S (1994) The cutaneous senses. In: Origins of Neuroscience pp 134-138. Oxford University Press US.

83. Frederick J, Buck ME, Matson DJ, Cortright DN (2007) Increased TRPA1, TRPM8, and TRPV2 expression in dorsal root ganglia by nerve injury. Biochemical and Biophysical Research Communications 358:1058-1064.

84. French AS, Torkkeli PH (1994) The Basis of Rapid Adaptation in Mechanoreceptors. News Physiol Sci 9:158-161.

85. Gamelin L, Boisdron-Celle M, Delva R, Guerin-Meyer V, Ifrah N, Morel A, Gamelin E (2004) Prevention of oxaliplatin-related neurotoxicity by calcium and magnesium infusions: a retrospective study of 161 patients receiving oxaliplatin combined with 5-Fluorouracil and leucovorin for advanced colorectal cancer. Clin Cancer Res 10:4055-4061.

86. Gasser HS, Erlanger J (1927) The role played by the sizes of the constituent fibers of a nerve trunk in determining the form of its action potential wave. Am J Physiol 80:522-547.

87. Gasser HS, Erlanger J (1922) A study of the action currents of nerve with the cathode ray oscillograph. Am J Physiol 62:496-524.

88. Gasser HS, Erlanger J (1929) The role of fiber size in the establishment of a nerve block by pressure or cocaine. Am J Physiol 88:581-591.

89. Gold MS, Shuster MJ, Levine JD (1996) Characterization of six voltage-gated K+ currents in adult rat sensory neurons. J Neurophysiol 75:2629-2646.

90. Greffrath W, Binzen U, Schwarz ST, Saaler-Reinhardt S, Treede RD (2003) Co-expression of heat sensitive vanilloid receptor subtypes in rat dorsal root ganglion neurons. Neuroreport 14:2251-2255.

91. Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. J Neurosci 22:6408-6414.

92. Gybels J, Handwerker HO, Van HJ (1979) A comparison between the discharges of human nociceptive nerve fibres and the subject's ratings of his sensations. J Physiol 292:193-206.

93. Hamalainen H, Vartiainen M, Karvanen L, Jarvilehto T (1982) Paradoxical heat sensations during moderate cooling of the skin. pp 77-81.

94. Harrison JLK, Davis KD (1999) Cold-evoked pain varies with skin type and cooling rate: a psychophysical study in humans. Pain 83:123-135.

95. Hensel H (1981) Thermoreception and temperature regulation. Monogr Physiol Soc 38:1-321.

96. Hensel H (1974) Thermoreceptors. Ann Rev Physiol 36:233-249.

97. Hensel H, Iggo A, Witt I (1960) A quantitative study of sensitive cutaneous thermoreceptors with C afferent fibres. J Physiol 153:113-126.

98. Hensel H, Kenshalo DR (1969) Warm receptors in the nasal region of cats. J Physiol 204:99-112.

99. Hensel H, Zotterman. Y (1951) The effect of menthol on the thermoreceptors. Acta Physiol Scand 24:27-34.

100. Hensel H, Zotterman.Y (1951) Action potentials of cold fibres and intracutaneous temperature gradient. J Neurophysiol 14:377-385.

101. Hensel H, Boman KKA (1960) Afferent impulses in cutaneous sensory nerves in human subjects. J Neurophysiol 23:564-578.

102. Hille B (1992) Ionic channels of excitable membranes. Sunderland, MA: Sinauer Associates.

103. Holmes J, Stanko J, Varchenko M, Ding H, Madden V, Bagnell C, Wyrick S, Chaney S (1998) Comparative neurotoxicity of Oxaliplatin, Cisplatin and Ormaplatin in a wistar rat model. Toxicological Sciences 46:342-351.

104. Iannetti GD, Zambreanu L, Tracey I (2006) Similar nociceptive afferents mediate psychophysical and electrophysiological responses to heat stimulation of glabrous and hairy skin in humans. J Physiol 577:235-248.

