Cold transduction mechanisms during peripheral neuropathic pain

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Ombretta Caspani
aus Mailand

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By Ombretta Caspani

from Milan

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ABBREVIATIONS

BDNF	brain derived neurotrophic factor
bp	base pairs
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
contra	contralateral side of the injury
DIG	digoxigenin
DNA	desoxyribonucleic acid
DRG	dorsal root ganglia
FITC	Fluorescein isothiocyanate
GDNF	glia cell line-derivated neurotrophic factor
HEK293	human embryonic kidney-derived cells
IB4	isolectin B4
IHC	immunohistochemistry
ipsi	ipsilateral side of the injury
L	lumbar DRG
mRNA	messenger ribonucleic acid
NF	neurofilaments
NFG	nerve growth factor
PCR	polymerase chain reaction
qRT-PCR	quantitative real time - PCR
RT-PCR	reverse transcriptase - PCR
SEM	standard error of the mean
SLP3	stomatin-like protein 3

SNL	spinal nerve ligation
TREK-1	TWIK-related K+ channel
TRP	transient receptor potential
TRPA1	member 1 of the TRP ankyrin family
TRPM8	member 8 of the TRP melastatin family
TRPV	TRP vanilloid family
TWIK	Tandem P domain weak inwardly rectifying K+ channel

1. INTRODUCTION

1.1 Somatosensation and nociception

Our peripheral sensory system allows us to experience many different kinds of sensations. A sensation starts with transduction, the process which converts stimulus energy into electrical energy. Working on my thesis I focused on the neurobiological logic behind the ability to detect touch and pain in mammals. These fundamental processes, termed somatosensation and nociception, respectively, allow for the detection of chemical, mechanical, and thermal stimuli, and can critically differentiate between innocuous and noxious stimuli.

Peripheral sensory neurons are the principle sensors of these stimuli and convert environmental cues into ascending neural activity. These neurons arise from cell bodies in the trigeminal or dorsal root ganglia (DRG) and innervate different regions of head and body respectively. The dorsal root ganglia are segmentally arranged adjacent to the spinal cord between the vertebrae. These pseudounipolar neurons send out one axon that divides into two branches, one is the fibre which projects out to the body's region and the other goes to the spinal cord. Sensory neurons are classified into three main groups according to their conduction velocity and hence the diameter and myelination proprieties of their axons. (figure 1A).

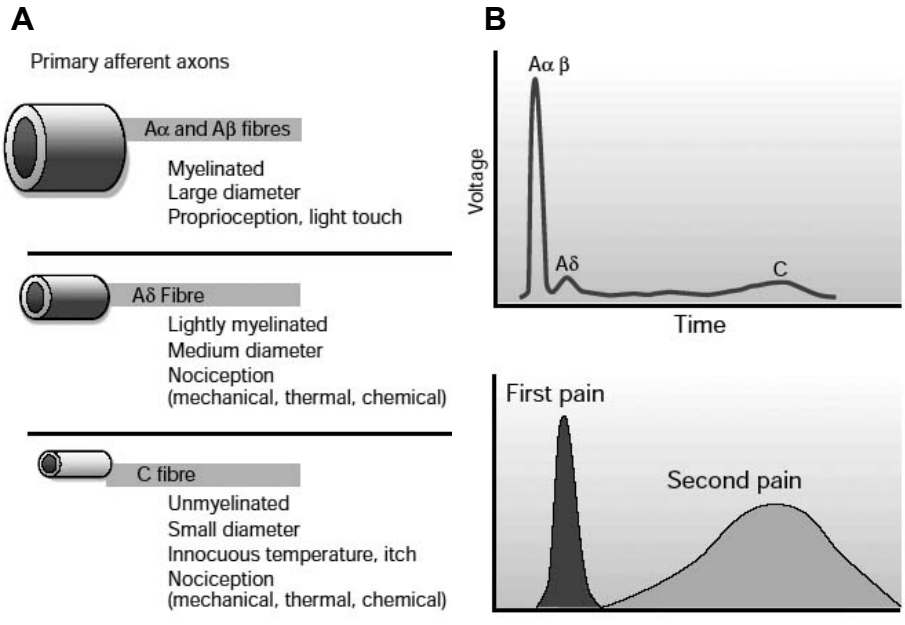

Figure 1: Different sensory neurons detect different types of sensation

A: Scheme of different classes of primary afferent sensory neurons;
B: Different compounds of action potential recording from a peripheral nerve.
(Modified from Fields, 1987)

The first group with the largest and heavily myelinated axons belong to the Aαβ class, conducting at a speed of over 10 m/sec; the second group are the Aδ fibres with thinly myelinated and medium diameter axons, propagating action potentials between 1-10 m/sec; the third group are the smallest unmyelinated C fibres with a conduction velocity of under 1 m/sec (Erlanger und Gasser, 1924; 1930; Gasser and Erlanger, 1927; Koltzenburg et al., 1997). This classification becomes crucial in the identification of the neuronal functions, in fact more than 80 percent of neurons with Aαβ fibres detect innocuous stimuli, which do not contribute to pain. They are the so-called proprioreceptors and mechanoreceptors. By contrast most of the C and Aδ sensory neurons detect stimuli capable of causing tissue damage and transmit signals that evoke pain. Specifically 90 percent of the C-fibres and 70 percent of the Aδ fibres sense noxious stimuli and take the name of nociceptors. It has long been assumed that Aδ and C nociceptors mediate 'first' and 'second' pain, respectively, namely the rapid, acute, sharp pain and the delayed, more diffuse, dull pain evoked by noxious stimuli (Julius and Basbaum, 2001; Hunt and Mantyh, 2001; Fang et al., 2005) (figure 1B).

C fibres represent 70 percent of all DRG neurons and consist of subgroups with different anatomical and functional properties; in particular two populations have been identified. One population of C fibres are the so-called peptidergic nociceptors, they express peptides such as substance P and calcitonin gene-related peptide (CGRP) and terminate in lamina I and in the outer lamina II of the spinal cord. The other population of C fibres are the IB4 positive nociceptors. Their name describes their ability to bind the isolectin B4 (IB4), and they terminate in the inner lamina II of the spinal cord. These two populations are influenced by different neurotrophic factors: nerve growth factor (NFG) promotes survival, growth and maintenance of the peptidergic nociceptors, while glia cell line-derivated neurotrophic factor (GDNF) regulates the IB4 positive nociceptor population

(Boucher and McMahon, 2001). Finally the different nociceptor populations have distinct electrophysiological proprieties, such that IB4 positive nociceptors have longer action potentials and smaller noxious heat responses (Stucky and Lewin, 1999).

1.2 Types of pain

Pleasure and pain are two major sensory emotions. While we usually seek pleasure from different activities, pain is something that normally signals impending tissue damage and thus evokes a strong avoidance. The Taxonomy Committee of the International Association for the Study of Pain has defined pain as 'unpleasant sensory and emotional experience associated with actual or potential tissue damage'. This definition includes the acute physiological nociceptive pain, which induces protective behavioural responses to save tissues from subsequent harm. In addition to physiological pain there is also what is called clinical pain which includes inflammatory and neuropathic pain.

Inflammatory pain is pain which occurs when tissue is inflamed or injured. Neuropathic pain is defined as "pain initiated or caused by a lesion or dysfunction in the peripheral or central nervous system" (Merskey and Bogduk, 1994). Both types of clinical pain are characterized by hypersensitivity at the site of damage and in adjacent normal tissue. Inflammatory pain hypersensitivity usually returns to normal if the disease process is controlled. Neuropathic pain persists long after the initiating event has healed (Woolf and Salter, 2000).

1.3 Neuropathic pain: etiology, symptoms and mechanisms

Neuropathic pain is a complex clinical syndrome which affects 5 % of the population in Europe, has a massive cost for the health care system and is personally devastating to the people who experience it (Breivik et al., 2006).

In patients with neuropathic pain a dysfunction or a lesion of the nervous system initiates complex mechanisms which produce distinct negative and positive sensory symptoms. These symptoms coexist in different combinations. Negative sensory symptoms involve a loss of sensitivity to stimuli in general and to painful stimuli in particular (hypoesthesia and hypoalgesia, respectively). Positive sensory

symptoms of neuropathic pain can be stimulus-independent. In this case sensations arise spontaneously without any peripheral stimulus, or they can be stimulus-dependent, in this case stimuli evoke stronger sensations than they normally do. Positive sensory symptoms include paresthesia (abnormal sensation, either evoked or spontaneous), dysesthesia (unpleasant, abnormal sensation, either evoked or spontaneous), hyperalgesia (increased response to a normally painful stimulus), and allodynia which is a painful response to a non-noxious stimulus (Serra, 1999).

Neuropathic pain may have multiple origins. Cancer is one of the major causes of neuropathic pain, but a variety of other neuropathologies may lead to neuropathic pain syndromes. The most common non-cancer neuropathic pain syndromes are listed in table 1 (Backonja and Serra, 2004).

Peripheral	Complex regional syndrome (type I and II)
	Posttraumatic nerve injury
	HIV sensory neuropathy
	Diabetic peripheral neuropathy
	Phantom limb pain
	Postherpetic neuralgia
	Trigeminal neuralgia
Central	Central poststroke pain
	Multiple sclerosis pain
	Spinal cord injury pain

Table 1. Noncancer neuropathic pain syndroms

Modified from Backonja and Serra, 2004

Clinicians used to classify neuropathic pain syndromes depending on the anatomical location and the underlying disease, but disease diagnosis by itself is not helpful in selecting the optimal pain treatment. In fact no one therapeutic drug class or agent is effective for all patients with neuropathic pain from a given etiology. Therefore scientists have proposed a new approach to the treatment of neuropathic pain, an approach based on the pathophysiological mechanisms.

Theoretically understanding the pathophysiological mechanisms, which underlie neuropathic pain symptoms may reveal novel targets for future putative analgesics (Woolf, 2004). In fact, each particular positive sensory symptom has a discrete pathophysiology, which may respond differentially to treatments targeting it. Therefore, it is of crucial importance to obtain a good understanding of the different pathophysiological mechanisms behind each of the symptoms and signs that constitute neuropathic pain.

The mechanisms which may contribute to neuropathic pain syndromes are multiple and complex, in big part still unknown and often controversial. Specifically, during peripheral neuropathic pain syndromes a lesion in a peripheral nerve triggers changes initially in the peripheral sensory system and successively in the central nervous system.

One change in the peripheral nervous system is the appearance of ectopic excitability. In fact, after peripheral nerve damage injured and neighbouring non-injured sensory neurons can generate ectopic discharges in the absence of any stimulus of the respective receptive field. Important factors causing the development of ectopic discharges are up-regulation of voltage gated sodium channels (Wood et al., 2004), and down-regulation of potassium channels (Everill and Kocsis, 1999). In addition, the inflammation process following nerve injury plays an important role in initiating the alteration of the sensory neurons. Indeed, activated macrophages infiltrate from endoneural blood vessels into the nerve and dorsal root ganglia, releasing proinflammatory cytokines (Sommer, 2003). These mediators induce ectopic activity in both injured and adjacent uninjured primary afferent nociceptors at the lesion site (Marchand et al., 2005).

Another important change is the phenotypic switch of the sensory neurons, so that neurons belonging to a specific neuronal subtype start expressing molecules which are normally distinctively expressed by other neuronal subtypes. For example, the neuromodulators BDNF (brain derived neurotrophic factor) and substance P are normally expressed only in C-fibres, but after peripheral nerve injury begin to be expressed in A fibre neurons (Noguchi et al., 1995; Fukuoka et al., 2001).

One more peripheral change is the degeneration of primary sensory neurons. In particular, peripheral nerve injury interrupts the contact of the cell bodies of dorsal

root ganglia with their peripheral targets, which are source of neurotrophic factors such as NGF or GDNF, as a result of that there are atrophic changes in the injured neurons followed by their death (Tandrup et al., 2000). Loss of neurons and the resulting imbalance contributes to the abnormal sensations.

As a consequence of the peripheral hyperactivity, subsequent changes occur in the spinal cord, such as dorsal horn neurons increasing their responsiveness to synaptic inputs. After peripheral nerve injury the inputs from the primary sensory neurons are stronger, consequently the central responsiveness increases (Woolf, 2004).

Additional to the increasing in the responsiveness of the dorsal horn neurons, another important central change, which occurs after nerve injury, is the apoptotic loss of inhibitory neurons in the superficial dorsal horn of the spinal cord (Moore et al., 2002).

Changes in the central nervous system might also be augmented by non-neural glial cells in the spinal cord. Peripheral nerve injury activates spinal cord glia and causes these cells to enhance pain by releasing proinflammatory cytokines and glutamate and BDNF (Wieseler-Frank et al., 2005; Coull et al., 2005).

Given the complexity of this pathology it is important to understand in detail the different underlying mechanisms. One aspect of the sensory system that has not been extensively studied in the context neuropathic pain is the transduction process, which is the starting point of sensation. The main reason for the lack of information on transduction during neuropathic pain is that until recently very little was known about the molecular mechanisms of transduction in mammals. In recent times several mammalian transduction molecules have been identified.

One example is stomatin-like protein 3 (SLP3). In the work done together with Gary Lewin's group in which I collaborated during my PhD (see appendix 1), we demonstrated that stomatin-like protein 3 (SLP3) is an essential component of the mammalian mechanotransduction complex, and we showed that in SLP3 knockout mice the mechanical hyperalgesia induced by chronic constriction injury (CCI) of the sciatic nerve was impaired in comparison with the wild type, while no impairment of CCI-induced thermal hyperalgesia were observed (appendix 1:

Wetzel et al., 2007). This makes SLP3 a possible important target against mechanical hyperalgesia. Moreover, this study displayed the importance of transduction mechanisms in neuropathic pain.
In my thesis I concentrated on cold allodynia. Patients with this symptom feel pain after stimuli which normally give a pleasant cool sensation. In particular, I focused on cold sensation and on specific transduction mechanisms of cold in animal models of neuropathic pain conditions and I investigated whether these proposed mechanisms are modified in the presence of cold allodynia.

1.4 Thermosensation and cold sensation

The ability to perceive temperature is fundamental for the survival of animals. Our perception of temperature allows us to differentiate between hot and cold and between innocuous and noxious, thus we can experience noxious warm (≥ 43°C), innocuous warm (32°C-42°C), innocuous cold, or cool (30°C-15°C) and noxious cold (below 15°C). (Dhaka et al., 2006).
We sense the temperature of our skin and surroundings using different populations of sensory neurons whose peripheral terminals can detect specific ranges of temperatures, and through action potentials send the signals to the central nervous system where they are integrated and interpreted to trigger appropriate reflexive and cognitive responses. These sensory neurons take the general name of thermoreceptors and, more specifically, warm or cold thermoreceptors.

The first studies on thermoreceptors were done using single-fibre recordings. The first publications appeared in the 1950s by Hensel and Zotterman (Hensel and Zotterman, 1951). They reported the temperature dependence of cutaneous C and Aδ fibres, which are part of specific thermosensor neurons present in nearly all vertebrates, but are especially important in mammals. These classic studies done in cats showed that cold and warm thermoreceptors fire action potentials with a static discharge frequency at steady-state temperature. In warm thermoreceptors, the discharge frequency increases steeply when the temperature rises from 30 °C to 43 °C and then falls off at higher temperatures; in cold thermoreceptors, the

discharge frequency rises as temperature drops from 40 °C to around 25 °C, and then decreases to a stationary frequency.

The instantaneous responses of warm and cold thermoreceptors to temperature changes are mirror images of one another. Warm thermoreceptors exhibit an increase in discharge frequency on heating, and a decrease on cooling; the opposite is true for cold thermoreceptors (Campero et al., 2001). This implies that cooling evokes a dual message to the brain: an increased activity of cold-sensitive fibres and a decreased activity of warm-sensitive fibres. Another interesting aspect of the thermoreceptor activity comes from looking at their steady-state discharge frequency at different temperatures, in fact a single thermoreceptor can have the same steady-state discharge rate at two different temperatures (Spray, 1986). This observation raises the question of how the sensation of different temperatures is decoded, and supports the hypothesis that diverse signals from varied type of neurons are integrated at the central level.
Since the publications of Hensel and Zotterman, a lot of studies have investigated thermoreceptors in different species, in particularly in primates (Iggo, 1969; Darian-Smith et al., 1973; Hensel and Iggo, 1971). More recently a non-invasive technique, microneurography, allowed us to obtain detailed information on human cold thermoreceptors (Campero et al., 1996; Campero et al., 2001). In two different studies Campero and colleagues examined cold-specific fibers and cold-sensitive polymodal nociceptors. In the first case they investigated the cold thermoreceptors described by Hensel and Zotterman, which were insensitive to mechanical stimuli and had temperature threshold for activation of 29°C. This study showed a very high sensitivity of the cold sensory system, in fact the perception of cool temperature occurs when the skin is cooled as little as 1 °C. (Campero, 2001). In the other study they selected polymodal fibers sensitive to mechanical, heat and cold stimuli and applied graded ramps of low temperature, between 20 to 0°C. The first action potential was recorded at a skin-thermode interface temperature of 10°C. Action potentials with higher discharge frequencies were recorded at lower temperature (Campero et al., 1996). However, the exact proportion of nociceptors that respond to noxious cold temperatures is not clear, with reported percentages ranging from 10 to 100% of Aδ- and C-fibers (Leem et al., 1993; Simone and Kajander, 1996 and 1997).

In the late 1990s, a number of laboratories started studying cold thermoreceptors using cultured dorsal root (DRG) or trigeminal (TG) ganglion neurons as in vitro models of afferent nerves: following excision of the DRG and loss of the axon, proteins normally destined for the receptor terminal begin to appear in the soma and can be studied with patch-clamp recording or calcium imaging. In contrast to fiber recordings, it is consistently reported that approximately 10–30% of these cells will respond to cold temperatures, with thresholds for activation ranging from ~30 to near 15°C (Reid and Flonta, 2001; McKemy et al., 2002; Thut et al., 2003).

Despite substantive advances at the physiological level, which have given a lot of information about the specialization of thermosensory functions, until recently very little was known about the molecular nature of mammalian thermotransduction. A major breakthrough in understanding thermal transduction came with the identification and characterization of the heat-activated ion channel TRPV1. This channel belongs to the transient receptor potential (TRP) family, and is active at temperature above 42°C. Experiments with null TRPV1 mice indicated a role of the channel in the noxious heat sensation and also in nociception and inflammation (Caterina et al., 1997 and 2000; Tominaga et al., 1998; Davis et al., 2000).

The transient receptor potential proteins are non-selective cation channels made up of four subunits having six transmembrane domains flanked by intracellular C- and N-terminal regions of variable length and a pore loop between transmembrane domain 5 and 6.

After the cloning of TRPV1, other transient receptor potential channels including TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1 were found to be activated by specific temperature range and proposed as likely thermosensors (figure 2). Hence these six channels are called with the general name of ThermoTRPs. All thermoTRPs are chemicalsensors too, and each of them is activated or modulated by different molecules. The chemical sensitivity of the transient receptor potential channels is a very important characteristic in understanding the details of our perception. In fact, chemical and thermal sensitivity of ion channels, which can trigger the sensory neurons, may explain why natural products can give feelings

similar to physical stimuli such as heat and cold. Thus capsaicin, the main ingredient in hot chilli peppers, elicits a sensation of burning pain and activates the noxious heat sensitive channel TRPV1.

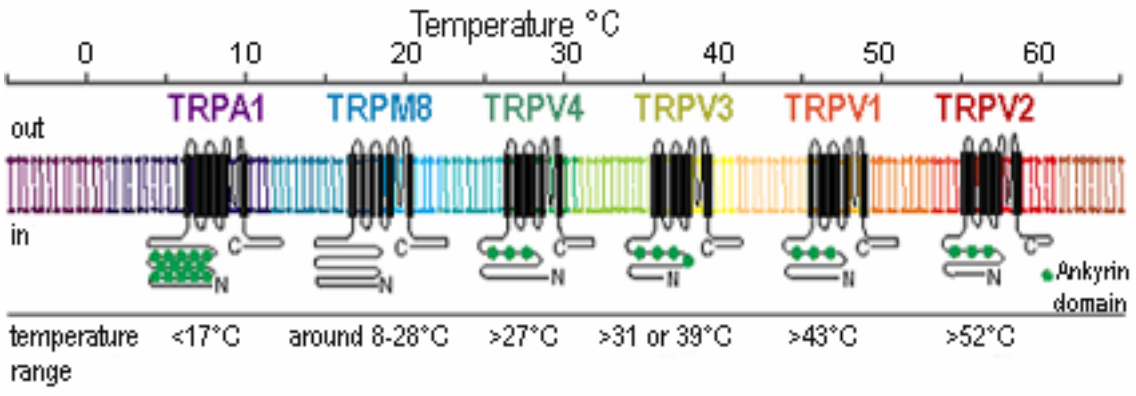

Figure 2: Schematic representation of the thermosensitive TRP channels.

Proposed membrane topology and functionally important domains of the channels are represented. They include six putative transmembrane units with a proposed pore region between transmembrane domains 5 and 6. The amino and carboxy termini are cytoplasmic and contain various interaction domains like variable number of ankyrin repeats. Listed below each channel are their reported thermal thresholds and the range of temperatures to which they respond. Together these channels can, theoretically, account for the detection of all commonly encountered thermal stimuli, from noxious cold to noxious heat.
Modified from Jordt et al., 2003

In the study of cold sensation, TRPM8, which is active at cool temperature, and TRPA1, which has also been postulated to activate at noxious cold temperature, have attracted the most attention. I will discuss these 2 channels in detail below. However, in the context of cold sensation in pathological conditions the two warm-activated transient receptor potential channels, TRPV3 and TRPV4 might also be important. Both these transient receptor potential channels share 40-50% homology with TRPV1 and were hypothesized to mediate the signalling of warm temperatures in ranges below that detected by TRPV1.

TRPV3 is activated by warm temperature threshold of 33°C, with increased responses to higher noxious thermal stimuli and enhanced current following repetitive heat stimulation (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002).

TRPV3 is also strongly activated and sensitised by camphor. Instead, camphor modulates warm sensation, consistent with its activity on TRPV3 (Green, 1990).
In addition, other natural compounds as irritants extracted from thyme, oregano and cloves activate TRPV3 (Xu et al., 2006).
In humans, TRPV3 is expressed in trigeminal and dorsal root ganglia neurons, spinal cord, brain, tongue and keratinocytes (Xu et al., 2002). In mice TRPV3 is prominently expressed in keratinocytes (Peier et al., 2002), although we found TRPV3 mRNA in dorsal root ganglia too.

TRPV4 is a polymodal receptor activated by warm temperature above 26°C, hypotonicity, protons, nitric oxide and several natural compounds such as endocannabinoids and arachidonic acid metabolites (Watanabe et al., 2002; Guler et al., 2002; Strotmann et al., 2000; Wissenbach et al., 2000; Liedtke et al., 2000; Watanabe et al., 2003; Vriens et al., 2005).
TRPV4 is expressed in several tissues, including the hypothalamus, keratinocytes, and primary sensory neurons (Guler et al., 2002; Liedtke et al., 2000; Alessandri-Haber et al., 2003).

In 2005 the laboratory of Patapoutian investigated the thermosensitivity in TRPV3 knockout mice, while the laboratory of Caterina investigated the thermosensitivity in TRPV4 knockout mice. In both studies, behavioural experiments showed impaired thermosensation in the knockout mice in comparison with the wild type, supporting the hypothesis of a contribution of both the ion channels in the molecular detection of thermal stimuli (Moqrich et al., 2005; Lee et al., 2005).

The strong expression of these two TRP channels in keratinocytes also raised the possibility that skin participates in mammalian thermosensation. Although analogies were found in the electrophysiological analyses of keratinocytes and TRPV3 and TRPV4 heterologuos expression cells, no communication systems between keratinocytes and sensory neurons have been found yet (Dhaka et al., 2006).

Besides the thermosensitive transient receptor potential ion channels, another thermosensory mechanism has been proposed to contribute to cold sensation, this

mechanism involves the two pore potassium channel TREK-1 (TWIK-related K+ channel; TWIK: Tandem P domain weak inwardly rectifying K+ channel).
TREK-1, which is highly expressed in dorsal root ganglia and hypothalamus, is strongly inhibited by cooling. This led to the hypothesis that cold could induce the closing of the background potassium channel causing indirect depolarization of sensory neurons (Maingret et al., 2000; Viana et al., 2002). However TREK-1-null mice do not exhibit any significant differences in response to cold in isolated skin-saphenous nerve preparations (Alloui et al., 2006). Indeed, C-fibers recorded from TREK-1 knockout mice are more sensitive to heat than wild types. This observation is consistent with cellular localization of this potassium channel, which is extensively co-localized with TRPV1 (Alloui et al., 2006). Thus, TREK-1 likely plays an important role in membrane excitability in sensory afferents, and its absence makes nociceptors more readily depolarized, however it does not appear to have a direct role in thermosensation.