105. Iggo A, Muir AR (1969) The structure and function of a slowly adapting touch corpuscle in hairy skin. J Physiol (Lond) 200:763-796.

106. Isbister GK, Kiernan MC (2005) Neurotoxic marine poisoning. Lancet Neurol 4:219-228.

107. Ishikawa K, Tanaka M, Black JA, Waxman SG (1999) Changes in expression of voltage-gated potassium channels in dorsal root ganglion neurons following axotomy. Muscle Nerve 22:502-507.

108. Jacobs SS, Fox E, Dennie C, Morgan LB, McCully CL, Balis FM (2005) Plasma and cerebrospinal fluid pharmacokinetics of intravenous oxaliplatin, cisplatin, and carboplatin in nonhuman primates. Clin Cancer Res 11:1669-1674.

109. Jamieson SM, Liu J, Connor B, McKeage MJ (2005) Oxaliplatin causes selective atrophy of a subpopulation of dorsal root ganglion neurons without inducing cell loss. Cancer Chemother Pharmacol 56:391-399.

110. Jarvilehto T, Hamalainen H, Soininen K (1981) Peripheral neural basis of tactile sensations in man: II. Characteristics of human mechanoreceptors in the hairy skin and correlations of their activity with tactile sensations. Brain Research 219:13-27.

111. Ji G, Zhou S, Kochukov MY, Westlund KN, Carlton SM (2007) Plasticity in intact A delta- and C-fibers contributes to cold hypersensitivity in neuropathic rats. Neuroscience 150:182-193.

112. Jiang CH, Mazieres L, Lindstrom S (2002) Cold- and menthol-sensitive C afferents of cat urinary bladder. J Physiol (Lond) 543:211-220.

113. Jordt SE, McKemy DD, Julius D (2003) Lessons from peppers and peppermint: the molecular logic of thermosensation. Current Opinion in Neurobiology 13:487-492.

114. Jorum E, Warncke T, Stubhaug A (2003) Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-D-aspartate (NMDA) receptor antagonist ketamine--a double-blind, cross-over comparison with alfentanil and placebo. Pain 101:229-235.

115. Julius D, McCleskey EW (2005) Cellular and molecular properties of primary afferent neurons. In: Wall and Melzack's Text book of pain (McMahon SB, Koltzenburg M, eds), Scotland: Churchill Livingston.

116. Kang D, Choe C, Kim D (2005) Thermosensitivity of the two-pore domain K+ channels TREK-2 and TRAAK. J Physiol 564:103-116.

117. Katsura H, Obata K, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Sakagami M, Noguchi K (2006) Antisense knock down of TRPA1, but not TRPM8, alleviates cold hyperalgesia after spinal nerve ligation in rats. Exp Neurol 200:112-123.

118. Kelland L (2007) The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 7:573-584.

119. Kiernan MC, Krishnan AV (2006) The pathophysiology of oxaliplatin-induced neurotoxicity. Curr Med Chem 13:2901-2907.

120. Kirchhoff C, Leah JD, Jung S, Reeh PW (1992) Excitation of cutaneous sensory nerve endings in the rat by 4-aminopyridine and tetraethylammonium. J Neurophysiol 67:125-131.

121. Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. J Comp Neurol 493:596-606.

122. Kocsis JD, Bowe CM, Waxman SG (1986) Different effects of 4-aminopyridine on sensory and motor fibers: pathogenesis of paresthesias. Neurology 36:117-120.

123. Koerber HR, Druzinsky RE, Mendell LM (1988) Properties of somata of spinal dorsal root ganglion cells differ according to peripheral receptor innervated. J Neurophysiol 60:1584-1596.

124. Koltzenburg M (2004) The role of TRP channels in sensory neurons. pp 216-213, discussion 213-20, 277-9.

125. Koltzenburg M, Stucky CL, Lewin GR (1997) Receptive Properties of Mouse Sensory Neurons Innervating Hairy Skin. J Neurophysiol 78:1841-1850.

126. Konietzny F (1984) Peripheral neural correlates of temperature sensations in man. Hum Neurobiol 3:21-32.

127. Konietzny F, Hensel H (1977) The dynamic response of warm units in human skin nerves. Pflugers Arch 370:111-114.

128. Kress M, Koltzenburg M, Reeh PW, Handwerker HO (1992) Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers in vivo and in vitro. J Neurophysiol 68:581-595.

129. Krishnan AV, Goldstein D, Friedlander M, Kiernan MC (2005) Oxaliplatin-induced neurotoxicity and the development of neuropathy. Muscle Nerve 32:51-60.

130. Krishnan AV, Goldstein D, Friedlander M, Kiernan MC (2006) Oxaliplatin and axonal Na+ channel function in vivo. Clin Cancer Res 12:4481-4484.

131. Kruger L, Perl ER, Sedivec MJ (1981) Fine structure of myelinated mechanical nociceptor endings in cat hairy skin. J Comp Neurol 198:137-154.

132. Kunesch E, Schmidt R, Nordin M, Wallin U, Hagbarth KE (1987) Peripheral neural correlates of cutaneous anaesthesia induced by skin cooling in man. Acta Physiol Scand 129:247-257.

133. Kuo MT, Chen HH, Song IS, Savaraj N, Ishikawa T (2007) The roles of copper transporters in cisplatin resistance. Cancer Metastasis Rev 26:71-83.

134. Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP (2006) TRPA1 Contributes to Cold, Mechanical, and Chemical Nociception but Is Not Essential for Hair-Cell Transduction. Neuron 50:277-289.

135. LaCroix-Fralish ML, Ledoux JB, Mogil JS (2007) The Pain Genes Database: An interactive web browser of pain-related transgenic knockout studies. Pain 131:3-4.

136. LaMotte RH, Campbell JN (1978) Comparison of responses of warm and nociceptive C-fiber afferents in monkey with human judgments of thermal pain. J Neurophysiol 41:509-528.

137. Lawson JJ, McIlwrath SL, Woodbury CJ, Davis BM, Koerber HR (2008) TRPV1 Unlike TRPV2 Is Restricted to a Subset of Mechanically Insensitive Cutaneous Nociceptors Responding to Heat. J Pain 9:298-308.

138. Lawson SN (1992) Morphological and biochemical cell types of sensory neurons. In: Sensory Neurons: Diversity, Development, and Plasticity (Scott SA, ed), pp 27-59. Oxford University Press.

139. Leem JW, Willis WD, Chung JM (1993) Cutaneous sensory receptors in the rat foot. J Neurophysiol 69:1684-1699.

140. Leffler A, Linte RM, Nau C, Reeh P, Babes A (2007) A high-threshold heat-activated channel in cultured rat dorsal root ganglion neurons resembles TRPV2 and is blocked by gadolinium. European Journal of Neuroscience 26:12-22.

141. Lehky TJ, Leonard GD, Wilson RH, Grem JL, Floeter MK (2004) Oxaliplatin-induced neurotoxicity: acute hyperexcitability and chronic neuropathy. Muscle Nerve 29:387-392.

142. Leonard G, Wright M, Quinn M, Fioravanti S, Harold N, Schuler B, Thomas R, Grem J (2005) Survey of oxaliplatin-associated neurotoxicity using an interview-based questionnaire in patients with metastatic colorectal cancer. BMC Cancer 5:116.

143. Lewin GR, Moshourab R (2004) Mechanosensation and pain. J Neurobiol 61:30-44.

144. Liapi A, Wood JN (2005) Extensive co-localization and heteromultimer formation of the vanilloid receptor-like protein TRPV2 and the capsaicin receptor TRPV1 in the adult rat cerebral cortex. European Journal of Neuroscience 22:825-834.