1.5 TRPM8

The first big step in understanding the molecular mechanisms of cold transduction was made in 2002 with the cloning by two independent laboratories of TRPM8, the sensor for cold and menthol (McKemy et al., 2002; Peier et al., 2002).

TRPM8 belongs to the melastatin subfamily of transient receptor potential channels (TRPM). These channels differ from many other TRP family members in that they possess a much longer amino terminal cytosolic domain that is devoid of ankyrin repeats.
In addition to menthol several other cooling agents, including icilin, eucalyptol, and WS-3 (N-Ethyl-p-menthane-3-carboxamide), activate TRPM8 in vitro (Mc Kemy et al., 2002; Behrendt et al., 2004). Icilin is considered a super-cooling agent since it has higher potency and efficacy than menthol in cellular and behavioral studies (Mc Kemy et al., 2002; Wei et al., 1983). Together with the number of cooling agents that activate TRPM8, several antagonists have been identified, including BCTC, thio-BCTC, capsazepine, and protons (Behrendt et al., 2004; Andersson et al., 2004).

Menthol is a cyclic terpene alcohol which gives leaves of the genus Mentha their distinct smell and flavor. It has been known for centuries that menthol produces a sensation of cold (Sprengell, 1735; Siegel, 1970). Moreover, cold and menthol both induce membrane depolarization and firing of action potentials in a subpopulation of sensory neurons (Hensel and Zotterman, 1951; Reid and Fonda, 2001). McKemy and colleagues cloned TRPM8 by using menthol as a stimulus to isolate a cDNA from rat trigeminal cDNA library that could confer menthol sensitivity in heterologous expression system (Mc Kemy et al., 2002).
Peier and colleagues cloned the same gene looking for TRP-related sequences expressed in mouse dorsal root ganglia neurons and testing them for thermal sensitivity (Peier et al., 2002).

Several *in vitro* findings supported the hypothesis that TRPM8 is an important molecular mediator of cold perception. In heterologous expression systems, TRPM8 was activated at a temperature threshold of ≅ 28 °C with currents increasing in magnitude down to 8°C. Thus, the temperature range detected by TRPM8 seems to include both innocuous cool (30-15°C) and noxious cold (under 15°C) (McKemy et al., 2002; Peier et al., 2002). TRPM8 heterologous expression systems presented several physical characteristics similar to the one of cold-menthol sensitive sensory neurons, these included outward rectification, ion selectivity, adaptation and the shift of activation temperature by high concentration of menthol (McKemy et al., 2002; Peier et al., 2002).
The percentages of TRPM8 expressing neurons and of cold-menthol sensitive neurons were similar too. In mice and rats TRPM8 is expressed in 5-10 % of dorsal root ganglia neurons, similar to the percent of cold-menthol sensitive neurons, which is around 7% (Peier et al., 2002; Reid and Fonda, 2001). Analogous to the cold-menthol sensitive neurons, TRPM8 expressing neurons are of small diameter, suggesting them to be C-fibers. Nevertheless TRPM8 is not co-expressed with any other known marker, such as neurofilament or CGRP and is not expressed in IB4 positive neurons either (Peier et al., 2002). Therefore, the neurons expressing TRPM8 belong neither to a class of myelinated A fibers nor to a class of afferents historically considered to be nociceptors.
There is no evidence for epidermal expression of TRPM8. However this protein was initially identified in a human prostate cancer line (Tsavaler et al., 2001),

suggesting that it may, under some circumstances, be expressed in non-neuronal epithelial cells.

Recently three independent research groups generated TRPM8 knockout mice and provided important outcomes regarding the participation of TRPM8 in cold transduction (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). *In vitro*, the percentage of cold sensitive sensory neurons from TRPM8 null mice was remarkably reduced in comparison with the wild type neurons, but not abolished, confirming the existence of TRPM8-dependent and TRPM8-independent mechanisms for cold transduction (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Moreover Bautista and colleagues investigated the cold sensitivity in the TRPM8 null neurons at two different temperature thresholds, 22°C and 12°C. According to their results in TRPM8 null mice the population of neurons with temperature threshold at 22°C was completely eliminated, while the population of neurons with temperature threshold at 12°C was unaltered in comparison with the wild type population. This seems to indicate that the sensitivity to cool temperature is completely dependent on TRPM8, while the TRPM8-independent mechanism of cold sensitivity is only involved in the noxious cold detection.
The role of TRPM8 in cold sensation was confirmed by behavioural experiments too. All three different TRPM8 knockout mice were deficient in avoiding certain temperatures although there were discrepancies regarding the affected temperature range (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Each study showed with behavioural experiments that TRPM8 knockout mice had grave impairments in cold perception, but at the same time they were able to discriminate very cold temperature. These results together with the *in vitro* analysis of sensory neurons make firmer the hypothesis that TRPM8 is a physiological transducer of cold, but that other mechanisms of cold transduction also exist.

A controversial point in the three different null TRPM8 mice and their analyses regards the menthol sensitivity. In calcium imaging analyses of cultured DRG neurons, Dhaka et al. and Colburn et al. found a massive reduction, but not elimination, of menthol sensitive neurons, moreover these remaining menthol

sensitive neurons in TRPM8 null mice were not cold sensitive. That would suggest that TRPM8 is not the only menthol sensor, and another menthol sensitive mechanism, which does not influence cold sensitivity, is present. On the contrary Bautista and colleagues reported a complete removal of menthol sensitive neurons in the TRPM8 null mice and they consequently proposed that TRPM8 is the sole and exclusive menthol sensor in the sensory neurons.
A recent study supports the hypothesis that menthol is not entirely selective for TRPM8, indeed Mahieu and colleagues demonstrated that menthol evoked Ca2+ release from intracellular stores via a TRPM8-independent pathway (Mahieu et al., 2007).

One issue regarding TRPM8, which has not yet found an answer, is whether TRPM8 has a role in the alteration of cold sensitivity during neuropathic pain. Contrasting results have emerged. Colburn and colleagues used chronic constriction injury (CCI) of the sciatic nerve to induce neuropathic pain in TRPM8 knockout and wild type mice and tested their cold sensitivity using the acetone test (see methods). While the wild type mice showed strong pain behaviors, TRPM8 null mice did not exhibit nocifencive behaviors, similar to the uninjured control mice (Colburn et al., 2007). In contrast, another group showed that intrathecal TRPM8 anti-sense did not alter cold plate lift responses following spinal nerve ligation in rats (Katsura et al., 2006).

1.6 TRPA1

Another exciting moment in the story of the transient receptor potential family was the cloning and characterization of TRPA1, which was proposed to be the transducer for noxious cold in sensory neurons (Story et al., 2003). Although this hypothesis opened a big unresolved controversy (see below), TRPA1 turned out to be an especially interesting channel.

TRPA1 is the sole mammalian member of the ankyrin subfamily of transient receptor potential channels (TRPA). This channel is characterized by a long amino terminal cytosolic domain containing fourteen ankyrin repeats and is expressed in trigeminal and dorsal root ganglia neurons and in the inner ear (Story et al., 2003;

Corey et al., 2004). In particular TRPA1 is expressed in small diameter sensory neurons and normally colocalized with TRPV1, which is a known marker for nociceptors.
Other characteristics of this cannel are consistent with a role of TRPA1 in nociception. For instance, TRPA1 is activated by pungent compounds of mustard oil, garlic, wintergreen oil, clove oil, ginger and cinnamon oil (Jordt et al., 2004; Bandell et al., 2004; Bautista et al., 2005; Macpherson et al., 2006) all of which induce acute painful burning or pricking sensation.

Story and colleagues cloned TRPA1 in 2003 using a genome-based approach similar to the one that identified TRPM8. They detected the channel in sensory neurons and observed that in heterologous expression systems TRPA1 was activated by cold with a temperature threshold of 17°C. Consequently they suggested this channel as a candidate receptor for noxious cold temperature (Story et al., 2003).
Successively this hypothesis was contradicted by other groups. One group was unable to activate TRPA1 with cold (Jordt et al., 2004), while another group, analyzing sensory neuron cultures, found no correlation between the noxious cold sensitive neurons and the neurons expressing TRPA1 (Babes et al., 2005).
The generation of TRPA1 null mice by two independent laboratories did not resolve the controversy, since one group observed no cold sensation deficits, while the second group did. Bautista and colleagues found that the percentage of cold-sensitive sensory neurons in culture, number of nocifensive responses to acetone-evoked cooling, paw-withdrawal latencies, and induction of shivering in a cold-plate model (20 to −10°C) were indistinguishable in wild type and TRPA1 knockout mice (Bautista et al., 2006). On the contrary, Kwan and colleagues did report a decreased number of paw lifts when TRPA1 null mice were placed upon a surface cooled to 0°C, as well as in the duration of paw shakes during acetone evaporative cooling. However, this significant difference in thermal sensitivity was only observed in female mice and not in males. (Kwan et al., 2006). Therefore even after the generation of TRPA1 knockout mice the controversy on the cold sensitivity of TRPA1 is still open.

In an extensive review on cold sensitivity and TRP channels, Gordon Reid pointed out that the cold responses in the TRPA1 expression system were different from the noxious cold responses recorded in sensory neuron cultures (Reid, 2005).
In a parallel project done during my PhD studies we investigated the mystery of TRPA1 cold activation in heterologous expression systems and we provided evidence indicating that calcium plays an important role in cold activation of TRPA1 (see appendix 2). First we showed that intracellular calcium directly activates TRPA1 and this activation is mediated by an EF-hand domain in the N-terminal. Second, we saw cold responses in control cells not expressing TRPA1; however the cold responses in TRPA1 expressing cells were significantly bigger. Third, we demonstrated that TRPA1 is not directly gated by cold but is activated by increased basal intracellular calcium in heterologous cells during cooling (appendix 2: Zurborg et al., 2007).
In apparent contrast to an indirect mechanism of TRPA1 cold activation, Sawada and colleagues reported cold activation of TRPA1 in single-channel recordings under calcium chelating conditions (Sawada et al., 2007). This would suggest that thermal activation of TRPA1 is similar to the majority of thermoTRPs in that it does not require cytoplasmic processes, although such processes can affect the resulting response. All these contrasts in the cold activation of TRPA1 keep the role of TRPA1 in thermosensation unclear and further studies are needed to clarify the issue.

Both the studies with TRPA1 knockout mice confirmed the role of TRPA1 in nociception to several painful agents. They tested the irritant components of mustard oil and garlic with behavioural experiments and calcium imaging in sensory neuron primary cultures. They observed a strong decrease in pain behaviors and near absence of activation of neurons by these compounds (Bautista et al., 2006; Kwan et al., 2006).
In addition, Bautista and colleagues tested whether TRPA1 could mediate the toxicity of acrolein. Acrolein is a highly poisonous aldehyde present in tear gas and is a toxic metabolite of chemotherapeutic agents. In heterologous expression systems TRPA1 is activated by acrolein, and in calcium imaging experiments on trigeminal cultures of wild type mice acrolein evoked responses within the subpopulation of mustard oil sensitive neurons, while in cultures from TRPA1 null

mice acrolein-evoked responses were completely absent, indicating that TRPA1 is an essential site for acrolein action (Bautista et al., 2006).
TRPA1 is also activated by the proalgesic inflammatory peptide bradykinin. The two knockout studies investigated whether the inflammatory pain caused by bradykinin is mediated via TRPA1. Indeed, in both studies TRPA1 null mice showed substantially decreased responses to bradykinin at the cellular and behavioural level (Bautista et al., 2006; Kwan et al., 2006). All these findings indicate that TRPA1 is an important component of the transduction machinery that depolarizes nociceptors in response to endogenous and environmental irritants or proalgesic agents.

Until now very few studies have focused on TRPA1 during clinical pain. Noguchi's laboratory reported that treatment with TRPA1 antisense oligodeoxynucleotides reduced behavioral hypersensitivity to cold after CFA-induced inflammation or sciatic nerve injury (Obata et al., 2005); and alleviated cold hyperalgesia induced by L5 spinal nerve ligation, probably resulting from an increase in TRPA1 protein level in the nearby uninjured L4 DRG (Katsura et al., 2006). However in order to define the role of TRPA1 in clinical states further detailed studies are needed.

1.7 Objectives

My Ph.D. thesis focuses on the intense pain arising from contact with cold temperatures (cold allodynia), which commonly develops during neuropathic pain.

The overall hypothesis of this dissertation was that nerve injury induces changes in the peripheral mechanisms of cold transduction which lead to cold allodynia.

To test this hypothesis I used the chronic constriction injury of the sciatic nerve to induce neuropathic pain in mice and I analysed the mechanisms of cold transduction by behavioural, molecular biological (qRT-PCR), cell biological (*in situ* hybridization and immunohistochemstry) and electrophysiological methods. Specifically I examined whether the nerve injury generates changes in:

- ⇨ the *in vivo* activity of the best candidate for cold transduction, TRPM8;
- ⇨ the DRG mRNA levels of several candidates of temperature transducers, TRPM8, TRPA1, TRPV3, TRPV4 and TREK-1;
- ⇨ the neuronal populations expressing TRPM8 in the DRG;
- ⇨ the neuronal populations expressing TRPA1 in the DRG;
- ⇨ the populations of *in vitro* cold sensitive DRG neurons;
- ⇨ the populations of *in vitro* TRPM8 agonist sensitive DRG neurons and their cold sensitivity;
- ⇨ the populations of *in vitro* TRPA1 agonist sensitive DRG neurons and their cold sensitivity;
- ⇨ the functional and anatomical properties of the *in vitro* cold sensitive DRG neurons.

Manuscript

Some of the work from this thesis is described in a manuscript, which is currently under revision.

2 ANIMALS, MATERIALS AND METHODS

2.1 Animals and animal housing

Wild type C57BL/6j mice were bred in FEM (Forschungseinrichtungen für experimentelle Medizin) and housed in cages lined with ground corncob bedding. Standard laboratory rodent chow and tap water were available *ad libitum*. Room temperature was maintained at 22°C and at a relative humidity between 40 % and 60 %. A 12/12 hr (8 a.m./8 p.m.) light/dark cycle was used. Mice experiments were approved by the animal care committee of the Senate of Berlin.

2.2 Material

2.2.1 Technical equipment and other materials

Cloning and Cell culturing

Thermoblock	Eppendorf,	Hamburg, Germany
Bacterial Shaker	GFL Gesellschaft für Labortechnik GmbH	Burgwedel, Germany
Bacterial incubator (Heraeus Function Line T12)	Heraeus Instruments	Hanau, Germany
Cell culture dishes	TPP Europe	Trasadingen, Switzerland
Cell culture flasks	Nunc GmbH	Wiesbaden, Germany
Cell culture plates	Sigma-Aldrich	Taufkirchen, Germany
Cell scraper	Sigma-Aldrich	Taufkirchen, Germany
Cell strainer (40 µm mesh)	BD Biosciences Discovery Labware	Franklin Lakes, NJ, USA
cellular incubator (6000)	Heraeus Instruments	Hanau, Germany
Kryo tubes	Nunc GmbH	Wiesbaden, Germany
Laminar Flow (Hera Safe18)	Heraeus Instruments	Hanau, Germany
Mikroskope (Axiovert 25)	Carl Zeiss Mikroskopie	Göttingen, Germany

Spectrophotometer (Gene Quant II RNA/DNA calculator)	Pharmacia Biotech	Hørsholm, Denmark

Electrophoresis and documentation

Electrophoresis apparatus (Power Pac 300)	Bio-Rad Laboratories	Munich, Germany
UV-Light (Macro Vue UV=25)	Hoefer	San Francisco, CA, USA
Transilluminator (RH-2)	Herolab	Wiesloch, Germany
Monitor (CDM 1003)	MONACOR INTERNATIONAL GmbH	Bremen, Germany
Video copy processor (PSI)	Mitsubishi	Japan

PCR

PCR tubes and caps	Applied Biosystems	Foster City, CA, USA
Thermocycler (Gene Amp 9700)	Applied Biosystems	Foster City, CA, USA
Thermocycler (Mastercycler personal)	Eppendorf	Hamburg, Germany
Thermocycler (LightCycler 1.5 Instrument)	Roche Diagnostics Corporation	Mannheim, Germany

In situ hybridization and immunohistochemistry

Cryostat (Microm HM560)	MICROM International GmbH	Walldorf, Germany
Fluorescence microscope (Axioskop2)	Carl Zeiss Mikroskopie	Göttingen, Germany
Lamp (ebq 100)	Leistungselektronik	Jena, Germany

	Jena GmbH	
AxioCam MRC	Carl Zeiss Mikroskopie	Göttingen, Germany
Hybridization oven (Hybaid shake 'n' Stack)	Thermo Electron Corporation	Waltham, MA, USA

Calcium imaging

Inverted microscope (Axiovert 200)	Carl Zeiss Mikroskopie	Göttingen, Germany
Polychrome V monochromator	Till Photonics GmbH	Gräfelfing, Germany
CCD Imago camera	Till Photonics GmbH	Gräfelfing, Germany
Peltier device	E.S.F electronic	Göttingen, Germany
Thermocouple	E.S.F electronic	Göttingen, Germany
Temperature controller (RDTC-1)	E.S.F electronic	Göttingen, Germany
VC-6 six channel valve controller	Warner Instrument Corporation	Hamden, CT, USA
Peristaltic pump	World Precision Instruments	Berlin, Germany

Others

Combi tips (10/50 ml)	Eppendorf	Hamburg, Germany
Heraeus centrifuges (Megafuge 3.0 R, Multifuge 4 KR, Variofuge 3. OR, table top Biofuges pico and fresco)	Heraeus	Hanau, Germany
pH-Meter (MP220)	Mettler-Toledo	Schwerzenbach, Switzerland

Pipettes (1-25 ml, one-way)	Sarstedt AG & Co.	Nümbrecht, Germany
Reaction tubes (0.5/1.5/2.0 ml)	Eppendorf	Hamburg, Germany
Tubes (15 and 50 ml)	Falcon	Heidelberg, Germany
Ultrapure Water Systems (Direct-Q™ 5)	Millipore GmbH	Eschborn, Germany
Vortex mixer (2TM Mixer 7-2020)	NeoLab	Heidelberg, Germany
Vortex mixer (Voretex-Genie)	Scientific Industries	Bohemia, NY, USA
Water bath (SUB14)	Grant Instruments Cambridge Ltd	Shepreth, Great Britain
Thermoblock	Eppendorf	Hamburg, Germany
Syringes	Braun	Melsungen, Germany
Needles	Becton-Dickinson GmbH	Heidelberg, Germany
Sterile filters	Millipore GmbH	Eschborn, Germany

2.2.2 Chemicals and reagents

Behavioural experiments

Acetone	Sigma-Adldrich	Taufkirchen, Germany
(-)-menthol	Sigma-Adldrich	Taufkirchen, Germany

Cloning and qRT-PCR

β-Mercaptoethanol	Carl Roth GmbH	Karlsruhe, Germany
RNeasy mini kit	Qiagen	Hilden, Germany
Titan One Tube RT-PCR Kit	Roche Diagnostics GmbH	Mannheim, Germany
Reverse trascriptase Polymerase RT-AMW	Roche Diagnostics GmbH	Mannheim, Germany
pGEM-T Easy Vector Systems	Promega	Mannheim, Germany
RNAse inhibitor	Roche Diagnostics GmbH	Mannheim, Germany

Primer oligo dT	Roche Diagnostics GmbH	Mannheim, Germany
Taq polymerase	Roche Diagnostics GmbH	Mannheim, Germany
Gel extraction kit (QIAquick)	Qiagen	Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen	Hilden, Germany
QIAfilter Plasmid Maxi Kit	Qiagen	Hilden, Germany
Luria Bertani (LB) Agar	GIBCO Invitrogen Corporation	Karlsruhe, Germany
Ampicillin	E. Merck AG	Darmstadt, Germany
dNTP (Deoxy-nucleotide triphosphate mix)	Roche Diagnostics GmbH	Mannheim, Germany
DTT (Dithiothreitol)	Sigma-Aldrich	Taufkirchen, Germany
Fast Start DNA Master SYBR Green I Kit	Roche Diagnostics GmbH	Mannheim, Germany
Oligo dT (primer dT for cDNA synthesis)	Roche Diagnostics GmbH	Mannheim, Germany

Primers

The primers were purchased from TIBMOLBIOL (Berlin, Germany)

name	sequence	product lenght	application
TRPA1 full length upper	ggc gtc cag gtg gag tca	3527	cloning + in situ hybridization
TRPA1 full lenght lower	ggg tga ggt tgc agg aac ta		
TRPM8 full lenght upper	gct tct gtc tcc caa gtg ct	3382	cloning + in situ hybridization
TRPM8 full lenght lower	gat ttg gtt tgt ccc cac aa		
TRPA1-LC upper	atg tcc aat cat gga gat gg	352	cloning + qRT-PCR
TRPA1-LC lower	caa gaa ggg tca tgg gta tg		

TRPM8-LC upper	agg ctg gag atg aga ttg tg	313	cloning + qRT-PCR
TRPM8-LC lower	ctg aag tgg gtg gag aag ag		
TRPV3-LC upper	gag cag aca gac atc act tcc	366	cloning + qRT-PCR
TRPV3-LC lower	gat ttc cag cac aga gtt atc c		
TRPV4-LC upper	aca aga gat gga gga gaa agg	353	cloning + qRT-PCR
TRPV4-LC lower	tcg gta gta gat gtc tct gaa gg		
TREK 1-LC upper	gac gac cat tgg att tgg	344	cloning + qRT-PCR
TREK 1-LC lower	gag tca gtt cct ggt tgt gg		
galanin-LC upper	cat tta gcg aca agc atg g	322	cloning + qRT-PCR
galanin-LC lower	agc aga gaa cag agg att gg		
GAPDH-LC upper	aca gtc aag gct gaa aat gg	357	cloning + qRT-PCR
GAPDH-LC lower	cat gag ccc ttc tac aat gc		
TRPA1probe upper	aaa gga gct aag ctg tgt aaa tc	902	cloning + in situ hybridization
TRPA1probe lower	gtg gag agg ggt cat ccc		
TRPM8probe upper	aag caa gat ccc ttg tgt ggt	570	cloning + in situ hybridization
TRPM8probe lower	gat ctg cat gtt ccg gta ca		
TRPA1 263 upper	cgt gtg aag tgc tga	546	cloning + in situ

	ata taa tgg		hybridization
TRPA1 263 lower	cca tca tgt cga tgt gtg c		
TRPA1 673 upper	aac ggc tac agc agg gag ac	414	cloning + in situ hybridization
TRPA1 673 lower	gga gca aat tca caa tgt tcc		
TRPM8 242 upper	ccc aga tca acc aaa atg ag	628	cloning + in situ hybridization
TRPM8 242 lower	act ggt gcg ctc aga gat g		

Agarose gel electrophoresis

Bromophenol blue	Sigma-Adldrich	Taufkirchen, Germany
Agarose LE	Roche Diagnostics GmbH	Mannheim, Germany
Ethidium bromide	Sigma-Adldrich	Taufkirchen, Germany
DNA molecular weight marker (IX, 72-1350 bp)	Roche Diagnostics GmbH	Mannheim, Germany
DNA molecular weight marker (XIV, 100 bp)	Roche Diagnostics GmbH	Mannheim, Germany
DNA molecular weight marker (XVII, 500 bp)	Roche Diagnostics GmbH	Mannheim, Germany

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DEPC (diethylpyrocarbonate)	Sigma-Adldrich	Taufkirchen, Germany
TISSUE PREPARATION:		
Paraformaldehyde (PFA)	Sigma-Aldrich	Taufkirchen, Germany
Sucrose	Carl Roth GmbH	Karlsruhe, Germany
2-methylbutane	Sigma-Adldrich	Taufkirchen, Germany

Frozen Section Medium	Richard-Allan Scientific	Kalamazoo, MI, USA
Polysin Microscope Slides	Menzel GmbH	Braunscheig, Germany
Coverslips	Carl Roth GmbH	Karlsruhe, Germany
PROBES PREPARATIONS:		
High Pure PCR Product Purification Kit	Roche Diagnostics GmbH	Mannheim, Germany
DIG RNA labeling mix	Roche Diagnostics GmbH	Mannheim, Germany
T7 RNA polymerase	Roche Diagnostics GmbH	Mannheim, Germany
SP6 RNA polymerase	Roche Diagnostics GmbH	Mannheim, Germany
DNAse I	Qiagen	Hilden, Germany
DOT-SPOT test		
Positively Charged Nylon membrane	Roche Diagnostics GmbH	Mannheim, Germany
Blocking reagent for nucleic acid hybridization	Roche Diagnostics GmbH	Mannheim, Germany
Anti-DIG conjugated to alkaline phosphatase	Roche Diagnostics GmbH	Mannheim, Germany
NBT/BCIP solution	Roche Diagnostics GmbH	Mannheim, Germany
STAINING:		
Proteinase K	Sigma-Aldrich	Taufkirchen, Germany
Formamide	Sigma-Aldrich	Taufkirchen, Germany
Triethanolamine	Sigma-Aldrich	Taufkirchen, Germany
Acetic anhydride	Sigma-Aldrich	Taufkirchen, Germany
Yeast tRNA	Sigma-Aldrich	Taufkirchen, Germany
Denhardt's solution	Sigma-Aldrich	Taufkirchen, Germany
CHAPS	Sigma-Aldrich	Taufkirchen, Germany

Anti-DIG antibody conjugated with horseradish peroxidase	Roche Diagnostics GmbH	Mannheim, Germany
Tyramide Signal Amplification System	PerkinElmer,	Boston, MA, USA
FITC-labeled IB4	Sigma-Aldrich	Taufkirchen, Germany
Anti-CGRP (polyclonal)	Sigma-Aldrich	Taufkirchen, Germany
Anti-NF200 (clone N52)	Sigma-Aldrich	Taufkirchen, Germany
Normal horse serum	Vector Laboratories Inc.	Burlingame, USA
Normal goat serum	Vector Laboratories Inc.	Burlingame, USA
Fluorescin anti-mouse IgG made in horse	Vector Laboratories Inc.	Burlingame, USA
Fluorescin anti-rabbit IgG made in goat	Vector Laboratories Inc.	Burlingame, USA
Mowiol 4-88	CALBIOCHEM	San Diego, USA

DRG cultures

Collagenase IV	Sigma-Aldrich	Taufkirchen, Germany
Trypsin	Biochrom	Berlin, Germany
DMEM/Hams-F12 medium	GIBCO Invitrogen Corporation	Karlsruhe, Germany
Heat-inactivated horse serum	Biochrom	Berlin, Germany
Glutamine	GIBCO Invitrogen Corporation	Karlsruhe, Germany
Glucose	Sigma-Aldrich	Taufkirchen, Germany
Penicillin and streptomycin	Biochrom	Berlin, Germany
Poly-L-lysine	Sigma-Aldrich	Taufkirchen, Germany

Calcium Imaging

Pluronic F-127	GIBCO InvitrogenCorporation	Karlsruhe,Germany
FURA-2/AM dye	GIBCO InvitrogenCorporation	Karlsruhe,Germany
Mustard oil	Sigma-Aldrich	Taufkirchen,Germany
(-)-menthol	Sigma-Aldrich	Taufkirchen,Germany
Calcium chloride ($CaCl_2$)	Sigma-Aldrich	Taufkirchen,Germany
Magnesium chloride ($MgCl_{2)}$	Sigma-Aldrich	Taufkirchen,Germany
Glucose	Sigma-Aldrich	Taufkirchen,Germany
HEPES	Sigma-Aldrich	Taufkirchen,Germany

Others

DMSO (dimethyl sulfoxide)	Sigma-Aldrich	Taufkirchen,Germany
Ethanol 99.9%	Carl Roth GmbH	Karlsruhe,Germany
Restriction enzymes	GIBCO InvitrogenCorporation	Karlsruhe,Germany
Boric acid	Sigma-Aldrich	Taufkirchen,Germany
Tris-Base and Tris-HCl (Trishydroxymethylaminomethane)	Sigma-Aldrich	Taufkirchen,Germany
Tris-HCl	Sigma-Aldrich	Taufkirchen,Germany
Glycerol	Sigma-Aldrich	Taufkirchen,Germany
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	Taufkirchen,Germany
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	Taufkirchen,Germany
Acetic acid	Sigma-Aldrich	Taufkirchen,Germany
NaCl	Sigma-Aldrich	Taufkirchen,Germany
Formaldehyde	Sigma-Aldrich	Taufkirchen,Germany
Maleic acid	Sigma-Aldrich	Taufkirchen,Germany
Tween20	Sigma-Aldrich	Taufkirchen,Germany
Formamide	Sigma-Aldrich	Taufkirchen,Germany
Potassium chloride (KCl)	Sigma-Aldrich	Taufkirchen,Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich	Taufkirchen,Germany
Monopotassium phosphate	Sigma-Aldrich	Taufkirchen,Germany

(KH_2PO_4)		
Hydrochloride (HCl)	Merck AG	Darmstadt,Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich	Taufkirchen,Germany
EDTA (Ethylendiaminetetraacetic acid)	Sigma-Aldrich	Taufkirchen,Germany
Glycine	Sigma-Aldrich	Taufkirchen,Germany
Isofluran Curamed	Rhodia Organic Fine Limited	Bristol, UK
Methanol	Carl Roth GmbH	Karlsruhe,Germany

2.2.3 Media, buffers and solutions

Agarose gel electrophoresis

1xTBE buffer	100mM Tris base, 100mM boric acid, 2.5mM EDTA,
Sample buffer	0.25% bromphenol blue, 50mM Tris pH 7.6, 60%glicerol,
Agarose gel	1 or 2%agarose, 1xTBE, 0.0005% ethidium bromide,

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Fixation buffer	4% PFA in PBS pH7.4
DEPC H_2O	0.1% DEPC
Carbonate buffer	60mM Na_2CO_3, 40mM $NaHCO_3$, pH 10.2
Hydrolysis-neutralization buffer	3M Sodium acetate, 1% (V/V) acetic acid; pH 6,0
Blocking solution	1% (W/V) blocking reagent for nucleic acid hybridization in maleic acid solution
Detection buffer	100mM Tris-HCl, 100mM NaCl; pH 9.5
RNA-dilution buffer	DEPC-H2O, 20xSSC and formaldehyde mixed in a volume ratio of 5 + 3 + 2
Washing buffer	100mM Maleic acid, 150mM NaCl; pH 7.5, 0.3%Tween20
70% ethanol	70%ethanol in DEPC-H_2O

Proteinase K buffer	50 mM Tris-HCl, pH 7.5, 5 mM EDTA
Maleic acid buffer	100mM Maleic acid, 150mM NaCl; pH7.5
Mowiol solution	12g Glycerin and 4.8g Mowiol 4-88 in 24ml 0.2M Tris/HCl pH 8.5 solution. Stir at 50°C for 30-40minutes. Clarify by centrifugation at 4000-5000 rpm for 20 min and store the surnatant at 4°C
20x SSC	3M NaCl, 0.3M Sodium Citrate
Hybridization buffer	50%deionized Formamide, 5xSSC, 1mg/ml Yeast tRNA, 100µg/ml Heparin, 1xDenhardt's solution, 0.1%CHAPS, 5mM EDTA, 0.1%Tween20
IB4-blocking buffer	PBS with 0,1mM $MgCl_2$, $MnCl_2$, $CaCl_2$
NF200-blocking buffer	7% horse serum in maleic acid solution
CGRP-blocking buffer	7% goat serum in maleic acid solution

DRG cultures

DRG medium	10% Horse Serum, 1mM glutamine, 100u penicillin/100µg/ml streptomycin, 0.8% glucose, in DMEM/HAM'S F12 without glutamine

Calcium Imaging

calcium imaging buffer (CIB)	140mM NaCl, 4mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM Glucose, 10mM HEPES, pH 7.4
Pluronic solution	20% Pluronic F-127 in DMSO
FURA solution	50 µg FURA-2/AM dye diluted in 10 µl Pluronic solution and 50 µl DMSO

Other

10x PBS (Phosphate Buffer Saline)	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4., pH7.5
DEPC-PBS	1ml DEPC in PBS, mix well and autoclave
10xTN (Tris Sodiumcloride solution)	0.1M Tris-HCl; 0,15M NaCl; Ph 7.5

2.3 Methods

2.3.1 Surgical procedures

All experiments were conducted on C57/B6 mice. A chronic constriction injury (CCI) of the sciatic nerve was used to model neuropathic pain. Briefly, the right sciatic nerve was exposed at mid-thigh level under isoflurane anaesthesia. 3 loose silk ligatures were tied around the nerve and the incision was closed.

2.3.2 Behavioural experiments

For all behavioural experiments, mice were habituated to the test procedure for 7 days before surgery. Responses were taken 1 day prior to surgery and at 2, 4, 7 and 14 days postoperative.

The acetone test was used to assess cold allodynia in mice. In this test, evaporative cooling of locally applied acetone is sufficient to evoke nociceptive behaviour in CCI mice. 40µl of acetone was applied to the dorsal hind paw ipsilateral to the injury and the behaviour was assigned an arbitrary score. A score of 0 indicated no response, 0.5, a licking response, 1, flinching and brushing of the paw, 2, strong flinching, and 3, strong flinching and licking. Behaviour was observed during the first 30 seconds after acetone application and measurements were repeated 2 times with a 1 minute interval to obtain a mean value.

To assess the effects of menthol on nociceptive behaviour in CCI mice, 40µl of 250mM (-)-menthol dissolved in 90%DMSO and 10%PBS or vehicle alone was applied to the dorsal surface of the ipsilateral hind paw. Mice were observed for 5 minutes and the time spent licking the hind paw was measured. Experiments were repeated in control mice.

2.3.3 Molecular biology

Standard methods were performed according to Sambrook and Russell (1989) and will not be described in detail (agarose gel electrophoresis, bacterial cultures, ethanol precipitations, restriction digests, etc.).

2.3.4 Cloning: TRPA1, TRPM8, TRPV3, TRPV4, TREK1, GAPDH and Galanin fragments

Dissection of DRG

After killing the mouse using CO_2 the whole spine was dissected. After laminectomy, DRG from all spinal levels were dissected out and were frozen immediately on dry ice.

RNA extraction using RNeasy mini kit

Isolation and cleaning of total RNA from DRG was performed in accordance to the manufacturer's instructions.

Amplification of full length sequences for TRPA1 and TRPM8

mRNA of TRPA1 and TRPM8 are 3527 and 3382 bp long, respectively; the amplification of such long fragments needed a long range PCR, which was done using the Titan One Tube RT-PCR Kit (Reverse Trascriptase PCR). The reaction mix was prepared according to the manufactor's instructions; the following PCR profile was used:

Cycles	Temperature and time profile		
1x	Reverse transcription step	47 °C	30 min
1x	Template denaturation	94 °C	2 min.
10x	Denaturation	94 °C	10 sec.
	Annealing	60 °C for TRPA1 65°C for TRPM8	30 sec.
	Elongation	68 °C	3 min.
30x	Denaturation	94 °C	10 sec.
	Annealing	60 °C for TRPA1 65°C for TRPM8	30 sec.
	Elongation	68 °C	3 min. +5sec. each cycle
1x	Prolonged elongation	68 °C	7 min.
		4°C	∞

Amplification of the gene fragments used for detection in qRT-PCR and for the preparation of *in situ* hybridization probes

For the amplification of all these smaller sequences the following procedures were used.

Synthesis of cDNA library from total RNA

cDNA library was synthesised with the following reaction:

Reaction mix			Temperature and time profile		
Extracted RNA	1 µg		Annealing oligo-RNA	25 °C	10 min
RNAse inhibitor	1 µl		Enzymatic reaction	42 °C	1 h
dNTP	2 µl		Enzymatic degradation	99 °C	5 min
primer oligo dT	2 µl			4°C	∞
RT-AMW Buffer	4 µl				
ReverseTrascriptase-AMW	0,8 µl				
H_2O					
Final volume	20 µl				

Amplification of segments of interest

The fragments of interest were amplified with the following PCR reaction:

Reaction mix		Cycles	Temperature and time profile		
Upstream primer	2 µl	1x	Denaturation	94 °C	2 min.
Downstream primer	2 µl	35x	Denaturation	94 °C	30 sec.
dNTP	1 µl		Annealing	58 °C	60 sec.
cDNA	4 µl		Elongation	72 °C	30 sec.
Buffer	5 µl	1x	Prolonged elongation	72 °C	5 min.
Taq polymerase	2 µl			4 °C	∞
H_2O					
Final volume	50 µl				

PCR-fragments purification and analysis

The PCR products were purified with agarose gel electrophoresis, gel extraction and ethanol purification. The PCR-fragments were then cloned into pGEM-Teasy

vector, in accordance to the manufacturer's instructions and analyzed by restriction digestion or sequencing performed by Agowa.

Determining nucleic acid concentrations and quality

The amount of single-stranded RNA or double-stranded DNA was measured photometrically at a wavelength of λ = 260 nm. The nucleic acid concentration was calculated as follows:

A_{260} x 50µm/ml for double-stranded DNA

A_{260} x 40µm/ml for single-stranded RNA

The quality of RNA and purity of DNA was verified by $A_{260\ nm}/A_{280\ nm}$ ratios. This ratio lies between 1.5 and 1.9 for intact RNA and between 1.75 and 2.0 for pure DNA.

Isolation of plasmids from bacteria

For a first analysis of different clones small amount of plasmid (small scale up to 20 µg) was obtained by using QIAprep Spin Miniprep Kit according to the manufactor's instructions.

For isolation of plasmid in a large scale (up to 500 µg) the QIAfilter Plasmid Maxi Kit was used. After the plasmid isolation ethanol precipitation was performed and plasmids were stored at -20°C.

2.3.5 Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from lumbar L3-L6 DRG of 1day neuropathic, 7days neuropathic, 14days neuropathic and healthy mice and cDNA was made.

DRG dissection, RNA extraction and cDNA synthesis were performed as described in the cloning.

Quantitative PCR was performed using the Light Cycler 1.5 instrument utilizing SYBR green to detect amplification. PCR primers were designed to amplify 350 bp regions from TRPM8, TRPA1, TRPV3, TRPV4, TREK1, galanin as a positive control and the housekeeping gene GAPDH as a reference. These primers were tested at a range of annealing temperatures and were found to amplify 1 PCR product as determined by melting curve analysis and agarose gel electrophoresis. A standard concentration curve for each cDNA was constructed from serial

dilutions of linearized plasmid DNA (10^2, 10^3, 10^4, 10^5 and 10^6 copies/µl). Experiments were performed in triplicate and data were normalized to GAPDH levels. Quantitative Real Time PCR data were analyzed using the LightCycler software 3.5 (Roche Diagnostics Corporation, Mannheim, Germany). Standard curves were obtained using the 'Fit Points' analysis method by plotting the cycle numbers needed to reach a defined level of fluorescence versus the standard concentrations from serially diluted DNA-standards.

Each assay was performed using the following reaction:

Reaction mix	
Upstream primer	2 µl
Downstream primer	2 µl
Fast start DNA Master buffer mix (FastStartTaqDNApolymerase+SYBR Greendye, dNTPmix)	2 µl
$MgCl_2$	2.4 µl (4mM)
H_2O	
Template (cDNA or linearized plasmid)	2 µl
Final volume	20 µl

According to the instructions of the manufacturer the temperature and time profiles were the following:

AnalysisMode	Cycle	Segment	Target Temperature	Hold time	Acquisition Mode
Pre-incubation					
None	1		95 °C	10 min.	None
Amplification					
Quantification	45	Denaturation	95 °C	10 sec.	None
		Annealing	56°C for TRPA1 and TRPM8 58°C for all other genes		None
		Extention	72 °C	14 sec.	Single
Melting Curve					

Melting Curve	1	Denaturation	95 °C	0 sec.	None
		Annealing	65 °C	15 sec.	None
		Melting	95 °C slope=0.1°C/sec.	0 sec.	Continuous
Cooling					
None	1		40 °C	30 sec.	None

2.3.6 *In situ* hybridization and immunohistochemistry

digoxygenin (DIG)labeled RNA probes preparation

For the preparation of each riboprobe the relative plasmid was linearized with the correct restriction reaction. Linearized plasmid was then purified with the High Pure PCR Product Purification Kit and used for the following reaction:

1 µg linearized DNA template

2 µl DIG RNA labeling mix

2 µl 10x transcription buffer

2 µl appropriate RNA polymerase (T7 or SP6 for sense or antisense probes)

add DEPC- H_2O to 20 µl (final reaction volume)

The reaction mixture was incubated at 37°C for 2 hours. Then the DNA template was eliminated by adding 2 U of DNAse I and incubating at 37 °C for 15 minutes. Finally the polymerase reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0).

For better penetration of the tissue the probes were hydrolyzed by adding two volumes of carbonate buffer and incubation for 1 hour at 60°C. The reaction was stopped by adding an equal amount of hydrolysis-neutralization buffer. Then the probe was purified by ethanol precipitation and resuspended in 100µl DEPC-H_2O.

DOT SPOT TEST for the estimation of the DIG-labeled RNA probe concentration

The estimation of the probe's concentration was performed in a side by side comparison of the DIG-labeled RNA probes with a DIG-labeled control RNA. The following dilution series of both were made in RNA-dilution buffer and spotted on a strip of positively charged nylon membrane:

Sample	Conc.of the control	Dilution of R0	RNA dilution buffer

R0	100 ng/µl		
R1	10 ng/µl	1 : 10	2µl R0 + 18µl
R2	1 ng/µl	1 : 100	2µl R1 + 18µl
R3	100 pg/µl	1 : 1 000	2µl R1 + 198µl
R4	10 pg/µl	1 : 10 000	2µl R2 + 198µl
R5	5 pg/µl	1 : 20 000	2µl R4 + 10µl
R6	1 pg/µl	1 : 100 000	2µl R4 + 18µl

The RNA spots were fixed to the membrane by crosslinking with UV light for 5 minutes. The membrane was transferred into a petri dish containing 20ml washing buffer and left shaking for 2 minutes. It was then pre-incubated for 30 minutes in 10ml blocking solution and successively incubated for 30minutes with anti-DIG alkaline phosphatase diluted 1:5000 in blocking solution. The membrane was then washed 2x15 minutes with washing buffer, equilibrated for 5 minutes in detection buffer and incubated overnight in the dark with a freshly prepared solution of 200µl NBT/BCIP solution in 10ml detection buffer. The membrane was finally washed in ddH_2O and the spot intensities of the control and sample dilutions were compared to estimate the concentration of the DIG-labeled RNA probes.

Tissue preparation*:*

Mice were deeply anaesthetized with isoflurane and the tissue was fixed by transcardial perfusion of fixation buffer. Lumbar L3-L6 DRG were dissected as described in the cloning, post-fixed for a further 2 hours in fixation buffer and cryoprotected overnight in 30% sucrose. The DRG were then frozen in 2-methylbutane on dry ice, blocked in Frozen Section Medium and stored at –80 °C.

in situ hybridization protocol*:*

10 µm thick cryosections were cut and taken up on Polysin Microscope Slides. Sections were allowed to dry for at least 1 hour at room temperature and successively 15 minutes at 50 °C. Sections were washed 2x5mininutes in RNAse free PBS and treated with 1µg/ml proteinase K for 5 minutes and washed again. The tissue was then preincubated in 0.1M triethanolamine for 3 minutes and successively acetylated with 0.25% (v/v) acetic anhydride in 0.1M triethanolamine

for 10 min. After 3x5minutes washing in RNAse free PBS the slides were placed in a humid box previously prepared for the prehybridisation by pouring a mix of 50% formamide and 4xSSC on the bottom of the chamber. Slides were covered with 750µl of hybridization buffer without probe and incubated for 3-4 hours at 56°C. For the hybridization solution, 150ng of each DIG-labeled cRNA per ml hybridization buffer were combined, denatured at 85°C for 4min and then immediately placed on ice. The prehybridization solution was gently poured off the slides and 200µl of hybridization solution were added to each. Slides were covered with pieces of parafilm, to ensure an even distribution of the probes and to protect from drying. The hybridization proceeded in the humid box at 56°C overnight.

The following day slides were placed in 2xSSC at 56°C so that the parafilm could lift off without force. They were then washed 4 times in 2xSSC at 56°C for 10 minutes, once in 2xSSC at 56°C for 45 minutes, once in 0.1xSSC at 56°C for 1 hour, once in 0.2xSSC at room temperature for 10 minutes, and once in Maleic Acid Solution at room temperature for 10 minutes.

The detection was started by placing the slides in blocking solution for 30 minutes at room temperature. They were then transferred to a humid box with H_2O on the bottom, where they were laid flat and then incubated with anti-DIG antibody conjugated with horseradish peroxidase diluted 1:100 in blocking solution for 30 minutes at room temperature. The slides were then washed 3x5minutes in maleic acid solution with tween, and signal was visualized using Tyramide Signal Amplification System according to the manufacturer's instructions. The slides were finally washed 3x5minutes in maleic acid solution and stained with FITC-labeled IB4 or immunohistochemstry.

FITC-labeled IB4 staining

Slides were incubated for 1 hour at room temperature with 10µg/ml of FITC-labeled IB4 dissolved in IB4-blocking buffer. Finally the slides were washed 3x5 minutes in IB4-blocking buffer, rinsed with H_2O and mounted with Mowiol solution.

Immunohistochemstry: staining with anti CGRP and anti-NF200

Slides were pre-incubated in the respective blocking buffer (CGRP-blocking buffer, or NF200 blocking buffer) for 30minutes at room temperature and successively incubated overnight at 4°C with primary antibody. The primary antibody were

diluted in the respective blocking buffer at 1:2000 dilution for anti-CGRP and at 1:4000 dilution for anti-NF200. The following day the slides were washed 5x5 minutes and incubated for 1 to 2 hours at room temperature with the respective secondary antibody at 1:250 dilution (anti-rabbit for anti-CGRP and anti-mouse for anti-NF200). Finally the slides were washed 3x5 minutes in maleic acid solution, rinsed with H_2O and mounted with Mowiol solution.

Counting

All cell counts and quantification were conducted by an observer blinded to the experimental condition.

2.3.7 DRG cultures

Lumbar L3-L6 DRG were dissected from CCI and control mice and incubated with 1 mg/ml collagenase IV and 0.05% trypsin for 30 min each at 37°C. The DRG were suspended in DMEM/Hams-F12. DRG were dissociated by passing them through 18G, 22G, 25G needles, and debris was removed using a 40µm cell strainer. Cells were plated in a droplet of medium on poly-L-lysine (100 µg/ml) coated coverslips and left to adhere for 30 minutes before the coverslip was flooded. Calcium Imaging experiments were conducted 1-3 hours after plating of cells.

2.3.8 Calcium Imaging

Ratiometric calcium imaging was performed using FURA-2/AM dye and analysed using Tillvision software (Till Photonics, Gräfelfing, Germany). Cells were loaded with 3µM Fura-2/AM for 30 minutes at room temperature and placed in a recording chamber containing calcium imaging buffer. Pairs of images were collected every 2 seconds at alternating exposures of 340nM and 380nM (exposure time 70ms) using a Polychrome V monochromator and CCD Imago camera. Following subtraction of background fluorescence the ratio of fluorescence at 340nm and 380nm was calculated.

Coverslips were superfused with CIB buffer at approximately 2ml/min. Drugs were applied via a gravity driven perfusion system that allowed rapid exchange of solutions. Cold stimuli were applied using a peltier device and temperature

changes were monitored with a thermocouple placed within the flow of buffer and close to the cells.

Following selection of a suitable field of view, coverslips were maintained at 31°C for 5 minutes. A cold stimulus was then applied which cooled the cells from ~31°C to ~8°C at ~1°C per second. After a recovery period of 5 minutes at 31°C, either (-)-menthol (100μM), or in separate experiments, mustard oil (50μM) was applied for 1 minute. Drugs were washed out for 3-5 minutes before 40mM KCl was applied for 10 seconds to determine the total number of living cells.

A response was designated as a 20% increase in fluorescence ratio from baseline. The number of responders was expressed as a percentage of KCl responsive cells. The maximum amplitude of the response, the temperature threshold of cold responses and the cell diameter were also determined.

2.3.9 Data calculation

If not specifically described, the extrapolation of the data was done using Microsoft Excel 2000 for Windows (Microsoft Corporation) and Sigmaplot Software (Systat, San Jose, CA).

2.3.10 Data analysis

Statistical analysis was performed using Sigmastat software (Systat, San Jose, CA). Significance was tested using a two-way repeated measures ANOVA followed by Student-Newman-Keuls test in behavioural experiments, and a one-way ANOVA followed by Dunnett's Method in expression and calcium imaging experiments.