145. Ling B, Coudore-Civiale MA, Balayssac D, Eschalier A, Coudore F, Authier N (2007) Behavioral and immunohistological assessment of painful neuropathy induced by a single oxaliplatin injection in the rat. Toxicology 234:176-184.

146. Long RR (1977) Sensitivity of cutaneous cold fibers to noxious heat: paradoxical cold discharge. J Neurophysiol 40:489-502.

147. Lynn B, Carpenter SE (1982) Primary afferent units from the hairy skin of the rat hind limb. Brain Res 238:29-43.

148. Lynn B, Shakhanbeh J (1988) Properties of A[delta] high threshold mechanoreceptors in the rat hairy and glabrous skin and their response to heat. Neuroscience Letters 85:71-76.

149. Ma QP (2001) Vanilloid receptor homologue, VRL1, is expressed by both A- and C-fiber sensory neurons. Neuroreport 12:3693-3695.

150. Machover D, az-Rubio E, de GA, Schilf A, Gastiaburu JJ, Brienza S, Itzhaki M, Metzger G, N'Daw D, Vignoud J, Abad A, Francois E, Gamelin E, Marty M, Sastre J, Seitz JF, Ychou M (1996) Two consecutive phase II studies of oxaliplatin (L-OHP) for treatment of patients with advanced colorectal carcinoma who were resistant to previous treatment with fluoropyrimidines. Ann Oncol 7:95-98.

151. Magerl W, Koltzenburg M, Schmitz JM, Handwerker HO (1996) Asymmetry and time-course of cutaneous sympathetic reflex responses following sustained excitation of chemosensitive nociceptors in humans. J Auton Nerv Syst 57:63-72.

152. Maingret F, Lauritzen I, Patel AJ, Heurteaux C, Reyes R, Lesage F, Lazdunski M, Honore E (2000) TREK-1 is a heat-activated background K(+) channel. EMBO J 19:2483-2491.

153. Mantyh PW (2006) Cancer pain and its impact on diagnosis, survival and quality of life. Nat Rev Neurosci 7:797-809.

154. McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52-58.

155. McIlwrath SL, Lawson JJ, Anderson CE, Albers KM, Koerber HR (2007) Overexpression of neurotrophin-3 enhances the mechanical response properties of slowly adapting type 1 afferents and myelinated nociceptors. European Journal of Neuroscience 26:1801-1812.

156. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM, Fanger CM (2007) TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci U S A 104:13525-13530.

157. Menendez L, Bester H, Besson JM, Bernard JF (1996) Parabrachial area: electrophysiological evidence for an involvement in cold nociception. J Neurophysiol 75:2099-2116.

158. Meyer R, Ringkamp.M, Campbell J, Srinivasa R (2005) Peripheral mechanisms of nociception. In: Wall and Melzacks Textbook of pain (Stephen McMahon, Martin Koltzenburg, eds), pp 3-34.

159. Mizuno A, Matsumoto N, Imai M, Suzuki M (2003) Impaired osmotic sensation in mice lacking TRPV4. Am J Physiol Cell Physiol 285:C96-101.

160. Mohammadi B, Mitrovic N, Lehmann-Horn F, Dengler R, Bufler J (2003) Mechanisms of cold sensitivity of paramyotonia congenita mutation R1448H and overlap syndrome mutation M1360V. The Journal of Physiology 547:691-698.

161. Moqrich A, Hwang SW, Earley TJ, Petrus MJ, Murray AN, Spencer KS, Andahazy M, Story GM, Patapoutian A (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. Science 307:1468-1472.

162. Munns C, AlQatari M, Koltzenburg M (2007) Many cold sensitive peripheral neurons of the mouse do not express TRPM8 or TRPA1. Cell Calcium 41:331-342.

163. Muraki K, Iwata Y, Katanosaka Y, Ito T, Ohya S, Shigekawa M, Imaizumi Y (2003) TRPV2 Is a Component of Osmotically Sensitive Cation Channels in Murine Aortic Myocytes. Circ Res 93:829-838.