3. RESULTS

3.1 Behavioural responses to cold and menthol

We used a chronic constriction injury of the sciatic nerve (CCI) to model neuropathic pain in mice. Cold allodynia is prevalent in this model (Lee et al., 1998). To assess the levels of cold allodynia in mice, behavioural experiments were performed in collaboration with Dr. Dominika Labuz from our department. We monitored the behaviours at the following time points after the injury: 2, 4, 7 and 14 days. The degree of cold allodynia was determined using the acetone test. From the first time point after the surgery pain behaviours such as licking, brushing and flinching of the paw were significantly higher in the ipsilateral side of operated mice compared to the control mice and this continued for at least 14 days (figure 3A). The behaviours at the contralateral side were similar to the control (data not shown).

We then recorded behavioural responses to the TRPM8 agonist menthol. The ion channel TRPM8 is an identified transducer for cold temperatures (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007), and we therefore investigated whether TRPM8 activation might be important for cold allodynia. We reasoned that if TRPM8 is important for the mechanism responsible for cold-evoked allodynia after injury, then activation of this ion channel with its agonist menthol should also evoke nociceptive behaviour.

We applied 40µl of menthol or vehicle to the ipsilateral paw of CCI or control mice and monitored behavioural responses (figure 3B). Vehicle application did not evoke nociceptive behaviour in CCI or control mice. Application of menthol in control mice led to a small increase in licking duration compared to vehicle, but this was not statistically significant ($P=0.0956$, 2 way repeated measure ANOVA). However, application of menthol in CCI mice led to a significant increase in licking duration ($P<0.001$ compared to menthol control mice, 2 way repeated measure ANOVA) that became apparent 2 days after injury and peaked 7 days after injury, paralleling the development of cold allodynia. In addition to licking of the treated paw, CCI mice also displayed behaviours such as flinching of the paw and brushing of the affected area that also developed with a similar time course to

cold-evoked behaviour. This suggests that activation of TRPM8 after nerve injury, either by menthol or cold, can evoke nociceptive behaviours.

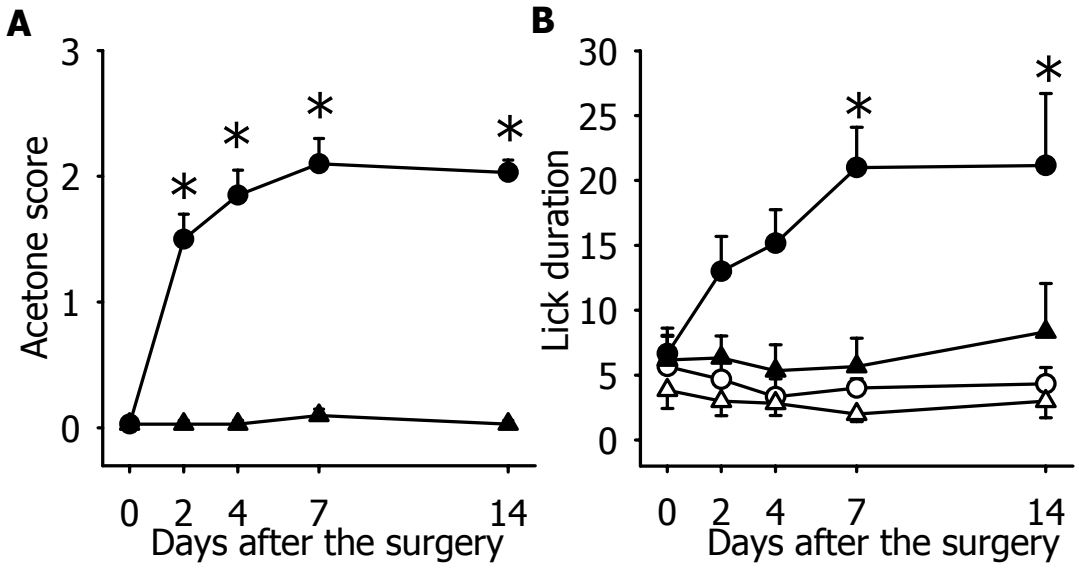


Figure 3: Behavioural testing for cold allodynia and menthol sensitivity in mice.

A, cold allodynia levels assess using the acetone test. Score where 0 = no response, 0.5 = licking, 1 = flinching and brushing of the paw, 2 = strong flinching, 3 = strong flinching and licking. Circles, CCI operated animals and triangles, control animals (n=6 animals for each group).

* indicates a significant difference $p<0.05$ CCI against control, two-way repeated measures ANOVA followed by Student-Newman-Keuls test.

B, Menthol evoked paw licking duration. Filled circles, CCI operated mice treated with menthol (250mM). Open circles, CCI mice with vehicle (90%DMSO, 10% PBS). Filled triangles, control mice menthol. Open triangles, control mice vehicle (n=6 animals for each group). All values are mean±SEM.

* indicates a significant difference $p<0.05$ CCI menthol against control menthol, two-way repeated measures ANOVA followed by Student-Newman-Keuls test.

3.2 Temperature sensing ion channels: mRNA quantification after nerve injury

Injury-induced alterations in the number of cold transduction molecules in sensory neurons could underlie the development of cold allodynia. We reasoned that all temperature transduction mechanisms which range from warm to noxious cold may contribute to the alteration of temperature sensitivity during cold allodynia, consequently we took into consideration five temperature sensing ion channels: the cool transducer TRPM8, the putative candidate for noxioums cold transduction TRPA1, the warm sensing ion channels TRPV3 and TRPV4, and the cold sensing potassium channel TREK-1.

In order to identify a possible role for these temperature sensing ion channels in neuropathic pain I measured the alteration in their gene expression by detecting mRNA levels in lumbar dorsal root ganglia (L3 to L6) of injured and control mice using Quantitative Real Time PCR (qRT-PCR).

Ipsilateral and contralateral DRG of injured mice at 1day, 7 days and 14 days after CCI-surgery were analysed in comparison to DRG of control mice.

3.2.1 TRPM8 mRNA quantification

The mRNA level of TRPM8 was lower in the ipsilateral side to CCI at 1 and 14 days after the surgery compared to the level in the control mice (figure 4).

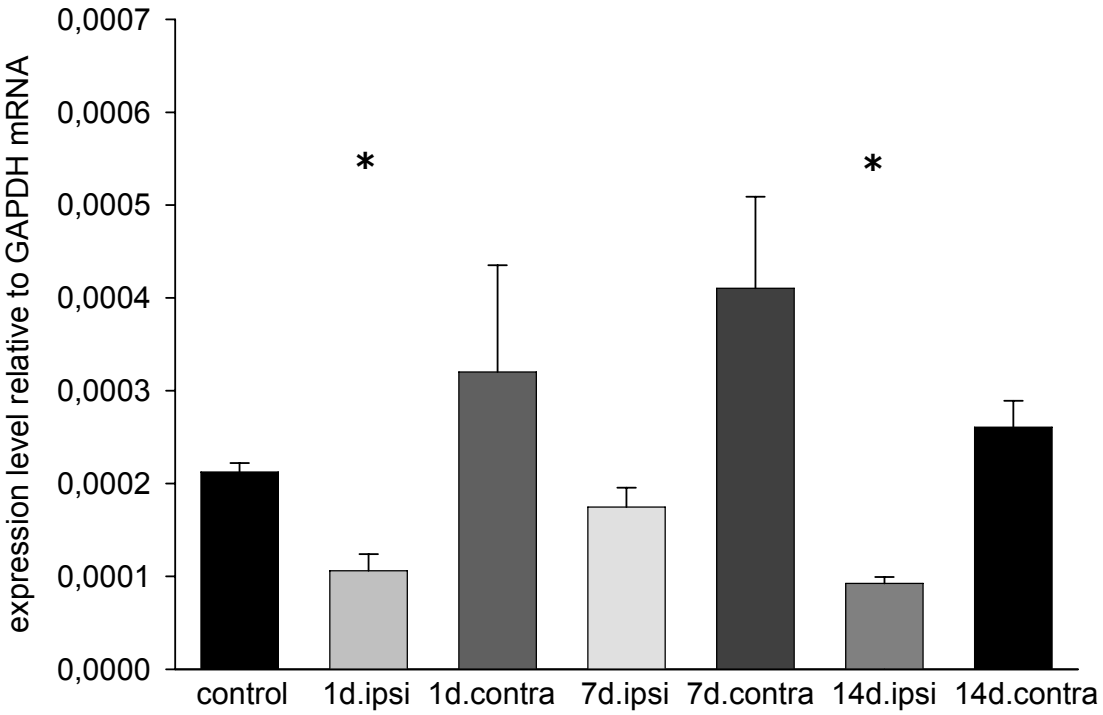


Figure 4: mRNA quantification for TRPM8 in DRG

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.2.2 TRPA1 mRNA quantification

The experiments for TRPA1 revealed a tendency to down-regulation in the mRNA level of the channel in the ipsilateral side at all time points after the nerve injury, although no significant difference was detected by the statistical analysis (figure 5).

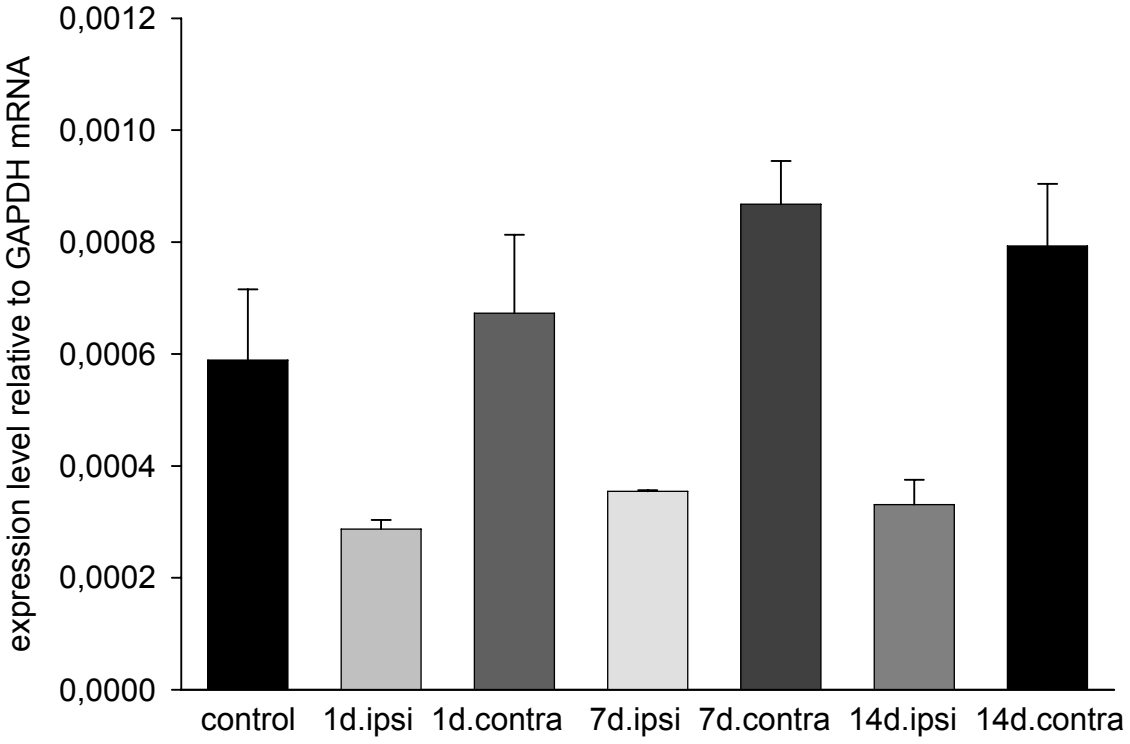


Figure 5: mRNA quantification for TRPA1 in DRG

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

3.2.3 TRPV3 mRNA quantification

The qRT-PCR for TRPV3 revealed lower mRNA expression in the ipsilateral side of injured mice at 1 and 7 days after the surgery compared to the control mice (figure 6).

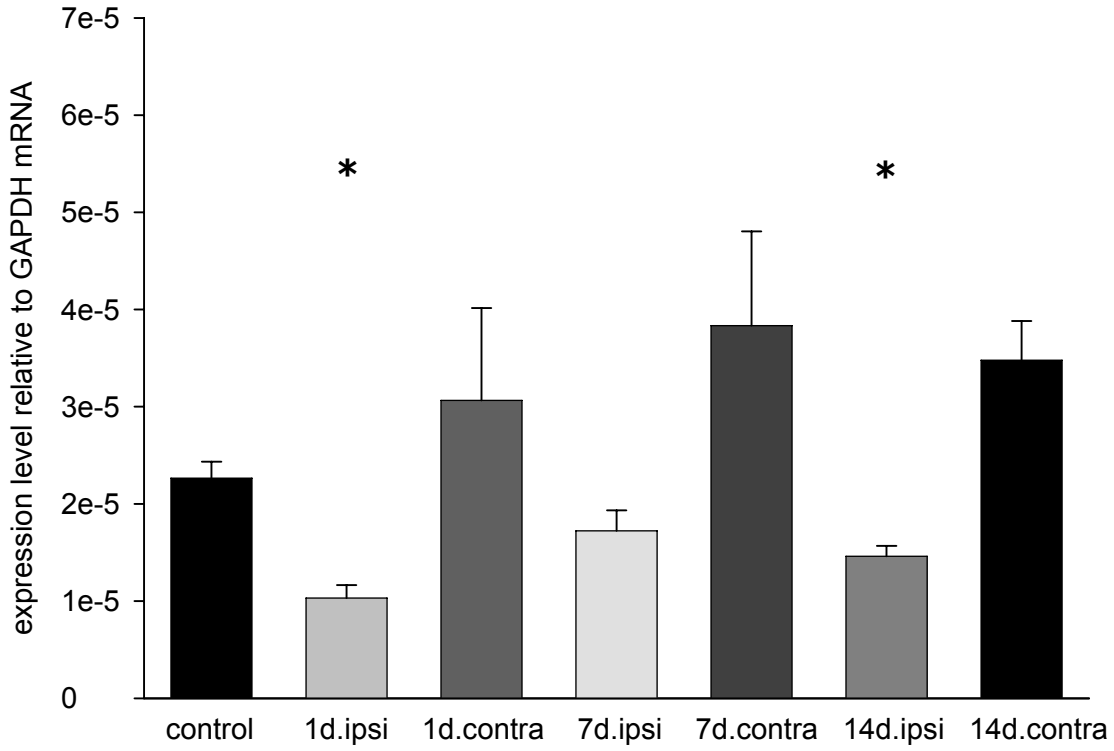


Figure 6: mRNA quantification for TRPV3 in DRG.

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.2.4 TRPV4 mRNA quantification

Statistically, no significant difference was detected in the TRPV4 mRNA levels between injured and control mice, however looking at the figure 7 a clear tendency to up-regulation appears in the contralateral side to CCI at all time points after the surgery.

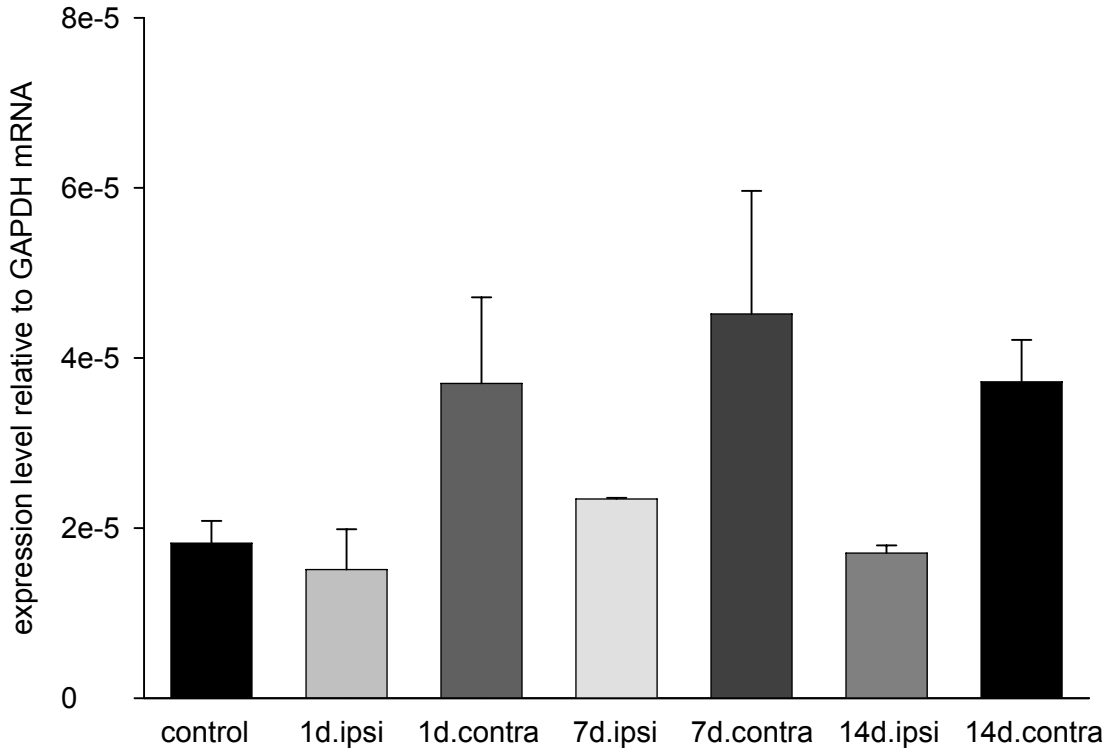


Figure 7: mRNA quantification for TRPV4 in DRG

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

3.2.5 TREK1 mRNA quantification

The mRNA level of TREK1 was lower in the ipsilateral side to CCI at 1 day after the surgery compared to the level in the control mice (figure 8).

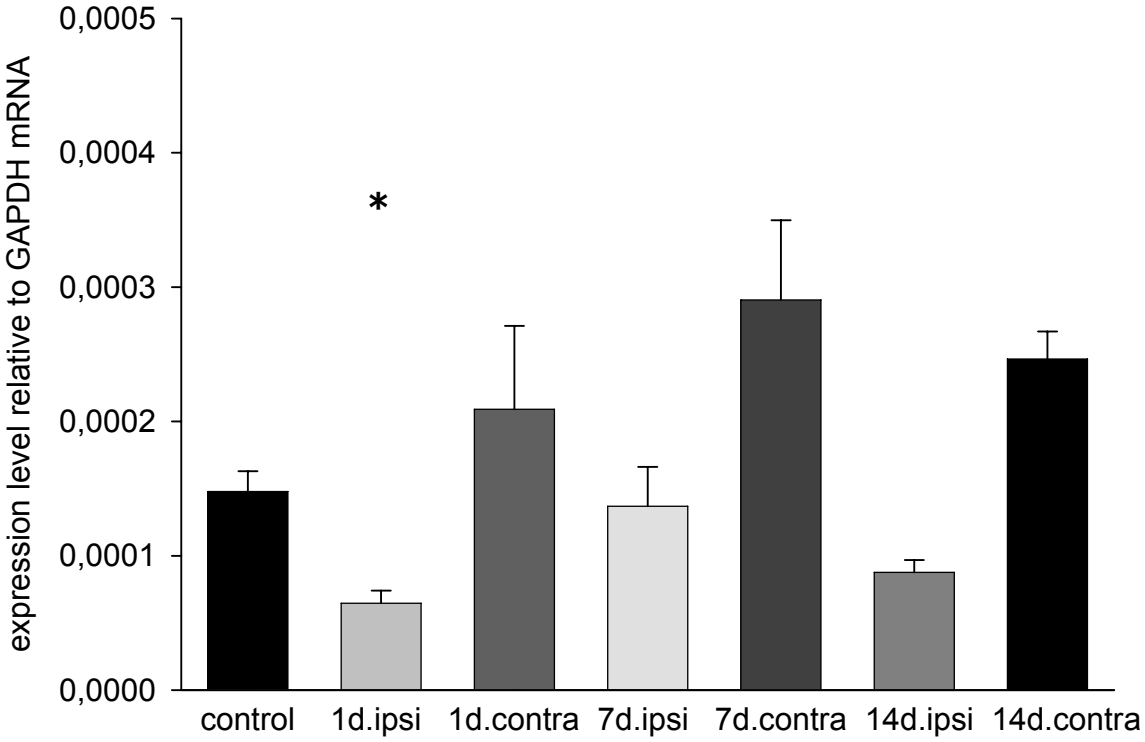


Figure 8: mRNA quantification for TREK1 in DRG.

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.2.6 GALANIN mRNA quantification

Because I wanted to test the validity of the method used for the qRT-PCR I used Galanin, which is known to be up-regulated after nerve injury, as a positive control (Nahin et al., 1994). Indeed the mRNA levels of Galanin in the ipsilateral side of CCI were considerably up-regulated compared to the levels in the contralateral side in both 7 and 14 days after the surgery (figure 9).

These results display the validity of the CCI model and the qRT-PCR technique used, confirming that they are feasible methods for measuring injury induced alterations in mRNA levels. Nevertheless they show a modest reduction in mRNA of the temperature sensing ion channels compared to the large 13-fold changes in expression seen with galanin.

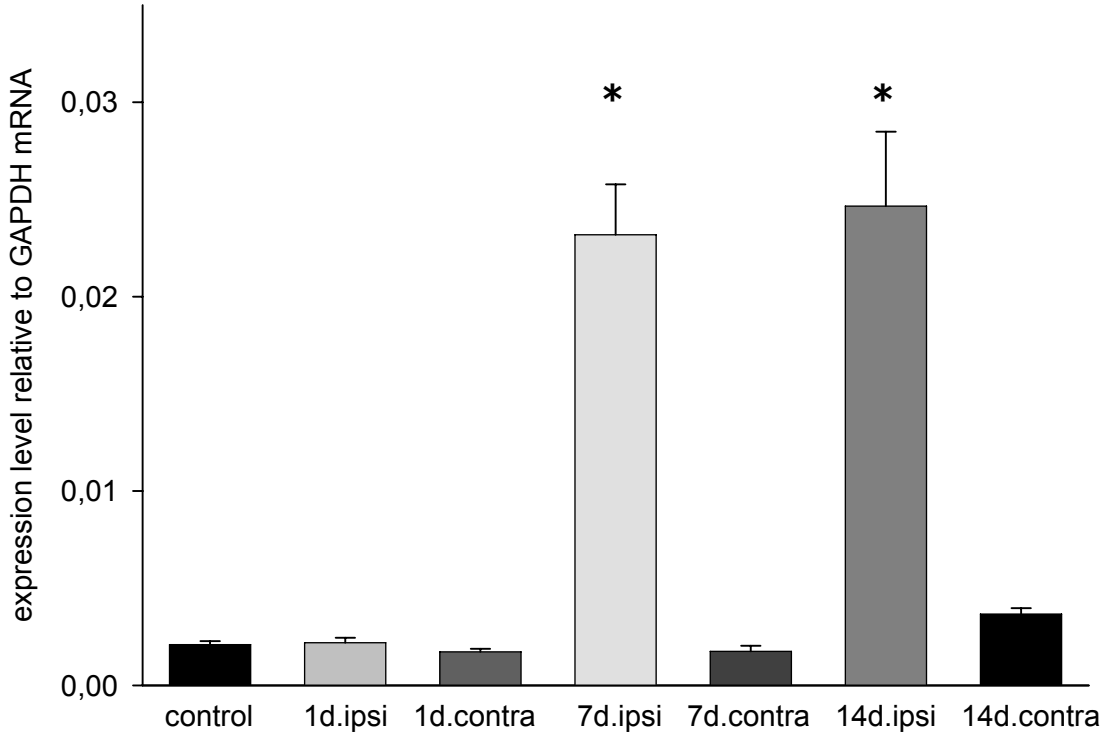


Figure 9: mRNA quantification for galanin in DRG

Levels are expressed relative to GAPDH in control animals and at 1, 7 and 14 days after the surgery (n=6 animals for each group). ipsi indicates ipsilateral to the injury, contra, contralateral. All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.3 Expression analysis of TRPM8 and TRPA1 in peripheral sensory neurons after nerve injury

Finding no considerable change in the expression levels of any of the measured temperature sensing ion channels I focused on the two major candidates for cold sensation TRPM8 and TRPA1, and analysed more in detail their expression after nerve injury.

With the intention of investigating changes in the subpopulation of TRPM8 and TRPA1 expressing neurons during neuropathic pain I performed double labelling with *in situ* hybridization and immunohistochemistry (IHC) in the lumbar DRG (L3 to L6) of injured and healthy mice. In addition to being a more sensitive method for quantification of mRNA levels, this technique also allowed me to identify the neuronal subtype expressing each channel and to determine whether this changed after injury.

In particular I labelled the neurons with RNA probes to detect TRPM8 mRNA or TRPA1 mRNA, and with antibodies against NF200 (neurofilaments) to detect non nociceptor neurons, or with antibodies against CGRP to detect peptidergic nociceptors, or with the IB4 which is a common marker for detecting non-peptidergic nociceptors. Figure 10 shows a typical example for each of the staining experiments.

In the *in situ* hybridization and immunohistochemistry analyses the ipsilateral DRG of injured mice at 7days and 14 days after the surgery were analysed in comparison with the DRG of healthy mice.

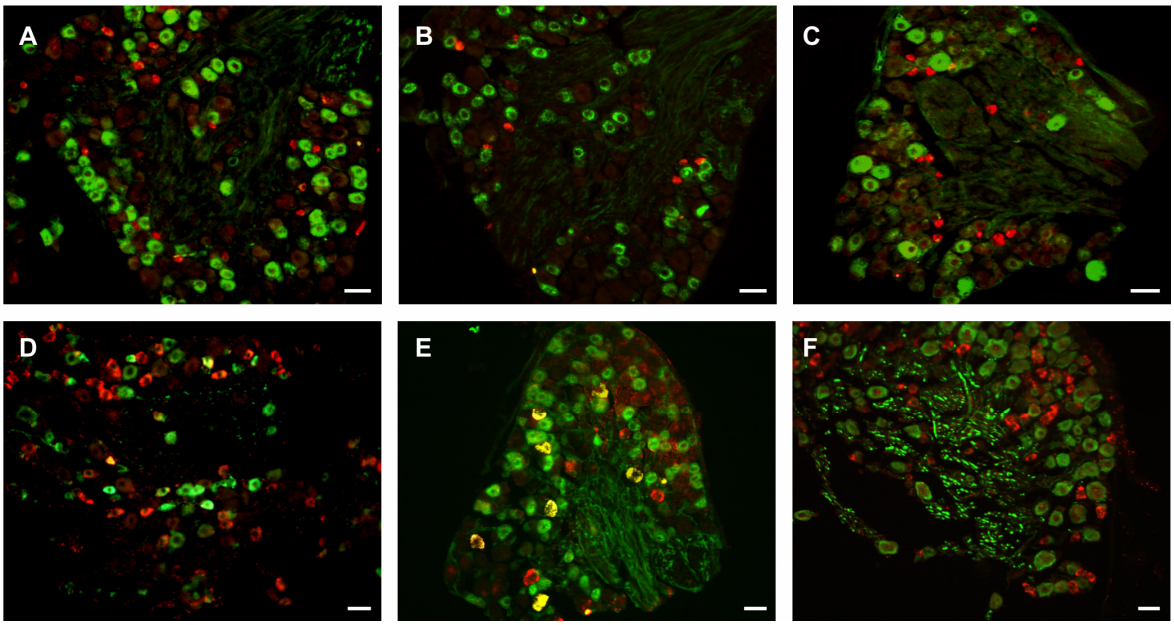


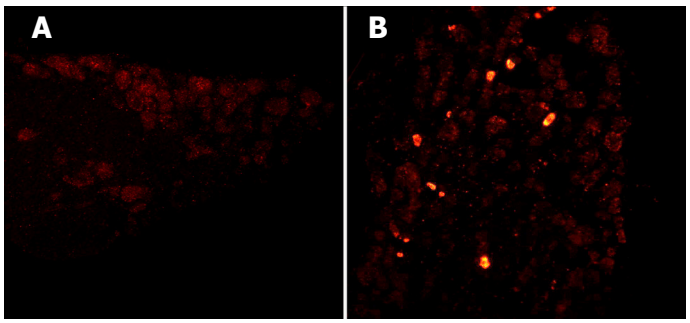
Figure 10: Distribution of TRPM8 and TRPA1 in specific subpopulations of DRG neurons.

A: TRPM8 + CGRP, **B**: TRPM8 + IB4; **C**: TRPM8 + NF200; **D**: TRPA1 + CGRP; **E**: TRPA1 + IB4; **F**: TRPA1 + NF200

The TRP channels are stained in red and the neuronal markers in green. Scale bar 40µm.

3.3.1 Expression of TRPM8 in peripheral sensory neurons after nerve injury

The *in situ* hybridization experiments for the detection of TRPM8 mRNA revealed a decrease in the percentage of TRPM8 expressing neurons after nerve injury. In fact almost 7 % of neurons in the DRG of healthy mice expressed TRPM8 mRNA, whereas at both time points after nerve injury less than 5 % of neurons in DRG express TRPM8 mRNA (figure 11).



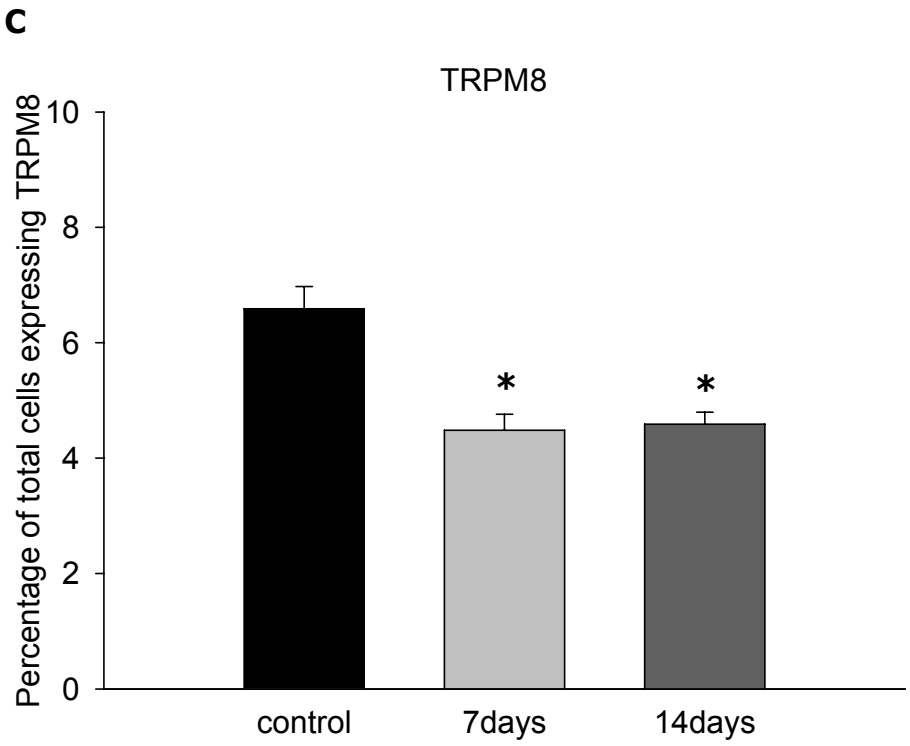
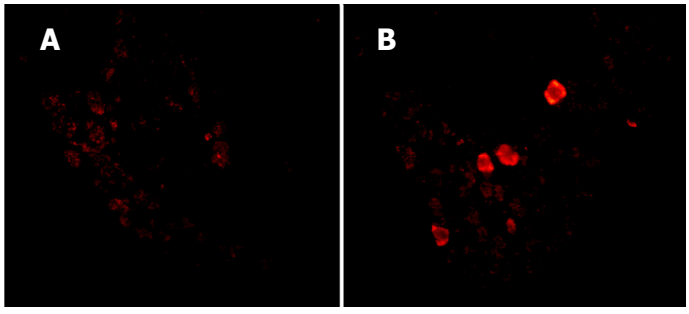


Figure 11: *In situ* hybridization with TRPM8 probes

A: negative control with sense probes.; **B**: TRPM8 staining with antisense probes. **C**: **TRPM8** expression in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with expression in DRG of healthy mice (n = 6 animals per group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.3.2 Expression of TRPA1 in peripheral sensory neurons after nerve injury

The *in situ* hybridization experiments for the detection of TRPA1 mRNA shown that nerve injury induced a decrease in the percentage of neurons expressing TRPA1. Almost 30 % of neurons in the DRG of healthy mice expressed TRPA1 mRNA, whereas at both time points after nerve injury only 20 % of neurons in DRG expressed TRPA1 mRNA (figure 12).



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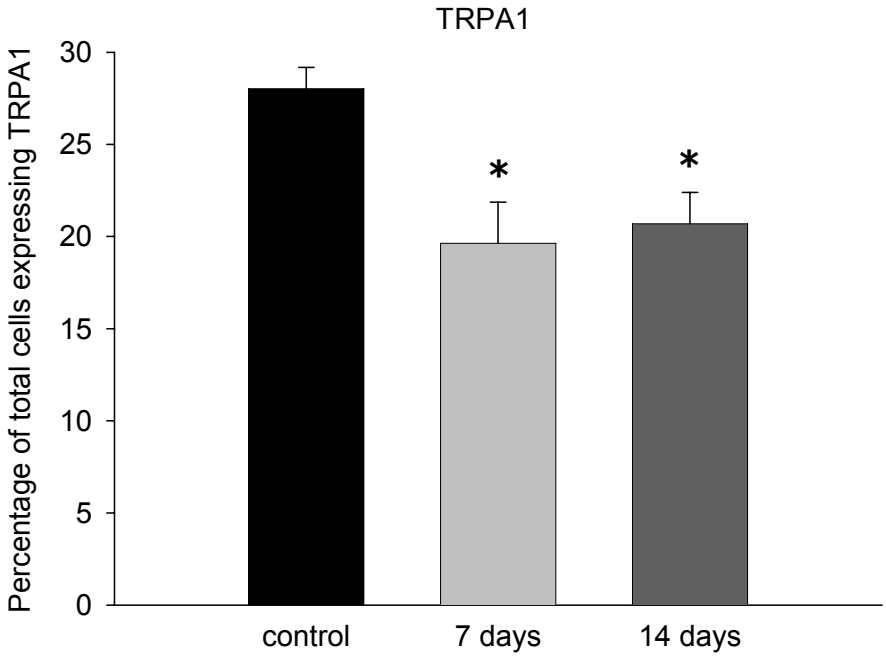

Figure 12: In situ hybridization with TRPA1 probes

A: negative control with sense probes; **B**: TRPA1 staining with antisense probes **C**: **TRPA1** expression in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with expression in DRG of healthy mice (n = 6 animals per group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.3.3 Expression of the known subpopulations of peripheral sensory neurons after nerve injury

To determine whether the decrease in TRPM8 mRNA and TRPA1 mRNA expression after nerve injury was a direct consequence of a change in the known subpopulations of peripheral sensory neurons I examined the expression of these subpopulations of neurons after nerve injury.

First of all I focused on the non-nociceptors, analysing the expression of NF200. 45 % of the neurons in the DRG express NF200. The IHC experiments revealed a small decrease in the percentage of non-nociceptors at 7 days after nerve injury, which disappears at 14 days after the surgery (figure 13).

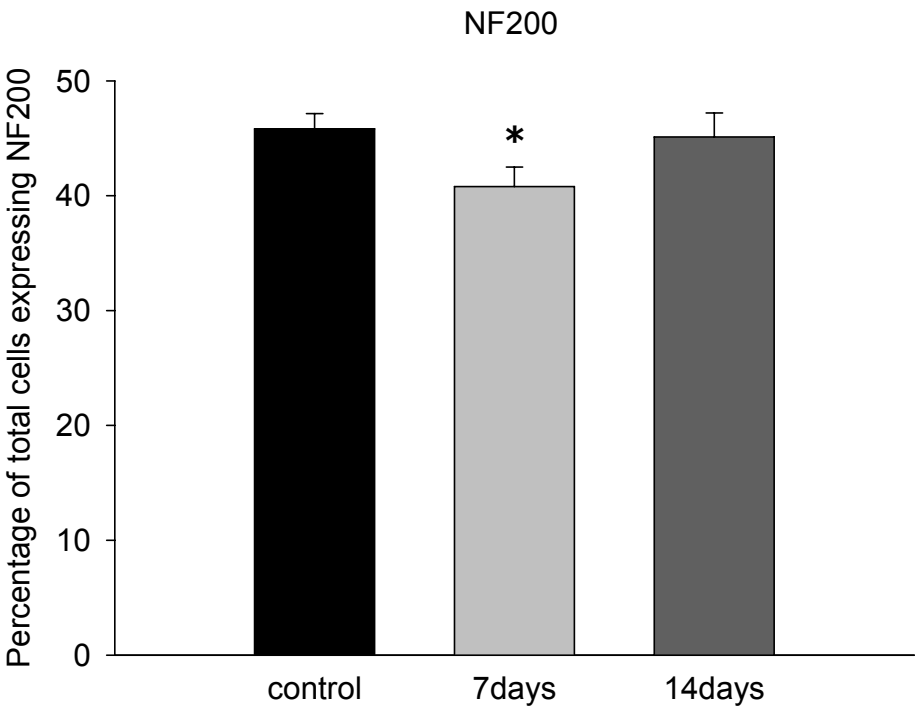

Figure 13: NF200 expression in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with expression in DRG of healthy mice (n=6 animals for each group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

I then focused on the subpopulation of peptidergic nociceptors. The IHC experiments for CGRP showed that circa 20 % of all neurons in the DRG express CGRP. At 7 days after nerve injury the percent of CGRP expressing cells decreased to 17 % of all neurons, but at 14 days after surgery the level of CGRP expressing cells went back to 20 % of all neurons as in the control group. (figure 14).

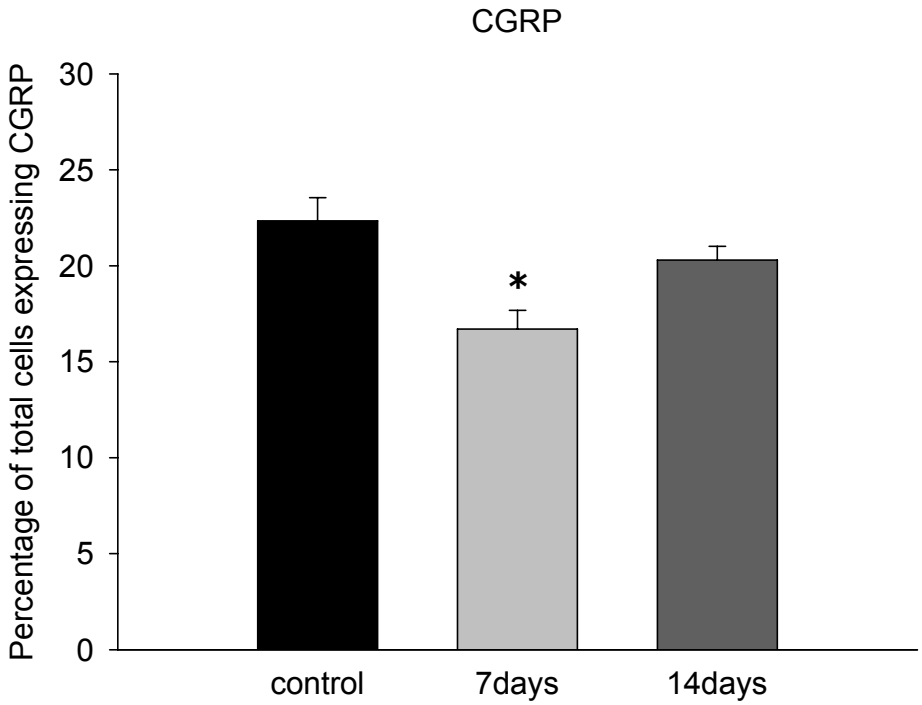

Figure 14: CGRP expression in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with expression in DRG of healthy mice (n=6 animals for each group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

Finally I concentrated on the subpopulation of the non-peptidergic nociceptors. The experiments labelling with IB4 revealed that IB4 bound 36% of all neurons in the control group. At 7 days after nerve injury this subpopulation of neurons decreased to 31 %, but at 14 days after nerve injury the percentage of IB4 labelled neurons went back to 35, similar to the control group (figure 15).

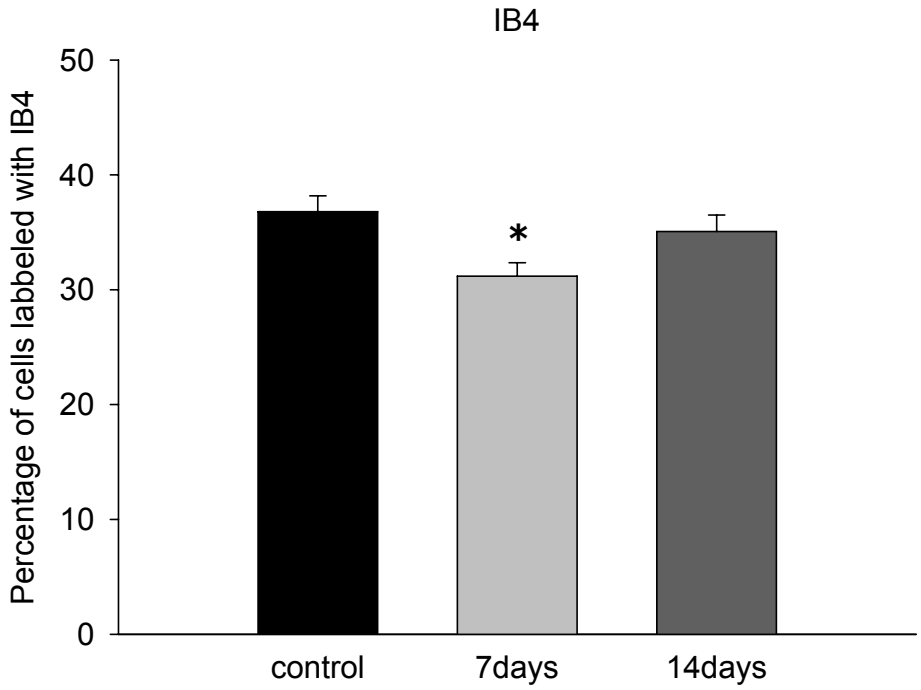

Figure 15: IB4 expression in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with expression in DRG of healthy mice (n=6 animals for each group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

Taking together the results for all three subpopulations of neurons, the expression analysis for the neuronal markers show no change in the percentage of all three subpopulations between control and 14 days after nerve injury, this proves that the decreases detected in the expression of TRPM8 mRNA and TRPA1 mRNA are not direct consequences of decrease in one or more subpopulation of neurons, but are a specific effect that nerve injury has on TRPM8 and TRPA1 expressing neurons.

3.3.4 Expression of TRPM8 in the known subpopulations of peripheral sensory neurons after nerve injury

To identify the subpopulation of neurons expressing TRPM8 and to investigate whether the decrease in the percentage of TRPM8 expressing neurons after nerve injury was associated with a shift of the TRPM8 expression in a different subpopulation of neurons I examined the double labelling of TRPM8 mRNA with the known neuronal markers .

First I examined the expression of TRPM8 mRNA in non-nociceptors. The double labelling experiments for TRPM8 and NF200 revealed that TRPM8 was not expressed in non-nociceptors and there was no change after nerve injury (figure 16).

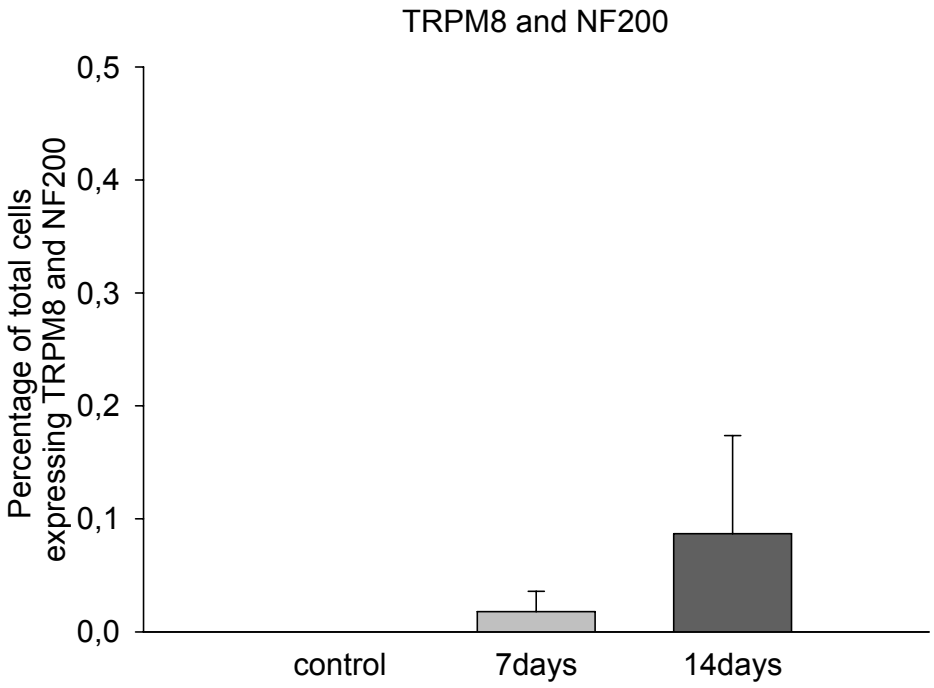

Figure 16: Co-localization of TRPM8 and NF200 in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3animals for each group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

I then looked at the expression of TRPM8 in peptidergic nociceptors. The double labelling experiments for TRPM8 and CGRP showed no change in the percentage of double labelled neurons indicating that after nerve injury there is not a shift of the TRPM8 expression in the subpopulation of peptidergic nociceptors. Moreover the co-expression of TRPM8 and CGRP was very small. In fact less than 0.2 % (±0.1) of all neurons in the DRG were double labelled with TRPM8 and CGRP, and only 0.8 % (±0.3) of CGRP labelled neurons were also labelled with TRPM8 mRNA indicating that the subpopulation of TRPM8 peptidergic nociceptors is almost non-existent. Finally only 3.0 % (±1.1) of TRPM8 mRNA expressing neurons were also labelled with CGRP, suggesting that TRPM8 is manly expressed in other subpopulation of neurons (figure 17).

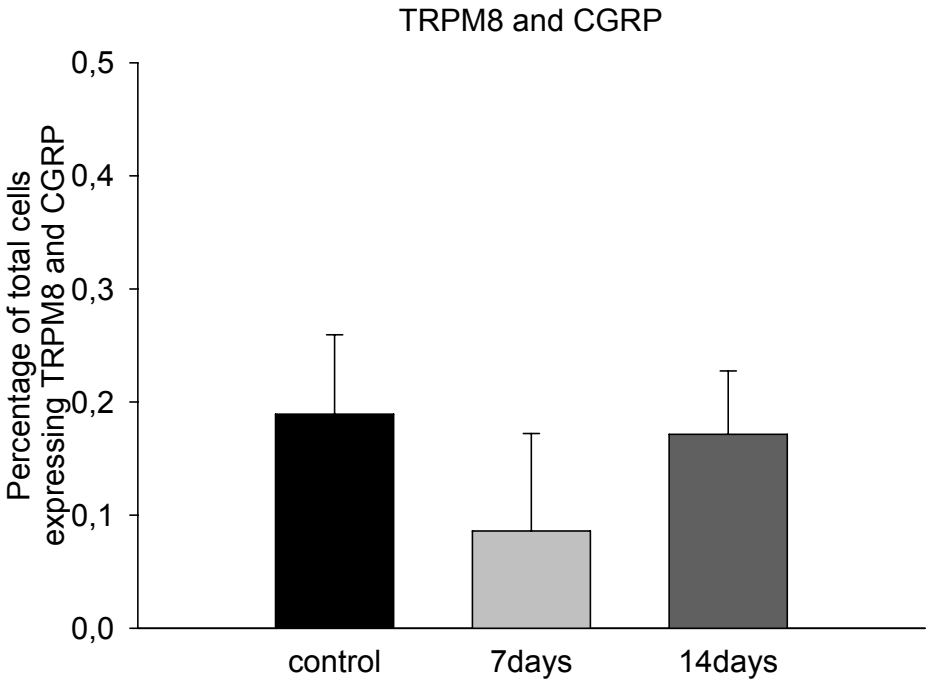

Figure 17: Co-localization of TRPM8 and CGRP in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3animals for each group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

Finally I examined the expression of TRPM8 in non-peptidergic nociceptors. The double labelling experiments for TRPM8 and IB4 shown that TRPM8 was not expressed in non-peptidergic nociceptors and there were no changes after nerve injury (figure 18).