164. Nagy I, Rang H (1999) Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. Neuroscience 88:995-997.

165. Namer B, Kleggetvait IP, Jorum E (2007) 458 Results from TRPM8 and TRPA1 activation through menthol and cinnamaldehyde in patients with cold allodynia following cold injury. European Journal of Pain 11:203.

166. Namer B, Seifert F, Handwerker HO, Maihofner C (2005) TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. [Miscellaneous Article]. Neuroreport 16:955-959.

167. Nealen ML, Gold MS, Thut PD, Caterina MJ (2003) TRPM8 mRNA is expressed in a subset of cold-responsive trigeminal neurons from rat. J Neurophysiol 90:515-520.

168. Neeper MP, Liu Y, Hutchinson TL, Wang Y, Flores CM, Qin N (2007) Activation Properties of Heterologously Expressed Mammalian TRPV2: Evidence for species dependence. J Biol Chem 282:15894-15902.

169. Nicholson GM, Walsh R, Little MJ, Tyler MI (1998) Characterisation of the effects of robustoxin, the lethal neurotoxin from the Sydney funnel-web spider Atrax robustus, on sodium channel activation and inactivation. Pflugers Archiv European Journal of Physiology 436:117-126.

170. Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M, Noguchi K (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. J Clin Invest 115:2393-2401.

171. Ochoa JL, Yarnitsky D (1994) The triple cold syndrome: Cold hyperalgesia, cold hypoaesthesia and cold skin in peripheral nerve disease. Brain 117:185-197.

172. Okazawa M, Inoue W, Hori A, Hosokawa H, Matsumura K, Kobayashi S (2004) Noxious heat receptors present in cold-sensory cells in rats. Neuroscience Letters 359:33-36.

173. Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 4:529-539.

174. Pearce JMS (2005) The Law of Specific Nerve Energies and Sensory Spots. European Neurology 54:115-117.

175. Pearn J (2001) Neurology of ciguatera. J Neurol Neurosurg Psychiatry 70:4-8.

176. Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A (2002a) A TRP channel that senses cold stimuli and menthol. Cell 108:705-715.

177. Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A (2002b) A heat-sensitive TRP channel expressed in keratinocytes. Science 296:2046-2049.

178. Perl ER (1968) Myelinated afferent fibres innervating the primate skin and their response to noxious stimuli. J Physiol 197:593-615.

179. Perl ER (2007) Ideas about pain, a historical view. Nat Rev Neurosci 8:71-80.

180. Perl ER, Kruger L (1996) Nociception and Pain: Evolution of Concepts and Observations. In: Pain and Touch (Lawrence K, ed), pp 179-211. San Diego: Academic Press.

181. Price DD, Hu JW, Dubner R, Gracely RH (1977) Peripheral suppression of first pain and central summation of second pain evoked by noxious heat pulses. Pain 3:57-68.

182. Price MP, Lewin GR, McIlwrath SL, Cheng C, Xie J, Heppenstall PA, Stucky CL, Mannsfeldt AG, Brennan TJ, Drummond HA, Qiao J, Benson CJ, Tarr DE, Hrstka RF, Yang B, Williamson RA, Welsh MJ (2000) The mammalian sodium channel BNC1 is required for normal touch sensation. Nature 407:1007-1011.

183. Rasband MN, Park EW, Vanderah TW, Lai J, Porreca F, Trimmer JS (2001) Distinct potassium channels on pain-sensing neurons. Proc Natl Acad Sci U S A 98:13373-13378.

184. Rau KK, Jiang N, Johnson RD, Cooper BY (2007) Heat Sensitization in Skin and Muscle Nociceptors Expressing Distinct Combinations of TRPV1 and TRPV2 Protein. J Neurophysiol 97:2651-2662.

185. Raymond E, Chaney SG, Taamma A, Cvitkovic E (1998) Oxaliplatin: a review of preclinical and clinical studies. Ann Oncol 9:1053-1071.