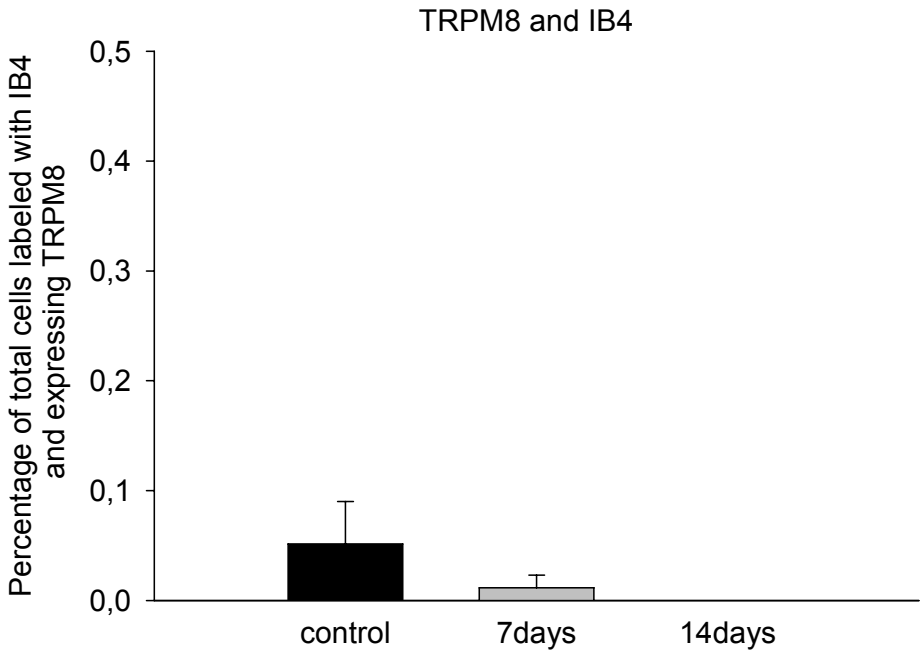

Figure 18: Co-localization of TRPM8 and IB4 staining in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3 animals for each group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

Taking together the results for the expression of TRPM8 in all three subpopulations of neurons, the analysis revealed that TRPM8 is not expressed in any of the analysed subpopulations of neurons, indicating that there is a specific subpopulation of peripheral sensory neurons which expresses TRPM8. Moreover the nerve injury does not induce a shift of the TRPM8 expression into any of the known subpopulations.

3.3.5 Expression of TRPA1 in the known subpopulations of peripheral sensory neurons after nerve injury

To identify the subpopulation of neurons expressing TRPA1 and to investigate whether the decrease in the percentage of TRPA1 expressing neurons after nerve injury was associated with a shift of the TRPA1 expression in a different subpopulation of neurons I examined the double labelling of TRPA1 mRNA and the known neuronal markers .

First I examined the expression of TRPA1 mRNA in non-nociceptors. The double labelling experiments for TRPA1 and NF200 showed no change in the percentage

of neurons co-expressing TRPA1 and NF200 (figure 19). Moreover this co-expression was very small. In fact less than 0.6 % (±0.17) of all neurons in the DRG were double labelled with TRPA1 and NF200, and only 1.2 % (±0.2) of NF200 labelled neurons were also labelled with TRPA1 mRNA indicating that the subpopulation of non nociceptors expressing TRPA1 is very small. Finally only 2.7 % (±0.7) of TRPA1 mRNA expressing neurons were also labelled with NF200, suggesting that TRPA1 is predominantly expressed in neurons with unmyelinated axons (i.e. the C-fibers).

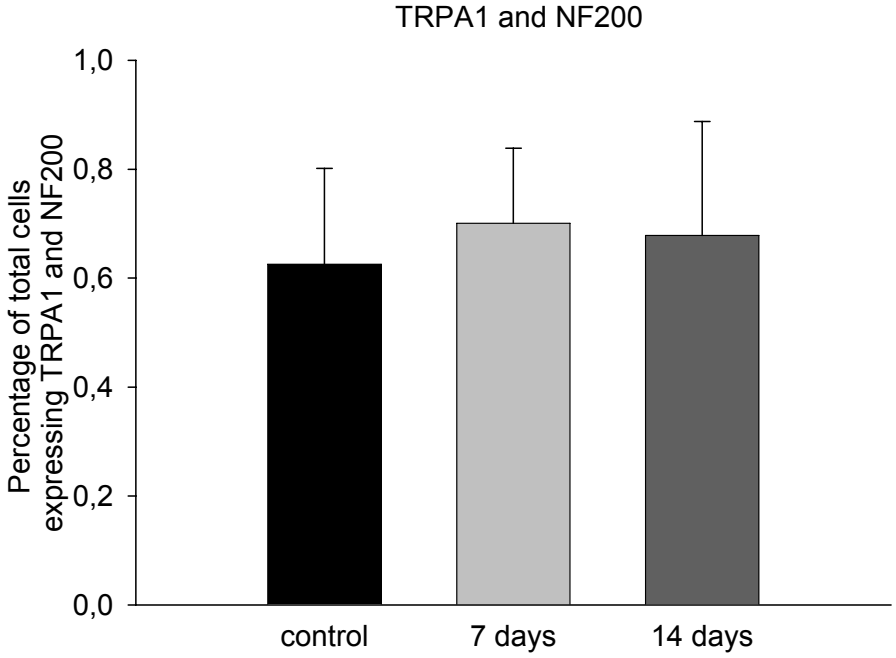

Figure 19: Co-localization of TRPA1 and NF200 in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3 animals for each group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

I then looked at the expression of TRPA1 in peptidergic nociceptors. The double labelling experiments for TRPA1 and CGRP showed that 2.5 % (±0.4) of all neurons in DRG co-expressed TRPA1 and CGRP, and no change in the percentage of this population was induced by nerve injury (figure 20). Besides, a detailed analysis of these data revealed that 9.7 % (±1.4) of TRPA1 expressing

neurons expressed CGRP too, indicating the presence of a population of TRPA1 expressing neurons which were peptidergic nociceptors, although this is not the predominant population of TRPA1 expressing neurons. Similarly 11 % (±1.9) of CGRP expressing neurons expressed TRPA1 too, revealing the presence of a significant, but not predominant population of peptidergic nociceptors which express TRPA1.

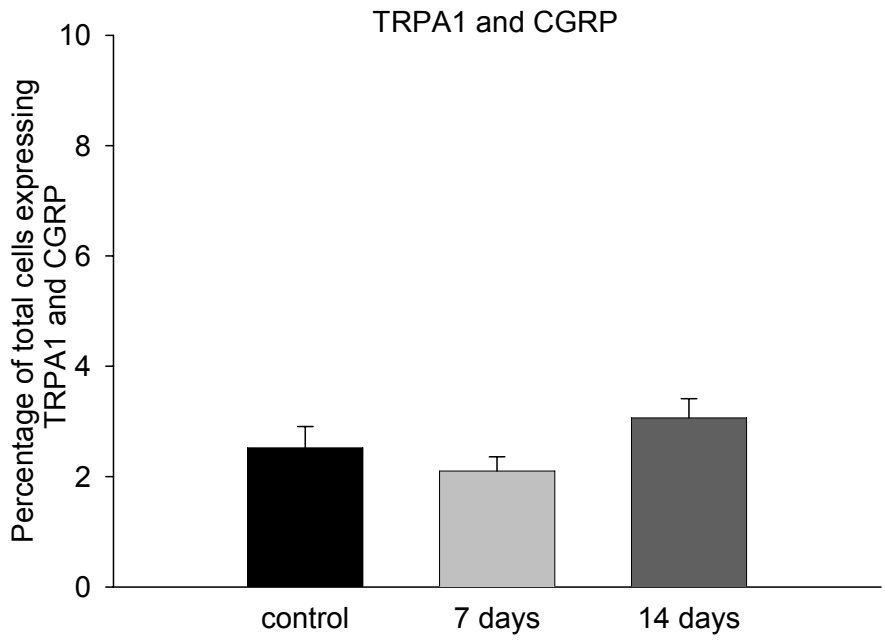

Figure 20: Co-localization of TRPA1 and CGRP in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3 animals for each group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

Finally I examined the expression of TRPA1 in non-peptidergic nociceptors. The double labelling experiments for TRPA1 and IB4 showed that the percentage of TRPA1 expressing neurons which were co-labelled with IB4 decreased at both time points after nerve injury (figure 21). These results suggest that the decrease of TRPA1 mRNA expressing neurons after nerve injury might be a consequence of decreased expression of TRPA1 in the IB4 positive population. Furthermore 74.4 % (±2.3) of TRPA1 expressing neurons were co-labeled with IB4, indicating that TRPA1 is mainly expressed in non peptidergic nociceptors. Similarly 72.51 %

(±2.5) of the IB4 positive neurons expressed TRPA1, bearing out the presence of a weighty population of non-peptidergic neurons which expressed TRPA1.

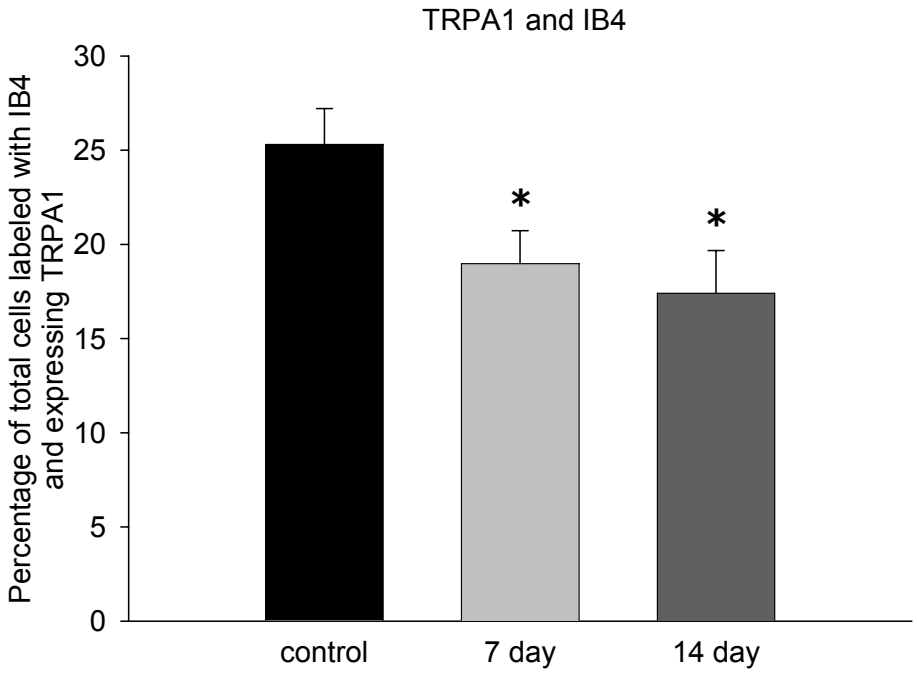

Figure 21: Co-localization of TRPA1 and IB4 in ipsilateral DRG of injured mice at 7 days and 14 days after surgery compared with the co-localization in DRG of healthy mice (n=3 animals for each group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

Taking together the results for the expression of TRPA1 in all three subpopulations of neurons it is evident that TRPA1 is mainly expressed in nociceptors. In particular circa 10 % of TRPA1 expressing neurons are peptidergic nociceptors, while circa 75 % are non-peptidergic nociceptors. Moreover nerve injury induced a decrease in the percentage of neurons expressing TRPA1, and this decrease only involves the population of non-peptidergic nociceptors.

3.4 Functional analysis of peripheral sensory neurons after nerve injury

To investigate the functional consequences of the nerve injury on the peripheral sensory neurons, I compared responses of cultured DRG neurons from neuropathic and healthy mice to cold and chemical stimuli using calcium imaging. Specifically I conducted experiments on neurons from the ipsilateral and the contralateral sides of mice at 7 and 14 days after nerve injury and on neurons from healthy mice. The responses of all four neuropathic groups were analysed in comparison with the responses of the healthy mice.

To evaluate changes in cold transduction I stimulated the cultured neurons using a 30-s cooling step from a base temperature of ≈31°C to ≈10°C and I related it to cold-sensitive TRP channel activities by stimulating the same cells with menthol, which is known to activate TRPM8, or with mustard oil, which is known to activate TRPA1. Figure 22 shows a typical experiment in which sensory neurons were tested for responses to cold, mustard oil and KCl, which is commonly used to select the alive cells. In this example, 3 of 12 neurons responded robustly to cold, 5 of 12 responded to mustard oil (and therefore presumably expressed TRPA1), and 1 of 12 cells responded to both stimuli. Similar experiments were performed using menthol to identify TRPM8 positive neurons. In the data analysis I considered responsive those cells which responded to the given stimulus with an increase of fluorescence over 20 %.

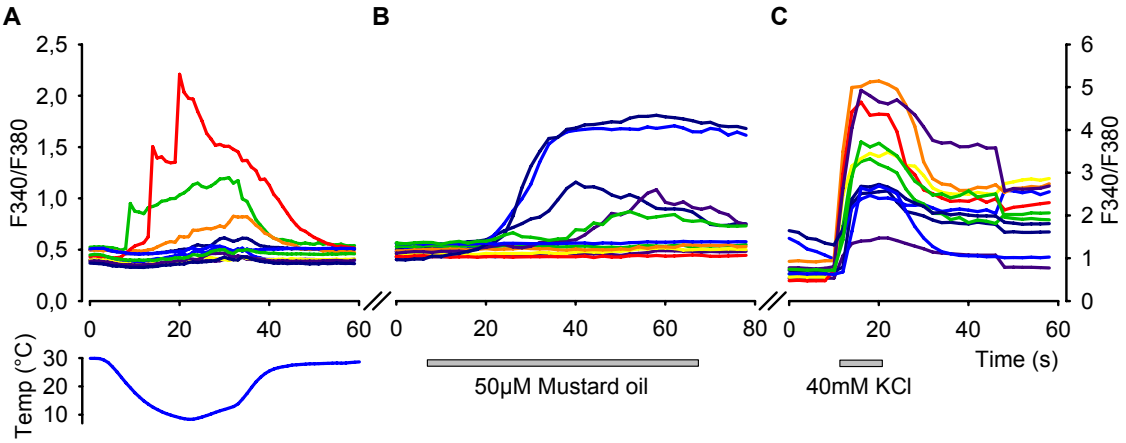

Figure 22: Representative recordings of Ca^{2+} transients in DRG neurons from control mice in response to ***A***, Cooling, ***B***, Mustard oil and ***C***, KCl (note different Y-axis scale for KCl).

3.4.1 Basic proportions of responsive neurons

The first step in the functional analysis of sensory neurons was looking whether the proportions of responding cells for each stimulus change after nerve injury.
The cooling step of the calcium imaging experiments revealed that 30 percent of the sensory neurons responded to cold and no significantly change was recorded in any of the neuropathic neurons (figure 23).

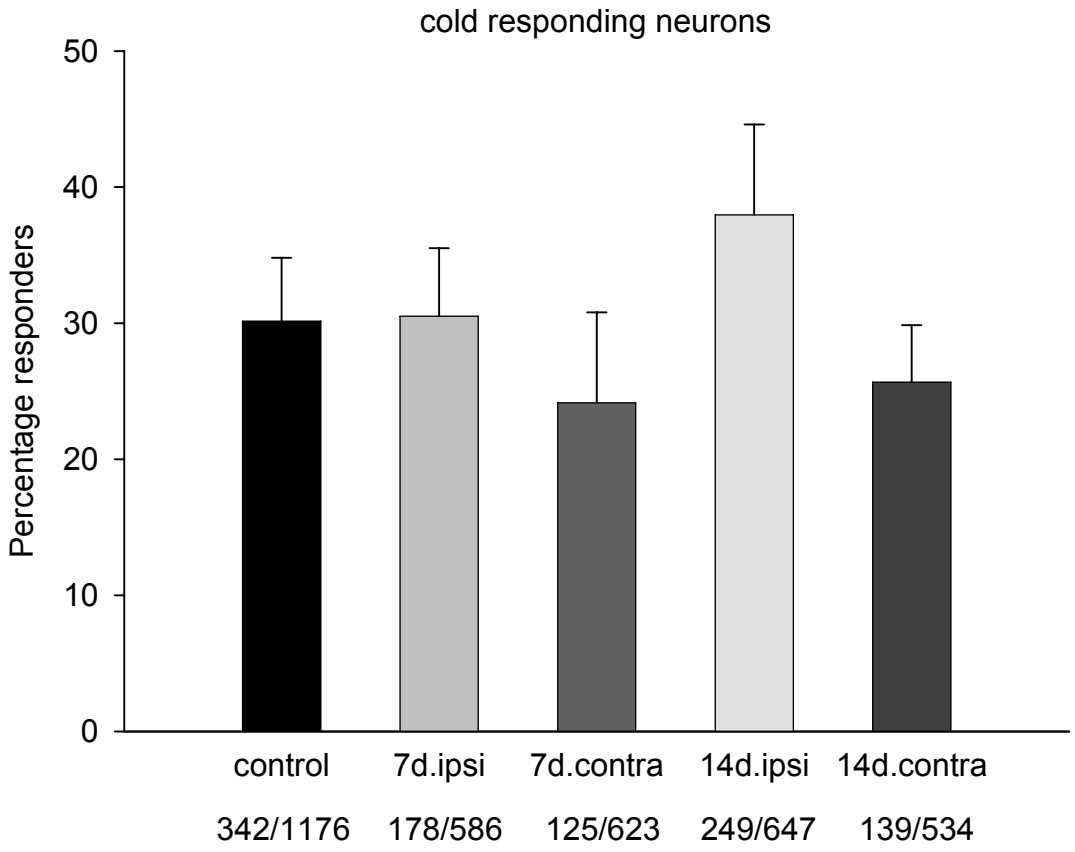

Figure 23: Percent of neurons responding to cold in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with percent in DRG of healthy mice (n = 10 to 16 animals per group). On the bottom are the number of responding neurons and the number of alive neurons per each group. All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

The menthol stimulus showed that 12 percent of the sensory neurons in the healthy mice responded to menthol and no significant change was recorded in any of the neuropathic neurons (figure 24).

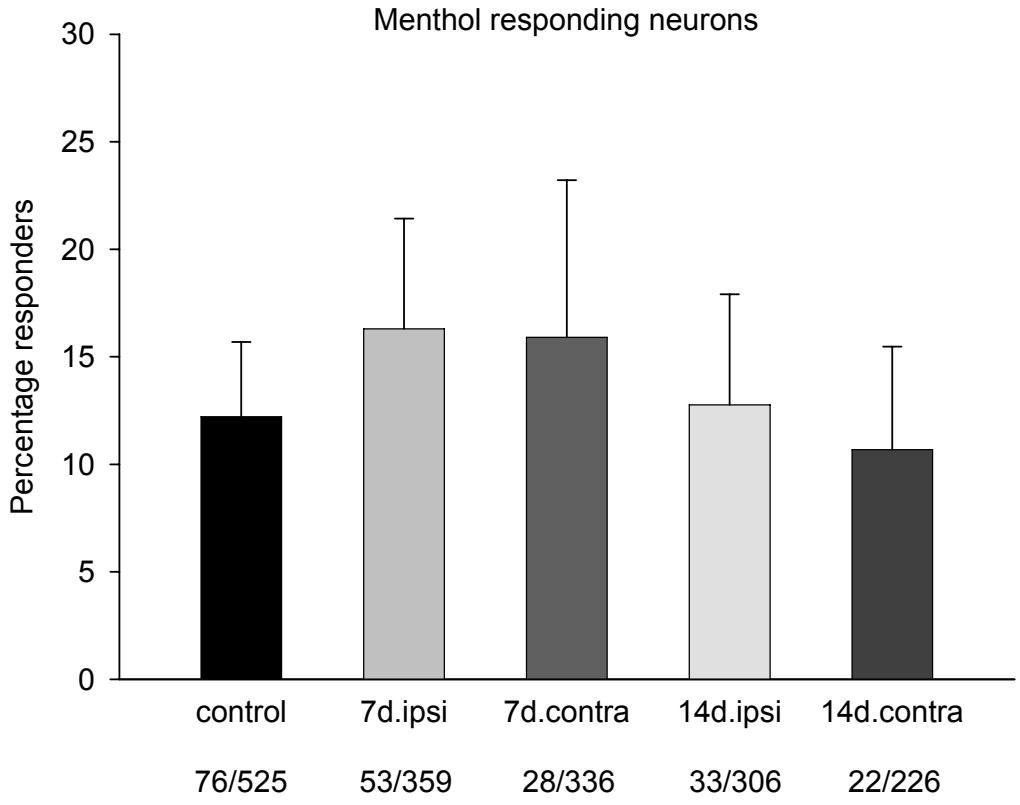

Figure 24: Percent of neurons responding to menthol in ipsilateral and contralateral DRG of injured mice at 7 days and 14 after surgery compared with percent in DRG of healthy mice (n = 7 to 11 animals per group). On the bottom are the number of responding neurons and the number of alive neurons per each group. All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

The mustard oil stimulus revealed a strong decrease in the proportion of responding neurons in the ipsilateral side of neuropathic mice at both time points after nerve injury. In fact in the healthy mice 45 percent of neurons responded to mustard oil, while in the ipsilateral side of injured mice only 18 percent of the neurons responded to mustard oil (figure 25). These results coincided with the outcomes of the *in situ* hybridization experiments showing the pathological consequences of altered TRPA1 gene expression after nerve injury.

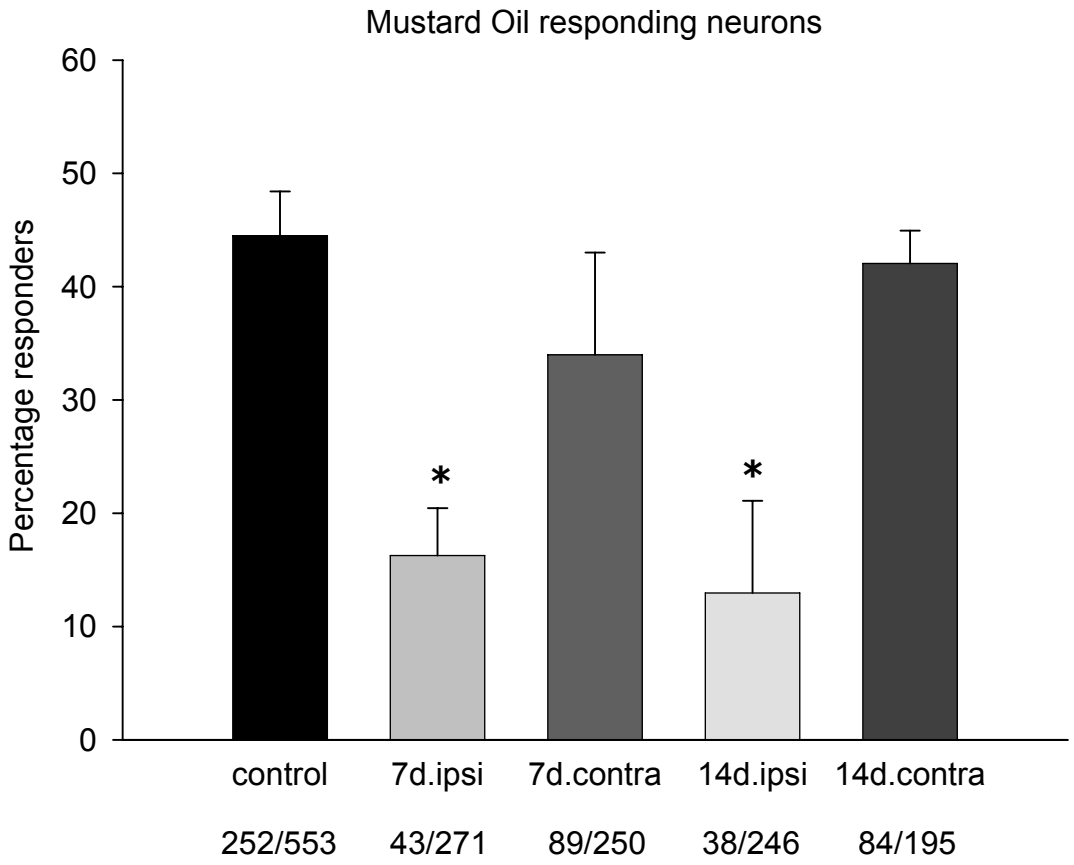

Figure 25: Percent of neurons responding to mustard oil in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with percent in DRG of healthy mice On the bottom are the number of responding neurons and the number of alive neurons per each group (n = 7 to 11 animals per each group). All values are mean ± SEM. * indicates a significant difference $p<0.05$ CCI against control, one-way ANOVA followed by Dunnett's method.