186. Reeh PW, Kocher L, Jung S (1986) Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat? pp 42-50.

187. Reeh PW (1986) Sensory receptors in mammalian skin in an in vitro preparation. Neurosci Lett 66:141-146.

188. Reid G, Flonta ML (2001) Cold transduction by inhibition of a background potassium conductance in rat primary sensory neurones. Neuroscience Letters 297:171-174.

189. Renganathan M, Cummins TR, Hormuzdiar WN, Waxman SG (2000) alpha -SNS Produces the Slow TTX-Resistant Sodium Current in Large Cutaneous Afferent DRG Neurons. J Neurophysiol 84:710-718.

190. Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, Meyer RA (2001) Capsaicin Responses in Heat-Sensitive and Heat-Insensitive A-Fiber Nociceptors. J Neurosci 21:4460-4468.

191. Rutter AR, Ma QP, Leveridge M, Bonnert TP (2005) Heteromerization and colocalization of TrpV1 and TrpV2 in mammalian cell lines and rat dorsal root ganglia. Neuroreport 16:1735-1739.

192. Saab CY, Cummins TR, Dib-Hajj SD, Waxman SG (2002) Molecular determinant of Nav1.8 sodium channel resistance to the venom from the scorpion Leiurus quinquestriatus hebraeus. Neuroscience Letters 331:79-82.

193. Safronov BV, Bischoff U, Vogel W (1996) Single voltage-gated K+ channels and their functions in small dorsal root ganglion neurones of rat. J Physiol 493 (Pt 2):393-408.

194. Sahara Y, Gotoh M, Konno K, Miwa A, Tsubokawa H, Robinson HP, Kawai N (2000) A new class of neurotoxin from wasp venom slows inactivation of sodium current. Eur J Neurosci 12:1961-1970.

195. Sangameswaran L, Delgado SG, Fish LM, Koch BD, Jakeman LB, Stewart GR, Sze P, Hunter JC, Eglen RM, Herman RC (1996) Structure and Function of a Novel Voltage-gated, Tetrodotoxin-resistant Sodium Channel Specific to Sensory Neurons. J Biol Chem 271:5953-5956.

196. Sanofi-aventis US (2006) ELOXATIN (oxaliplatin injection) Prescribing information.

197. Santini D, Vincenzi B, La Cesa A, Casale M, Salvinelli F, Tonini G (2003) Recurrent Episodes of Involuntary Masticatory Spasms Induced by Continuous Infusion of Oxaliplatin. J Natl Cancer Inst 95:1555-1556.

198. Scadding JW, Koltzenburg M (2005) Painful peripheral neuropathies. In: Wall and Melzack's Textbook of Pain (McMahon SB, Koltzenburg M, eds), pp 973-999. Philadelphia: Elsevier.

199. Schäfer K, Braun HA, Isenberg C (1986) Effect of menthol on cold receptor activity. Analysis of receptor processes. J Gen Physiol 88:757-776.

200. Schmidt R, Schmelz M, Forster C, Ringkamp M, Torebjork E, Handwerker H (1995) Novel classes of responsive and unresponsive C nociceptors in human skin. J Neurosci 15:333-341.

201. Schnorf H, Taurarii M, Cundy T (2002) Ciguatera fish poisoning: a double-blind randomized trial of mannitol therapy. Neurology 58:873-880.

202. Screnci D, McKeage MJ, Galettis P, Hambley TW, Palmer BD, Baguley BC (2000) Relationships between hydrophobicity, reactivity, accumulation and peripheral nerve toxicity of a series of platinum drugs. Br J Cancer 82:966-972.

203. Shimosato G, Amaya F, Ueda M, Tanaka Y, Decosterd I, Tanaka M (2005) Peripheral inflammation induces up-regulation of TRPV2 expression in rat DRG. Pain 119:225-232.

204. Shin JB, Martinez-Salgado C, Heppenstall PA, Lewin GR (2003) A T-type calcium channel required for normal function of a mammalian mechanoreceptor. Nat Neurosci 6:724-730.

205. Simone DA, Kajander KC (1996) Excitation of rat cutaneous nociceptors by noxious cold. Neurosci Lett 213:53-56.

206. Simone DA, Kajander KC (1997) Responses of cutaneous A-fiber nociceptors to noxious cold. J Neurophysiol 77:2049-2060.

207. Slugg RM, Meyer RA, Campbell JN (2000) Response of Cutaneous A- and C-Fiber Nociceptors in the Monkey to Controlled-Force Stimuli. J Neurophysiol 83:2179-2191.

208. Spike RC, Puskar Z, Andrew D, Todd AJ (2003) A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. Eur J Neurosci 18:2433-2448.

209. Spray DC (1986) Cutaneous temperature receptors. Annu Rev Physiol 48:625-638.

210. Stokes AJ, Shimoda LMN, Koblan-Huberson M, Adra CN, Turner H (2004) A TRPV2-PKA Signaling Module for Transduction of Physical Stimuli in Mast Cells. J Exp Med 200:137-147.

211. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112:819-829.

212. Strachan LC, Lewis RJ, Nicholson GM (1999) Differential Actions of Pacific Ciguatoxin-1 on Sodium Channel Subtypes in Mammalian Sensory Neurons. J Pharmacol Exp Ther 288:379-388.

213. Stucky CL, Lewin GR (1999) Isolectin B4-Positive and -Negative Nociceptors Are Functionally Distinct. J Neurosci 19:6497-6505.

214. Suzuki M, Mizuno A, Kodaira K, Imai M (2003) Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 278:22664-22668.

215. Szolcsanyi J, Anton F, Reeh PW, Handwerker HO (1988) Selective excitation by capsaicin of mechano-heat sensitive nociceptors in rat skin. Brain Res 446:262-268.

216. Takashima Y, Daniels RL, Knowlton W, Teng J, Liman ER, McKemy DD (2007) Diversity in the neural circuitry of cold sensing revealed by genetic axonal labeling of transient receptor potential melastatin 8 neurons. J Neurosci 27:14147-14157.

217. Tamura S, Morikawa Y, Senba E (2005) TRPV2, a capsaicin receptor homologue, is expressed predominantly in the neurotrophin-3-dependent subpopulation of primary sensory neurons. Neuroscience 130:223-228.

218. Todd.J, Koerber.R (2005) Neuroanatomical substrates of spinal nociception. In: Wall and Melzack's Text book of Pain (McMahon SB, Koltzenburg M, eds), Scotland: Churchill Livingston.

219. Tominaga M, Caterina MJ (2004) Thermosensation and pain. J Neurobiol 61:3-12.

220. Torebjork HE (1974) Afferent C units responding to mechanical, thermal and chemical stimuli in human non-glabrous skin. Acta Physiol Scand 92:374-390.

221. Torebjork HE, Hallin RG (1970) C-fibre units recorded from human sensory nerve fascicles in situ. A preliminary report. Acta Soc Med Ups 75:81-84.

222. Torebjork HE, Hallin RG (1973) Perceptual changes accompanying controlled preferential blocking of A and C fibre responses in intact human skin nerves. Exp Brain Res 16:321-332.

223. Treede RD, Meyer RA, Raja SN, Campbell JN (1995) Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin. J Physiol 483 (Pt 3):747-758.

224. Treede RD, Meyer RA, Campbell JN (1998) Myelinated Mechanically Insensitive Afferents From Monkey Hairy Skin: Heat-Response Properties. J Neurophysiol 80:1082-1093.

225. Vallbo AB, Hagbarth K-E (1968) Activity from skin mechanoreceptors recorded percutaneously in awake human subjects. Experimental Neurology 21:270-289.

226. Vallbo AB, Johansson RS (1984) Properties of cutaneous mechanoreceptors in the human hand related to touch sensation. Hum Neurobiol 3:3-14.