3.4.2 Proportions of double responsive neurons

In order to investigate the possible role of TRPM8 as a cold sensor I examined whether the menthol sensitivity matched with cold sensitivity. Indeed in healthy mice 72% (+-8.3) of the menthol responding neurons had cold sensitivity too. This good match of cold sensitivity in the menthol responding neurons supports the hypothesis that TRPM8 may play an important role as cold sensor, at the same time the fact that 28% of the menthol sensitive neurons did not have cold sensitivity suggests that the presence of a menthol sensor is not sufficient to give cold sensitivity to all neurons. Moreover looking at the proportions of cold responsive neurons (30% of all DRG neurons figure23) and menthol responsive neurons (12% of all DRG neurons figure24) it is clear that there is a big population

of cold responsive neurons which do not have menthol sensitivity, suggesting the existence of other mechanisms for cold transduction. Therefore I continued the functional analysis of sensory neurons after nerve injury considering the two different populations of cold responsive neurons, the menthol sensitive population, which corresponds to circa 40 percent of all cold responsive neurons, and the menthol insensitive population, which corresponds to circa 60 percent of all cold responsive neurons. Finally these experiments revealed that in the DRG of healthy mice 10 percent of all neurons were menthol sensitive and cold responsive while 19 percent of all neurons were menthol insensitive and cold responsive, and no difference was recorded after nerve injury in any of the two populations of cold responsive neurons (figures 26 and 27).

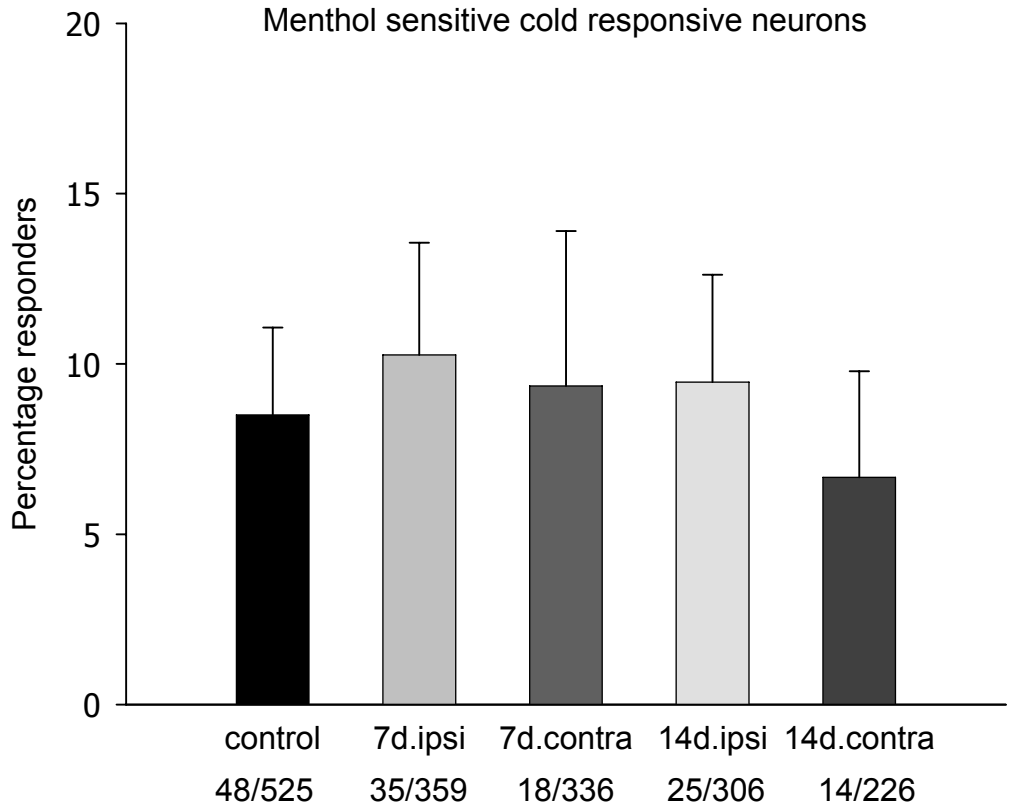

Figure 26: Percent of neurons responding to cold and menthol in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with percent in DRG of healthy mice

On the bottom are the number of responding neurons and the number of alive neurons per each group (n = 7 to 11 animals per group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

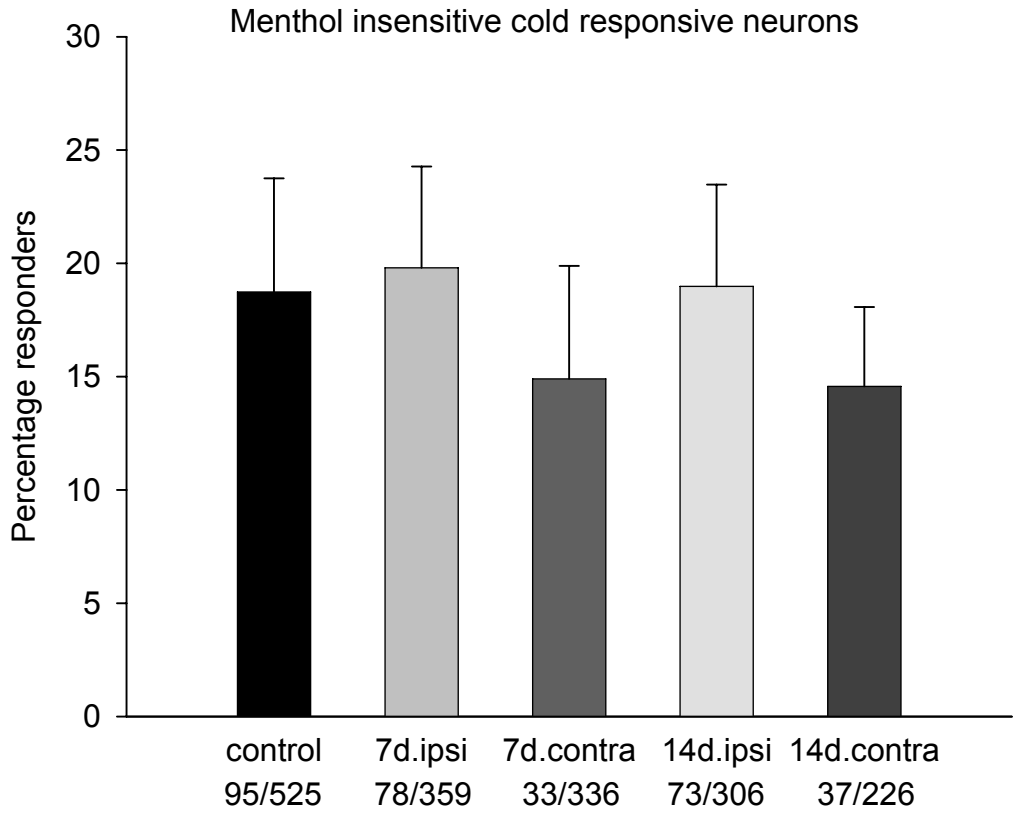

Figure 27: Percent of neurons responding to cold and insensitive to menthol in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with percent in DRG of healthy mice.
On the bottom are the number of responding neurons and the number of alive neurons per each group (n = 7 to 11 animals per group). All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

In the same way that was done for TRPM8 I investigated the possible role of TRPA1 as a cold sensor examining the match between mustard oil sensitivity and cold sensitivity. In healthy mice only 17,8 % (±2,0) of mustard oil responding cells had cold sensitivity too. This small match of cold sensitivity in the mustard oil responding neurons is against the hypothesis of TRPA1 as cold sensor. In fact the population of cold responsive neurons with mustard oil sensitivity was quite small; it was less than 10 percent of all DRG neurons in the healthy mice and in the contralateral side after nerve injury, while it was circa 5 percent of all the neurons in the ipsilateral side of mice after nerve injury (not significant), indicating a trend which follows the decrease of the mustard oil responsive neurons (figure 28).

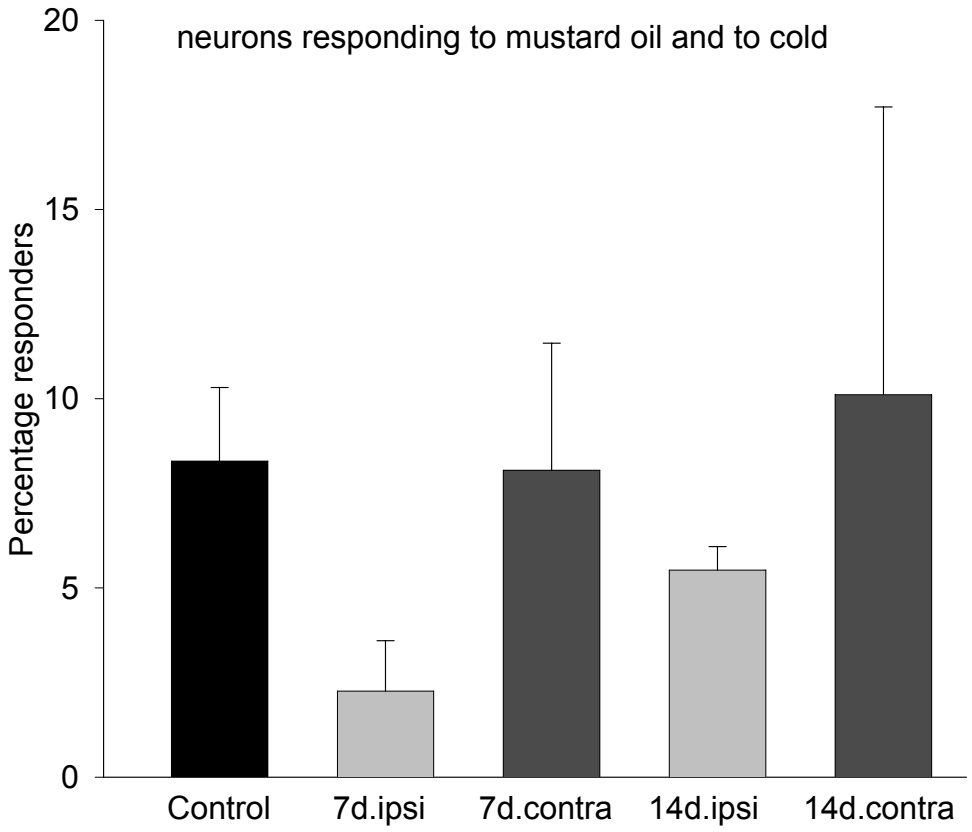

Figure 28: Percent of neurons responding to cold and mustard oil in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with percent in DRG of healthy mice (n=3 animals per group).
All values are mean ± SEM. No significant difference was detected, one-way ANOVA.

3.4.3 Properties of cold responses: temperature threshold and magnitude of the response.

Although no difference was recorded in the proportion of cold responsive neurons after nerve injury, alteration in the cold response properties of these neurons may occur, therefore I analysed whether after nerve injury changes in temperature threshold and in magnitude of the cold responses appeared.

In healthy mice the temperature threshold of all cold responsive neurons had an average value of 18.8 °C (± 0.66) and no significant difference was recorded in any of the neuropathic groups (Table 2, 1st column).

Temperature Threshold (°C)			
	CR	MS-CR	MI-CR
Control	18.8 ± 0.7	20.3 ± 0.9	18.0 ± 0.7
7days ipsi	17.0 ± 1.2	19.3 ± 1.9	16.0 ± 1.3
7days contra	17.5 ± 0.5	19.1 ± 2.2	17.4 ± 1.6
14days ipsi	18.5 ± 1.1	21.5 ± 2.1	16.3 ± 1.1
14days contra	17.1 ± 0.8	23.2 ± 1.2	16.5 ± 1.0

Table 2: Temperature threshold of cold responsive neurons in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with healthy mice.
In the first column are the values for all cold responding neurons (CR), in the second column are the values for the menthol sensitive responding neurons (MS-CR) and in the third column are the values for the menthol insensitive cold responding neurons (MI-CR). All values are mean ± SEM, n = 5 to 15 animals per group. No significant difference was detected between neuropathic and control mice, one-way ANOVA.

I then analysed the temperature threshold of the two different populations of cold responsive neurons. In the healthy mice the average temperature threshold was 20.3 °C (± 0.9) in the population of menthol sensitive cold responsive neurons and 18.00 °C (± 0.7) in the population of menthol insensitive neurons. No significant difference was recorded after nerve injury in any of the two groups of cold responsive neurons (Table 2, 2nd and 3rd column).

I then considered the size of the cold responses calculating the average of their maximum amplitude. Interestingly, the size of the responses was significantly larger in menthol sensitive cold responding neurons compared to menthol insensitive ($p<0.05$, T-test), although again, this was not altered post injury (Table 3)

Maximum Amplitude of response (% of increase)			
	CR	MS-CR	MI-CR
Control	84.7 ± 11.1	110.9 ± 17.2	58.9 ± 15.1
7days ipsi	79.1 ± 18.7	116.3 ± 26.1	51.4 ± 7.1
7days contra	85.7 ± 12.4	120.1 ± 29.1	83.0 ± 42.5
14days ipsi	52.6 ± 7.0	66.6 ± 21.6	43.9 ± 5.8
14days contra	53.1 ± 7.8	111.0 ± 13.1	40.6 ± 9.6

Table 3: Size of the cold responses in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with healthy mice
In the first column are reported the values for all cold responding neurons (CR), in the second column are the values for the menthol sensitive responding neurons (MS-CR) and in the third column are the values for the menthol insensitive cold responding neurons (MI-CR). All values are mean ± SEM, n = 5 to 15 animals per group. No significant difference was detected between neuropathic and control mice, one-way ANOVA.

3.4.4 Size of the neurons in DRG after nerve injury

Sensory neurons can be classified by their cell size with smaller neurons (<25 μm in the mouse) being predominantly nociceptive. Thus I analysed the size of the neurons by measuring the average neuronal diameter.

First I examined whether there was a general change in the size of neurons after nerve injury looking at the average diameter of all analysed neurons for each group and no difference was recorded in any of the neuropathic mice compared to the healthy mice (table 4, column 1).

I then examined the cold responsive neurons. The average diameter of all cold responsive neurons was 19.1 μm (±0.6). No difference was recorded between the size of the two populations of cold responsive neurons, and no change was recorded after nerve injury (table 4, columns 2, 3 and 4).

Interestingly the size of mustard oil responsive neurons (23.03 μm ±1.27) was significantly bigger than the size of cold responsive neurons ($p < 0.05$, T-test). No difference was recorded in the size of mustard oil responsive neurons from injured mice compare to the neurons from healthy mice (table 4, column 5).

	Size of neurons (μm)				
	All neurons	CR	MS-CR	MI-CR	MO
Control	21.5 ± 0.5	19.1 ± 0.6	18.8 ± 0.4	19.5 ± 1.1	23.0 ± 1.3
7days ipsi	20.9 ± 0.8	19.5 ± 1.2	16.9 ± 1.3	22.0 ± 2.1	23.0 ± 0.1
7days contra	21.1 ± 0.5	17.3 ± 0.8	17.2 ± 1.0	17.3 ± 1.2	21.1 ± 1.0
14days ipsi	22.7 ± 1.0	19.0 ± 1.7	20.3 ± 2.8	18.5 ± 2.7	22.5 ± 0.8
14days contra	21.5 ± 0.5	18.0 ± 1.4	16.2 ± 1.5	18.7 ± 1.9	22.8 ± 1.1

Table 4: Average diameter of sensory neurons in ipsilateral and contralateral DRG of injured mice at 7 days and 14 days after surgery compared with healthy mice
In the first column are reported the values for all sensory neurons, in the second column are the values for all cold responding neurons (CR), in the third and fourth columns are the values for the menthol sensitive (MS-CR) and menthol insensitive (MI-CR) cold responding neurons, in the last column are the values for the mustard oil responding neurons (MO). All values are mean ± SEM, n = 5 to 15 animals per group. No significant difference was detected between neuropathic and control mice, one-way ANOVA.

In conclusion the functional analysis was quite surprising because although the behavioural experiments clearly showed increase in cold and menthol sensitivity after nerve injury, no change appeared either in the cold responsive neurons or in the menthol responsive neurons. Additionally no correlation appeared between TRPA1 and cold sensitivity, suggesting that TRPA1 is not a cold transducer. Finally the reduction in the percentage of mustard oil responsive neurons confirms the results of the expression analysis suggesting that nerve injury induces a decrease in TRPA1 expression in DRG.

4. DISCUSSION

In the present study we explored the role of the mechanisms of cold transduction in the development of cold allodynia following nerve injury. We showed that the TRPM8 agonist menthol evokes nociceptive behaviour after nerve injury that parallels the emergence of cold-evoked hypersensitivity. We then investigated the possible peripheral mechanisms behind these changes *in vivo*. We first analysed the mRNA expression levels of five different temperature sensitive ion channels, which have been proposed as temperature transducers. After nerve injury there were only small changes in the expression levels of these ion channels in the DRG. We consequently focused on the two main candidates for cold transduction, TRPM8 and TRPA1 examining in depth their expression in the DRG, and analysing the receptive properties of cold sensitive sensory neurons after nerve injury. Unexpectedly, we observed a reduction in percentage of neurons expressing the TRP channel mRNA and no difference in the functional properties of cold sensitive DRG cells. These results suggest that cold allodynia does not arise directly from an amplification of these mechanisms of peripheral cold transduction.

4.1 Cold and menthol evoked behaviours after nerve injury

4.1.1 Menthol evokes nociceptive behaviour post-injury

The psychophysical effects of menthol have been well documented in humans. Menthol is known for its ability to evoke a cooling sensation when applied to the skin (Green, 1992; Cliff and Green, 1994; Yosipovitch et al., 1996), and high concentrations (30-40% w/v) produce burning pain and cold hyperalgesia (Wasner et al., 2004; Namer et al., 2005; Hatem et al., 2006). There are fewer studies addressing the in vivo effects of menthol in animal models, presumably because of the difficulty in designing behavioural assays to assess innocuous stimuli, and because higher nociceptive concentrations of menthol induce ataxia when injected in mice (Dhaka et al., 2007).

In my experiments, I applied 250mM (3.9% w/v) (-)-menthol to a localized area of the hind paw. I observed no ataxia and indeed control mice behaved in much the

same way as vehicle treated animals. After nerve injury however, the same concentration of (-)-menthol evoked strong nociceptive responses.

Menthol is also known to have analgesic actions in a number of painful conditions. A recent report demonstrated that activation of TRPM8 by (-)-menthol and other agonists led to marked analgesia in the CCI model of neuropathic pain in rats (Proudfoot et al., 2006). One major difference between that study and mine was that considerably lower concentrations of (-)-menthol were used (4mM compared to 250mM used here). It is therefore likely that low concentrations of (-)-menthol (4mM) evoke anti-nociception, while higher concentrations (250mM) are pro-nociceptive after injury. Intriguingly, in healthy human volunteers, concentrations similar to the one used here (320mM-630mM) induce a cooling sensation (Green, 1992; Yosipovitch et al., 1996), whereas concentrations of 1.9M-2.6M are required to evoke pain (Wasner et al., 2004; Namer et al., 2005; Hatem et al., 2006). An important question is whether the concentration of menthol required to evoke pain is also lowered in neuropathic pain patients.

4.1.2 TRPM8 triggers nociceptive behaviours to cold and menthol stimuli after nerve injury.

A remarkable point revealed from the topical application of menthol is that the nociceptive responses evoked by menthol developed with a time course similar to acetone-induced cold behaviour. This indicates that activation of TRPM8 in CCI mice triggers nociceptive behaviour and that TRPM8 might be a key component of cold hypersensitivity. In agreement, Colburn et al. (2007) reported that TRPM8 knockout mice display reduced acetone responses after nerve injury, suggesting that in neuropathic pain states TRPM8 acts as the predominant sensor for cool-evoked pain. Additionally another recent study showed that cold allodynic response in CCI animals was significantly attenuated by capsazepine, a blocker for both TRPM8 and TRPV1 receptors, but not by the selective TRPV1 antagonist I-RTX (5-iodoresiniferatoxin) (Xing et al., 2007), indicating a close connection between TRPM8 and cold allodynia.

4.2 Expression of temperature sensitive ion channel mRNA after nerve injury

4.2.1 Nerve injury induces small changes in the mRNA expression levels of temperature sensitive ion channels in the DRG

One of the main mechanisms underlying neuropathic pain is an alteration of gene expression in the DRG. We reasoned that after nerve injury increased expression of molecules involved in cold transduction could contribute to cold allodynia by amplifying responses to cold stimuli.

In a first approach we took into consideration five different molecules which have been proposed as temperature transducers, and I analysed their mRNA levels in the DRG using qRT-PCR. The first two of these molecules are TRPM8 and TRPA1, which are considered to be the main candidates in the mechanisms of cold transduction. Two others are the warm activated channels TRPV3 and TRPV4 which we considered might become important in cold transduction too after injury. The last of these molecules is the cold sensing potassium channel TREK-1, which has been proposed to contribute to cold sensation through an indirect transduction mechanism.

The qRT-PCR experiments detected a relatively low level of mRNA for these ion channels in the DRG of control mice, which correlates well with the selective expression of these channels within small subpopulations of neurons. TRPA1 has the highest mRNA expression level with 280900 copies / μg of RNA, the mRNA expression levels of TRPM8 is about one third of TRPA1 corresponding to 106400 copies / μg of RNA and TREK-1 is expressed at a level of 72830 copies / μg of RNA. Murine TRPV3 and TRPV4 are mainly expressed in keratinocytes and other studies failed in detecting them in DRG (Peier et al., 2002; Alessandri-Haber et al., 2003; Guler et al., 2002; Liedtke et al., 2000; Suzuki et al., 2003). Interestingly, the qRT-PCR revealed the presence of these two ion channels in the DRG, although at very low levels, which corresponded to 11262 copies / μg of RNA for TRPV3 and to 9008 copies / μg of RNA for TRPV4.

Rather surprisingly, the expression analysis with qRT-PCR showed a tendency to up-regulation of TRPV4 mRNA in the contralateral side of the injury and to down-regulation of all other channels in the ipsilateral side of the injury. In particular, the mRNA levels for TRPM8 and TRPV3 were significantly less in the ipsilateral side to CCI at 14 days after the surgery.

Another study has examined the expression of the TRP channel mRNA after nerve injury in rat by using a ribonuclease protection assay (Frederick et al., 2007). Frederick and colleagues reported no change for TRPV3 and a small up-regulation for TRPM8 and TRPA1 in the ipsilateral side of the injury. Their technique was not sensitive enough to detect TRPV4. This is in contrast to my results, where I observed a down-regulation of the channels. However the positive control I used and the ability to detect the low TRPV4 mRNA levels showed the validity and the higher sensibility of the method I utilized. Nevertheless one common point in both studies is that injury induced changes in expression of the temperature sensitive ion channels are relatively minor, especially when compared to other molecules such as galanin. It is therefore uncertain whether such small changes would have much significance for cold sensation.

4.2.2 The percentages of TRPA1 and TRPM8 expressing neurons decrease after nerve injury

Finding no predominant change in the mRNA expression levels of any temperature sensitive ion channels, we further focused on the cold sensitive ion channels TRPM8 and TRPA1 and analysed their expression in detail using the combination of *in situ* hybridization and immunohistochemstry. This technique allowed me to examine the expression of the channels in specific subtypes of sensory neurons. Indeed one potential mechanism that could account for cold allodynia is a novel expression of cold sensor molecules in a previously cold-insensitive subpopulation. A similar “phenotypic switch” has been described for a subpopulation of A-fibres which display an atypical expression of TRPV1 after injury (Rashid et al., 2003). We therefore investigated the expression pattern of TRPM8 and TRPA1 in myelinated neurons, non-peptidergic nociceptors and peptidergic nociceptors.

In agreement with other studies my staining experiments clearly showed that TRPM8 is expressed in a specific subclass of small sized neurons that are not co-labelled with any of the common neuronal markers (CGRP, IB4 and NF200) (Mc Kemy et al., 2002; Peier et al., 2002). The percentage of TRPM8 expressing neurons, which I found to be 7%, also accords with most of the previous studies

(Peier et al., 2002; Xing et al., 2007) albeit another group reported higher numbers corresponding to 25 % of all the DRG neurons (Kobayashi et al.; 2005); these discrepancies probably reflect the variability derived from diverse methods used for staining and counting. However the higher percentage reported by Kobayashi et al. (2005) disagrees with the number of cold-menthol sensitive neurons (around 10%) revealed from electrophysiological studies and as far as there is no explanation for this discrepancy, 7% of DRG neurons seems to be a feasible number for TRPM8 expression.