227. Vallbo AB, Olausson H, Wessberg J (1999) Unmyelinated afferents constitute a second system coding tactile stimuli of the human hairy skin. J Neurophysiol 81:2753-2763.

228. Vallbo AB, Hagbarth KE, Wallin BG (2004) Microneurography: how the technique developed and its role in the investigation of the sympathetic nervous system. J Appl Physiol 96:1262-1269.

229. Van HJ, Gybels JM (1972) Pain related to single afferent C fibers from human skin. Brain Res 48:397-400.

230. Viana F, de la PE, Belmonte C (2002) Specificity of cold thermotransduction is determined by differential ionic channel expression. Nat Neurosci 5:254-260.

231. von DS, Eckel F, Wagenpfeil S, Mayr M, Stock K, Kullmann F, Obermeier F, Erdmann J, Schmelz R, Quasthoff S, Adelsberger H, Bredenkamp R, Schmid RM, Lersch C (2007) Carbamazepine for prevention of oxaliplatin-related neurotoxicity in patients with advanced colorectal cancer: final results of a randomised, controlled, multicenter phase II study. Invest New Drugs 25:173-180.

232. Vydyanathan A, Wu ZZ, Chen SR, Pan HL (2005) A-Type Voltage-Gated K+ Currents Influence Firing Properties of Isolectin B4-Positive But Not Isolectin B4-Negative Primary Sensory Neurons. J Neurophysiol 93:3401-3409.

233. Waddell PJ, McCarthy PW, Reeh PW (1989) The effect of 4-aminopyridine on action potential shape and firing pattern of rat dorsal root ganglion neurones. Pflugers Arch 414 Suppl 1:S131-S132.

234. Wahren LK, Torebjork E, Jorum E (1989) Central suppression of cold-induced C fibre pain by myelinated fibre input. Pain 38:313-319.

235. Wasner G, Schattschneider J, Binder A, Baron R (2004) Topical menthol--a human model for cold pain by activation and sensitization of C nociceptors. Brain 127:1159-1171.

236. Webster RG, Brain KL, Wilson RH, Grem JL, Vincent A (2005) Oxaliplatin induces hyperexcitability at motor and autonomic neuromuscular junctions through effects on voltage-gated sodium channels. Br J Pharmacol 146:1027-1039.

237. Wilson RH, Lehky T, Thomas RR, Quinn MG, Floeter MK, Grem JL (2002) Acute Oxaliplatin-Induced Peripheral Nerve Hyperexcitability. J Clin Oncol 20:1767-1774.

238. Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24:6410-6415.

239. Woolf C, Salter M (2005) Plasticity and pain: role of the dorsal horn. In: Wall and Melzack's Textbook of Pain (McMahon SB, Koltzenburg M, eds), pp 91-105. Philadelphia: Elsevier.

240. Yang E-K, Takimoto K, Hayashi Y, de Groat WC, Yoshimura N (2004) Altered expression of potassium channel subunit mRNA and [alpha]-dendrotoxin sensitivity of potassium currents in rat dorsal root ganglion neurons after axotomy. Neuroscience 123:867-874.

241. Yarnitsky D, Ochoa JL (1990) Release of cold-induced burning pain by block of cold-specific afferent input. Brain 113 (Pt 4):893-902.

242. Zhou L, Zhang CL, Messing A, Chiu SY (1998) Temperature-Sensitive Neuromuscular Transmission in Kv1.1 Null Mice: Role of Potassium Channels under the Myelin Sheath in Young Nerves. J Neurosci 18:7200-7215.

243. Zimmermann K, Leffler A, Babes A, Cendan CM, Carr RW, Kobayashi Ji, Nau C, Wood JN, Reeh PW (2007) Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. Nature 447:856-859.

244. Zotterman.Y (1953) Special senses: thermal receptors. Annu Rev Physiol 15:357-372.