Some disparities between different studies emerge about the expression of TRPA1 too. TRPA1 was first found in 3,6 % of all DRG neurons (Story et al., 2003); this differs with our and other studies, which showed TRPA1 expression in circa 30% of the DRG neurons (Bautista et al., 2005; Kobayashi et al., 2005), and again this higher percentage matches well with mine and other electrophysiological analyses, which demonstrated that around 30% of the neurons are sensitive to TRPA1 agonists (Bautista et al., 2005; Bautista et al., 2006; Sawada et al., 2007).

A very interesting aspect of my results regards the population of neurons expressing TRPA1. I found two different populations of TRPA1 expressing neurons, the larger one is IB4 positive and corresponds to 25 % of all DRG neurons, the second population is CGRP positive and only covers 3 % of DRG neurons.

In contrast to my data in the first study on TRPA1 Story and colleagues found that almost all of the TRPA1 expressing neurons belong to the CGRP positive population (Story et al., 2003), but looking carefully at mine and their results a common point becomes visible; the size of the population of the TRPA1 expressing neurons which is CGRP positive is the same in both studies (around 3% of all DRG neurons), this raises the question as to whether their technique was not able to detect TRPA1 in CGRP negative neurons. Another study described a high co-expression between TRPA1 and CGRP although they did not report the exact percentage corresponding to this population (Bautista et al., 2005). However, our is the only study which investigated the double staining of TRPA1 and IB4 in the DRG and our data agree with a report from Koltzenburg and colleagues in which they combined calcium imaging with vital IB4 staining to study

the developmental expression of TRP channels in subpopulations of sensory neurons. In agreement with our results they found TRPA1 to be expressed in both populations of neurons, IB4 positive and IB4 negative, and they clearly showed that the IB4 positive population was far bigger than the IB4 negative one (Hjerling-Leffler et al., 2007).

After nerve injury, I observed that the number of TRPM8 and TRPA1 positive neurons is significantly decreased and I found no evidence of new expression in different sensory neuron subtypes.

There have been some studies on the expression of TRP channels in neuropathic pain models which report that TRPA1 expression is increased in uninjured, spared neurons (Obata et al., 2005). These studies used the L5 spinal nerve ligation (SNL) model where neurons in the L5 DRG are injured, but neighbouring fibres of the sciatic nerve that extend to the L4 DRG are separate and spared. In this model, both TRPA1 and TRPM8 were down-regulated in the L5 DRG in agreement with our data, but TRPA1 was weakly up-regulated in the uninjured L4 DRG. It was argued that this up-regulation in uninjured neurons could be a potential mechanism for cold hypersensitivity. However, I detected no up-regulation of TRPA1 after nerve injury, even though I measured robust cold allodynia. Therefore, if an up-regulation of TRPA1 is responsible for cold allodynia, then this could be a specific aspect of the SNL model of neuropathic pain.

Another two studies have investigated the expression of TRPM8 using the CCI model. In contrast to my results they found an increase in the expression of TRPM8 after CCI. Specifically one group found that after CCI the TRPM8 immunoreactive positive neurons in the L5 DRG ipsilateral to the injury increase from 7.2 ±0.7 % to 15.0 ±0.8 %, moreover they reported an increase in the cold and menthol sensitivity of the capsaicin sensitive population of DRG neurons ipsilateral to the injury. This would suggest that the increase of TRPM8 expression involves the TRPV1 positive population of neurons (Xing et al., 2007). The other group used an antibody raised to two different areas of human TRPM8 to analyse the co-expression of TRPM8 with the neuronal markers peripherin and NF200 after CCI; they did not report the percentages in terms of all DRG neurons, but in naïve rats they detected TRPM8 immunoreactivity in 8.3 ±0.2 % of the peripherin positive neurons and in 1.3 ±0.5 % of the NF200 positive neurons, while after CCI

these percentages increase to 15.5 ± 0.8 % and 7.9 ±1.2 %, respectively (Proudfoot et al., 2006). There are three main differences between our and these studies:

1. The first difference is in the method; I detected the presence of TRPM8 using RNA probes, while they used antibodies against TRPM8. In the method I utilized, a negative control with sense probes clearly showed that there were no false positives, which are often present in immunostaining with antibodies. Indeed, they observed a small percent of co-expression with neurofilament 200, which was absolutely absent in mine and other stainings (Peier et al., 2002). Additionally the possibility of false negatives in my experiment was minimized using three different probes for TRPM8 (including the full-length probe) and hydrolysing them. Another possibility could be that nerve injury would induce an increase in the half-life of TRPM8 rather then in its gene expression.
2. A second difference between these and our studies is in the lumbar DRG analysed. I stained all the lumbar DRG connected with the sciatic nerve (L3 to L6) while in the other studies L5 or L5 and L6 were stained. Considering this difference it is possible that nerve injury simultaneously provokes an increase of TRPM8 expression in the lower DRG L5 and L6 and decrease in the upper DRG L4 and L3. However our study stands for the complete outline of the sensory neurons connected to the sciatic nerve. Further studies on the expression of TRPM8 in all the 4 lumbar DRG are needed to assess this question.
3. Finally the two previous studies used the CCI model in rat while I used it in mice; this raises the question whether different mechanisms could develop in the different species after nerve injury.

However all studies, which have detected the percentage of TRPM8 expressing neurons after nerve injury, only reported small changes. Thus, it seems unlikely that such small variations could explain the big alterations in cold sensation induced by nerve injury.

4.3 Sensory neuron function after nerve injury

4.3.1 The percentage of menthol sensitive neurons does not change after nerve injury

I identified TRPM8 positive neurons by applying (-)-menthol to dissociated DRG cells. Interestingly, 12% of all DRG neurons responded to menthol in culture, which was greater than expected from my expression analysis and about a quarter of all menthol sensitive neurons did not respond to cold. One explanation for this discrepancy is that menthol is not entirely selective for TRPM8. Indeed, menthol has recently been demonstrated to evoke Ca2+ release from intracellular stores via a TRPM8-independent pathway (Mahieu et al., 2007), and to activate TRPA1 at low concentrations (Karashima et al., 2007). Thus a TRPM8 independent mechanism could account for the cold-insensitive, menthol responsive cells observed here. In fact, the percentage of neurons responding to both stimuli, menthol and cold, is 8.5, which matches well with my expression experiments for TRPM8. Additional evidence for this comes from two studies with TRPM8 knock out mice, in which the percentage of menthol sensitive neurons was decreased, but not abolished (Dhaka et al., 2007; Colburn et al., 2007).

In my analysis of menthol sensitive neurons, no variations emerged after CCI compared to the control, this is in contrast to the study of Xing and colleagues. In two separate tests they analysed neurons from L5 DRG and Dil-retrograde-labelled DRG neurons that innervate the skin of the hindlimbs. In both tests the population of menthol sensitive neurons which are cold and capsaicin sensitive increased in the ipsilateral side of the injury. As discussed above, one possible explanation for this difference between our and this study is that I used mice while Xing and colleagues used rats and there may be species differences in the role of TRPM8 in peripheral sensory neurons following nerve injury. It would be interesting examine this possible species differences in a future step.

4.3.2 The percentage of mustard oil sensitive neurons decreases after nerve injury

TRPA1 containing neurons were identified by their response to mustard oil and in these cells I observed no correlation between cold and mustard oil sensitivity. I applied cold stimuli as low as 8°C, which were well below the proposed temperature threshold for TRPA1 (Story et al., 2003; Sawada et al., 2007), but I

was unable to activate the majority of mustard oil sensitive neurons. Indeed over 80% of all the mustard oil sensitive neurons were insensitive to cold. We recently reported that TRPA1 is activated by intracellular Ca2+ and that cold sensitivity occurs indirectly through an increased Ca2+ concentration in HEK293 cells (Zurborg et al., 2007). However, cold does not evoke a general increase in Ca2+ concentration in sensory neurons (as occurs in HEK293 cells), thus even an indirect role for TRPA1 in cold sensation seems unlikely. Consequently, I did not examine behavioural responses to mustard oil in CCI mice and I continued the functional analysis by designating cold responsive neurons as either menthol-sensitive, or menthol-insensitive as has been reported in previous studies (Nealen et al., 2003; Thut et al., 2003; Babes et al., 2004).

Intriguingly, I found that mustard oil responses were strongly down-regulated after injury. It is difficult to gauge the functional significance of TRPA1 down-regulation and its contribution to the pathophysiology of neuropathic pain. However, voltage-gated Ca2+ currents are also diminished after nerve injury (Hogan, 2007) and this has been proposed to lead to increased excitability in injured sensory neurons via reduced activation of Ca2+ activated K+ currents. Since TRPA1 is gated by, and is permeable to Ca2+ (Zurborg et al., 2007), it is possible that reduced expression of the channel might also contribute to diminished Ca2+ currents and enhanced excitability after injury.

4.3.3 Cold sensitivity does not change after nerve injury

In the present study, 30% of acutely dissociated DRG neurons were cold-sensitive. This percentage is higher than in studies where neurons were maintained in cultures for more than a few hours (Peier et al., 2002; Story et al., 2003), but in agreement with work on freshly dissociated cells which reported 29% of mouse (Stucky et al., 2004) and 22% of rat DRG cells (Babes et al., 2004) to be cold-sensitive. This proportion is also in good agreement with the percentage of cold-sensitive neurons estimated from teased fibre recording of peripheral nerves in rodents (Koltzenburg, 2004). It is therefore possible that acutely dissociated neurons reflect the native cold sensitivity of neurons more accurately than cells maintained for longer periods in culture.

A surprising aspect of my results is that I observed no differences in any parameters of cold sensitivity in the primary cultures of DRG post-injury. This is in contrast to a study examining cold sensitivity in the SNL model of neuropathic pain (Djouhri et al., 2004). It was reported that while cold responses remained constant in injured neurons, a proportion of uninjured neurons of the L4 DRG developed sensitivity to cold after injury. This novel population of cold sensing neurons could potentially contribute to cold allodynia in the SNL model; however my data suggests that this is unlikely to be an universal mechanism for cold hypersensitivity. I observed no change in cold sensitivity of DRG neurons in CCI mice even though animals displayed cold allodynia. Similarly, in an experimental neuroma model in mice, Roza et al. (2006) demonstrated that cold sensitivity of axotomized fibers does not change from pre-injury levels. While my data do not exclude the possibility that changes in cold sensitivity might occur in the peripheral terminals of sensory neurons, it does indicate that alterations in the number of cold sensitive DRG neurons is not a predominant mechanism for cold allodynia in neuropathic pain.

4.4 Potential mechanisms for cold allodynia

Superficially, my *in vivo* and *in vitro* data are not entirely consistent. *In vivo* I observed a robust appearance of cold allodynia and a large increase in menthol sensitivity after nerve injury. When I looked at the peripheral mechanisms of cold transduction *in vitro*, I found down-regulation rather then up-regulation of both TRPM8 and TRPA1 and no changes in the functional proprieties of cold sensitive sensory neurons. These findings pose a fascinating question: How therefore does cold allodynia arise?

Several different mechanisms could play a role in the origin of cold allodynia. All we looked at was the peripheral nervous system, but after a nerve injury many important changes also occur in the central nervous system (in both the brain and spinal cord).

In the brain a modification in the central processing of cold stimuli could underlie the emergence of cold hypersensitivity after nerve injury. In a recent study Seifert and Maihofner assessed whether cold pain and cold allodynia produce different

brain responses. They used fMRI (functional magnetic resonance imaging) to examine brain activation to cold stimuli in human volunteers before and after the induction of cold allodynia by 40% of menthol. Indeed they showed that during cold allodynia the central processing in cold pain recruited the bilateral dorsolateral prefrontal cortex and the midbrain, which are regions not normally associated with cold sensation (Seifert and Maihofner, 2007).

At the spinal cord level different changes could also trigger cold allodynia and several mechanisms such as structural reorganization and increased responsiveness of dorsal horn neurons, loss of inhibitory neurons in the spinal cord and activation of spinal cord glia have been postulated to contribute to neuropathic pain states (Woolf, 2004; Woolf and Scholz, 2007).

In the present study I have not investigated the central mechanism of cold hypersensitivity, but some considerations bring us to hypothesize a potential central mechanism of cold allodynia. We and other groups have found that there are at least two different types of cold sensitive neurons, the TRPM8 positive and the TRPM8 negative ones (Thut et al., 2003; Babes et al., 2004). TRPM8 positive neurons transmit cold and menthol sensitivity and interestingly cold and menthol are known since antiquity to inhibit pain (Sprengell, 1735; Siegel, 1970). According to my results, nerve injury simultaneously increases the pain behaviours to menthol and cold stimuli and decreases the expression of TRPM8. Keeping in mind all the above arguments a new hypothesis comes into view; the decrease of TRPM8 expression after nerve injury could potentially lead to a lack of pain inhibition, which could promote more pain.

No direct study has been done to investigate this new hypothesis, but several reports lend support to the mechanism.

More than 40 years ago, the gate control theory of pain (Melzack & Wall, 1965) proposed that inhibitory neurons in the superficial dorsal horn of the spinal cord control the relay of nociceptive signals from the periphery to higher areas of the central nervous system. Specifically, a number of human studies have shown that cold pain sensation is under inhibitory control. Yarnitsky and Ochoa showed that

cold stimuli evoke burning and stinging pain rather than coolness when A-fibres are blocked, which indicates that cold sensitive A-fibres activate inhibitory inputs to pain transmission under normal condition (Yarnitsky and Ochoa, 1990).
In a recent study using mice engineered to express the farnesylated enhanced green fluorescent protein from the TRPM8 locus, staining experiments showed an overlap between TRPM8 and GAD65 in the lamina I of the dorsal horn of the spinal cord (Dhaka et al., 2008). GAD65, the smaller isoform of the enzyme glutamic acid decarboxylase, synthesizes the inhibitory neurotransmitter GABA (γ-amino butyric acid). GAD65 positive neurons may form axo–axonic synapses on TRPM8 expressing primary afferent terminals, which would confirm a direct connection between TRPM8 and inhibitory neurons. An interesting future step could be to investigate this inhibitory connection after nerve injury.

Additionally, a study on painful human teeth with irreversible pulpitis and cold hyperalgesia showed that TRPM8 expressing nerve area was significantly less in the cold sensitive painful molar compared with healthy controls (Alvarado et al., 2007). The authors proposed that TRPM8 is most likely not involved in cold-mediated noxious pulpal pain mechanisms, but a similar mechanism to the one we propose to underlie cold allodynia in neuropathic pain could also explain the emergence of cold hyperalgesia in teeth by the removal/reduction of TRPM8-mediated inhibitory inputs.

However to confirm our new hypothesis further studies are needed which would examine the inhibitory pathways and investigate the connections with TRPM8-expressing cold sensitive primary afferents. Additionally, a thorough investigation of cold transduction in both mice and rats could reveal important species differences.

5. SUMMARY

My Ph.D. thesis focuses on a common symptom of neuropathic pain: cold allodynia. Patients suffering from cold allodynia feel pain after stimuli which normally give a pleasant cool sensation. Despite the significant incidence and the important impact of this symptom, the molecular and physiological mechanisms underling alterations in cold sensitivity are still unknown. Recently, much progress has been made in our understanding of cutaneous thermal sensitivity, and different ion channels have been proposed as candidate of thermotrasduction in the sensory neurons. Above all two non-selective cation channels TRPM8 and TRPA1 have attracted much attention as potential sensors of cold. The goal of my Ph.D. project was to investigate whether changes in the mechanisms of temperature transduction might underlie cold hypersensitivity in a mouse model of neuropathic pain (the CCI model). To accomplish my task I used three different approaches.

In the first approach I examined the functional traits of TRPM8, the main candidate of cold transducer, after nerve injury *in vivo*. The idea for this analysis is based on the findings that TRPM8 is activated by two different stimuli, cold and menthol. (Mc Kemy et al., 2002; Peier et al., 2002). Therefore we reasoned that if changes in TRPM8 underlie alteration of cold sensitivity after nerve injury, the same changes should occur in menthol sensitivity too. To check this hypothesis I first established a new behavioural assay to test the menthol sensitivity in mice, and indeed I observed that menthol evokes strong nociceptive behaviour after nerve injury. I then aligned the results of the menthol assay with the measurements of cold sensitivity. The increase in menthol sensitivity developed with the same time course of the emergence of cold allodynia, this suggests that activation of TRPM8 in CCI mice triggers nociceptive behaviour and that TRPM8 might be a key component of cold hypersensitivity.

The second approach explored the expression of TRPM8 and TRPA1 in the dorsal root ganglia after nerve injury. For this analysis I performed two different techniques. I first quantified the mRNA levels of the TRP channels using qRT-PCR, and I then used a combination of *in situ* hybridization and

immunohistochemstry to identify the neuronal populations expressing the TRP channels. Surprisingly, after nerve injury I observed decrease in the expression of TRPM8 as well as of TRPA1 in both the mRNA levels and the percentage of expressing neurons.

The last approach involved an *in vitro* functional analysis of sensory neurons. My goal was to investigate the cold sensitivity and the function of the TRPM8 and TRPA1 after nerve injury. I carried out calcium imaging experiments in acutely dissociated DRG neurons to analyse the populations of neurons sensitive to cold, menthol (the TRPM8 agonist) and mustard oil (the TRPA1 agonist). The major findings of these experiments are:

- There is no relation between cold sensitivity and TRPA1 expression (only 18% of TRPA1 expressing neurons are cold sensitive). This confirms our previous work, where we reported that TRPA1 is not a cold sensor (appendix 2).
- The percentage of mustard oil sensitive neurons decreases after nerve injury, which agrees with my expression analysis for TRPA1.
- After nerve injury no difference appears in the percentage as well as in the properties of the cold sensitive neurons, and this is valid for both the menthol sensitive and menthol insensitive populations of cold sensitive neurons.

In conclusion my results did not confirm our initial hypothesis and cold allodynia in this mouse-model does not result from an increase in the expression and function of the cold sensitive TRP channels. Nevertheless TRPM8 seems to have a role in the emergence of cold allodynia, and we suggest that this role might be explained by changes in the spinal connections of the TRPM8 expressing neurons. As yet this hypothesis has not been proven.

6. ZUSAMMENFASSUNG

Meine Doktorarbeit beschäftigt sich mit einem bekannten und verbreiteten Symptom neuropathischer Schmerzen: der Kälteallodynie. Patienten, die unter Kälteallodynie leiden, empfinden einen normalerweise angenehm kühlen Stimulus als schmerzhaft. Trotz des häufigen Auftretens und der bedeutenden Belastung dieses Symptoms, sind die den Änderungen in der Kälte-Sensitivität zugrundeliegenden molekularen und physiologischen Mechanismen bisher unbekannt. In letzter Zeit wurden wichtige Fortschritte zum Verständnis der thermalen Sensitivität der Haut gemacht und in diesem Zusammenhang verschiedene Ionenkanäle als mögliche Kandidaten für thermische Transduktion in sensorischen Neuronen vorgeschlagen.
Besondere Aufmerksamkeit erlangten zwei nicht-selektive Ionenkanäle- TRPM8 und TRPA1- als mögliche Kälte-Sensoren. Ziel meiner Doktorarbeit war es, zu untersuchen, ob eine Veränderung im Mechanismus der thermischen Transduktion verantwortlich für Kälte-Hypersensitivität in einem neuropathischen Schmerzmodel (CCI-Model) in der Maus ist. Hierfür nutzte ich drei verschiedene Ansätze.

Im ersten Ansatz untersuchte ich die funktionellen Eigenschaften von TRPM8, dem wahrscheinlichsten Kälte-Sensor Kandidaten, nach einer Verletzung eines Nervens *in vivo*. Die Idee für diese Analyse basiert auf dem Befund, dass TRPM8 durch zwei Stimuli aktiviert werden kann, Kälte und Menthol (Mc Kemy et al., 2002; Peier et al., 2002). Falls Änderungen in der TRPM8 Expression Abweichungen in der Kälte-Sensitivität hervorrufen sollten, erwarteten wir, dass diese Abweichungen auch in der Menthol-Sensitivität auftreten. Um diese Hypothese zu überprüfen, etablierte ich einen neuen Verhaltenstest für Menthol-Sensitivität in Mäusen und konnte in der Tat beobachten, dass Menthol nach Verletzung eines Nervens ein starkes Schmerzverhalten hervorruft. Anschließend verglich ich die Ergebnisse des Mentholtests mit den Messungen der Kälte-Sensitivität. Der Anstieg in der Menthol-Sensitivität trat gleichzeitig mit der Entstehung der Kälteallodynie auf, was darauf hinweist, das die Aktivierung von TRPM8 in den CCI Mäusen das Schmerzverhalten zu beeinflussen scheint und TRPM8 eine Schlüsselkomponente der Kälte-Hypersensitivität sein könnte.

Der zweite Ansatz untersuchte die Expression von TRPM8 und TRPA1 in den dorsalen Hinterwurzelganglien nach Verletzung des Nerven. Für diese Analyse nutzte ich zwei unterschiedliche Techniken.
Zuerst quantifizierte ich die mRNA-Levels von TRP-Kanälen mittels qRT-PCR und benutzte anschließend eine Kombination aus *in situ* Hybridization and Immunhistochemie, um die TRP-Kanal exprimierende Neuronenpopulation zu identifizieren. Überraschenderweise beobachtete ich nach Verletzung des Nervens eine Abnahme von TRPM8 und TRPA1, sowohl in den mRNA-Levels als auch in der Prozentzahl der exprimierenden Neurone.

Der letzte Ansatz beinhaltete eine funktionelle *in vitro* Analyse der sensorischen Neurone. Mein Ziel war es, die Kälte-Sensitivität und die Funktion von TRPM8 und TRPA1 nach Verletzung des Nervens zu untersuchen. Ich führte “calcium imaging” Experimente in entnommenen DRG Neuronen durch, um die Neuronenpopulation zu analysieren, die sensitiv auf Kälte, Menthol (TRPM8 Agonist) und Senföl (TRPA1 Agonist) reagiert.
Die wichtigsten Ergebnisse dieser Experimente sind:

- Es gibt keine Beziehung zwischen Kälte-Sensitivität und TRPA1 Expression (nur 18% der TRPA1 exprimierenden Neurone sind kältesensitiv). Das bestätigt frühere Arbeiten, in denen wir zeigten, dass TRPA1 kein Kälte-Sensor ist (Appendix 2).
- Die Prozentzahl Senföl-sensitiver Neurone nahm nach Verletzung des Nervens ab, dies stimmt mit meiner Expressionsanalyse für TRPA1 überein.
- Nach Verletzung des Nervens trat kein Unterschied in der Prozentzahl sowie den Eigenschaften von kältesensitiven Neuronen auf, dies gilt für mentholsensitive und nicht-mentholsensitive Populationen kältesensitiver Neurone.

Zusammenfassend bestätigen unsere Ergebnisse unsere ursprüngliche Hypothese nicht. Die Kälteallodynie im genannten Mausmodel resultiert nicht aus einem Anstieg in der Expression und Funktion der kältesensitiven TRP-Kanäle. Nichtsdestotrotz scheint TRPM8 eine Rolle in der Entstehung der Kälteallodynie

zu spielen und wir glauben, diese Rolle könnte anhand von Veränderungen in den spinalen Verbindungen TRPM8-exprimierender Neurone erklärt werden. Diese Hypothese ist bisher nicht untersucht.

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9. DECLARATION

I hereby declare that the composition of this thesis and work presented in it is entirely my own with the exception of the acetone test, which was done in collaboration with Dr. Dominika Labuz. Some of the work from this thesis is described in a manuscript, which is currently under revision.

Berlin, March 2008 Ombretta Caspani

10. LEBENSLAUF

Aus rechtlichen Gründen wird mein Lebenslauf nicht veröffentlicht.

11. APPENDIX

Some of the work from this thesis is described in a manuscript, which is currently under revision. Additionally, as I discussed in the introduction, during my Ph.D. I collaborated at other two projects, which ended in the following publications:

Appendix 1:

A stomatin-domain protein essential for touch sensation in the mouse.
Christiane Wetzel, Jing Hu, Dieter Riethmacher, Anne Benckendorff, Lena Harder, Andreas Eilers, Rabih Moshourab, Alexey Kozlenkov, Dominika Labuz, Ombretta Caspani, Bettina Erdmann, Halina Machelska, Paul A. Heppenstall & Gary R. Lewin
Nature, 2007. Vol. 445: 206-209.

Appendix 2:

Direct activation of the ion channel TRPA1 by Ca(2+).
Sandra Zurborg, Brian Yurgionas, Julia A Jira, Ombretta Caspani & Paul A Heppenstall
Nat Neurosci, 2007. Vol.10: 277-279.