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EXPRESSION AND FUNCTION OF TRP CHANNELS IN PERIPHERAL SENSORY NEURONS

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Stockholm 2009

Published by Karolinska Institutet. Printed by Intellecta.

ISBN 978-91-7409-445-9

ABSTRACT

Transient receptor potential (TRP) channels comprise a family of multifunctional proteins which has since its discovery been of particular interest in sensory biology even though they are also frequently expressed in non-neuronal cell types. It is evident that TRP channels play an important role in the function of sensory neurons, not at least by being sensors of external stimuli. Direct activation by thermal and chemical stimuli has been shown while their involvement in mechanosensation is still elusive. In addition many TRP channels also seem to have more modulatory roles in the cell by for example adjusting intracellular Ca^{2+} levels in response to activation of other membrane proteins.

This thesis presents new findings on the expression and function of TRP channels in peripheral sensory neurons. Using quantitative real-time PCR the developmental expressions of all 28 vertebrate TRP channels were studied. Results showed that most channels are expressed in adult thoracic and lumbar dorsal root ganglia (DRG) and in nodose ganglia (NG). The most common expression pattern displayed low levels at early embryonic development followed by progressively increasing levels at later stages of development, reaching its highest expression at postnatal and adult stages. Cellular localisation studies of selected channels in the DRG further revealed expression in different neuronal subtypes. TRPC1 and TRPC2 were primarily found in large sized mechanosensitive neurons, TRPC3 was exclusively found in non-peptidergic nociceptors and TRPM8 was found in a very limited population of small sized neurons, not co-labelling with any of the tested markers for neuronal subtypes. These findings open for potential new, previously undefined roles, for some of the TRP channels in sensory neurons.

To elucidate new functional roles, an expression study measuring differential regulation of all TRP channels in rats during development of neuropathic pain was conducted. Downregulation was evident for TRPM6, A1, V1, M8, C3, C4 and C5 while upregulation was seen for TRPML3. TRPM6, C3, C4, C5 and ML3 have never previously been associated with pain and are therefore considered to be important new findings.

Since the identification of TRPC1 as a stretch sensor in liposomes from oocytes this finding has been questioned due to difficulties in confirmation of these results in heterologous cell systems. To further explore this issue we used native neurons from DRG to study the possible involvement of TRPC1 in mechanosensitivity. In neurons subjected to short hairpin RNA (shRNA) mediated knockdown of TRPC1, the response to cell stretch was shown to be reduced by 65%. These data further emphasise the indication that TRPC1 is important, either directly or indirectly, in the activation mechanism of mechanosensation.

In conclusion, this thesis show that most of the 28 vertebrate TRP channels are expressed in peripheral sensory ganglia and have specific neuronal subtype expression patterns. Furthermore, the data suggest potential new roles related to pain pathophysiology for several TRP channels and further strengthen the hypothesis of TRPC1 involvement in mechanosensation.

LIST OF PUBLICATIONS

I. **Susanne Elg**, Frédéric Marmigère, Jan P. Mattsson and Patrik Ernfors
Cellular subtype distribution and developmental regulation of TRPC channel members in the mouse dorsal root ganglion.
Journal of Comparative Neurology, 2007, 503, 35-46

II. **Susanne Staaf**, Frédéric Marmigère, Jan P. Mattsson and Patrik Ernfors
Dynamic expression of the TRPM subgroup of ion channels in developing mouse sensory neurons.
Submitted

III. **Susanne Staaf**, Sandra Oerther, Guilherme Lucas, Jan P. Mattsson and Patrik Ernfors
Differential regulation of TRP channels in a rat model of neuropathic pain.
Pain, Accepted 2009

IV. **Susanne Staaf**, Ingela Maxvall, Ulrika Lind, Johanna Husmark, Jan P. Mattsson, Patrik Ernfors and Stefan Pierrou
Down regulation of TRPC1 by shRNA reduces mechanosensitivity in mouse dorsal root ganglion neurons in vitro.
Neuroscience Letters, 2009, 457, 3–7

CONTENTS

LIST OF ABBREVIATIONS

ANS	autonomic nervous system
BK_{Ca}	large conductance calcium-activated potassium channels
CCI	chronic constriction injury
CGRP	calcitonin-gene related peptide
CNS	central nervous system
DRG	dorsal root ganglion
E	embryonic day
GDNF	glial cell derived neurotrophic factor
GPCR	gene protein coupled receptor
IB4	isolectin B4
NF200	neurofilament 200
NG	nodose ganglion
NGF	nerve growth factor
P	postnatal day
PNI	partial nerve injury
PNS	peripheral nervous system
Ret	rearranged during transfection
RTK	receptor tyrosine kinase
SNI	spared nerve injury
SNL	spinal nerve ligation
TG	trigeminal ganglion
Trk	tropomyosin receptor kinase
TRP	transient receptor potential channel
Va	varitint waddler mouse

1 INTRODUCTION

1.1 THE PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system (PNS) is composed of sensory and motor neurons. The sensory neurons (afferents) perceive sensory stimuli upon changes in the internal and external environment using the sensory receptors, i.e. chemosensors, mechanosensors, nociceptors, photosensors and thermosensors, in the receptive fields and convey the information further on to the central nervous system (CNS) for interpretation. The motor neurons (efferents) run from the CNS to the muscles and glands to execute physiological responses to the original stimuli. The PNS can be subdivided into the somatic and the autonomic nervous system (Bear et al., 1996).

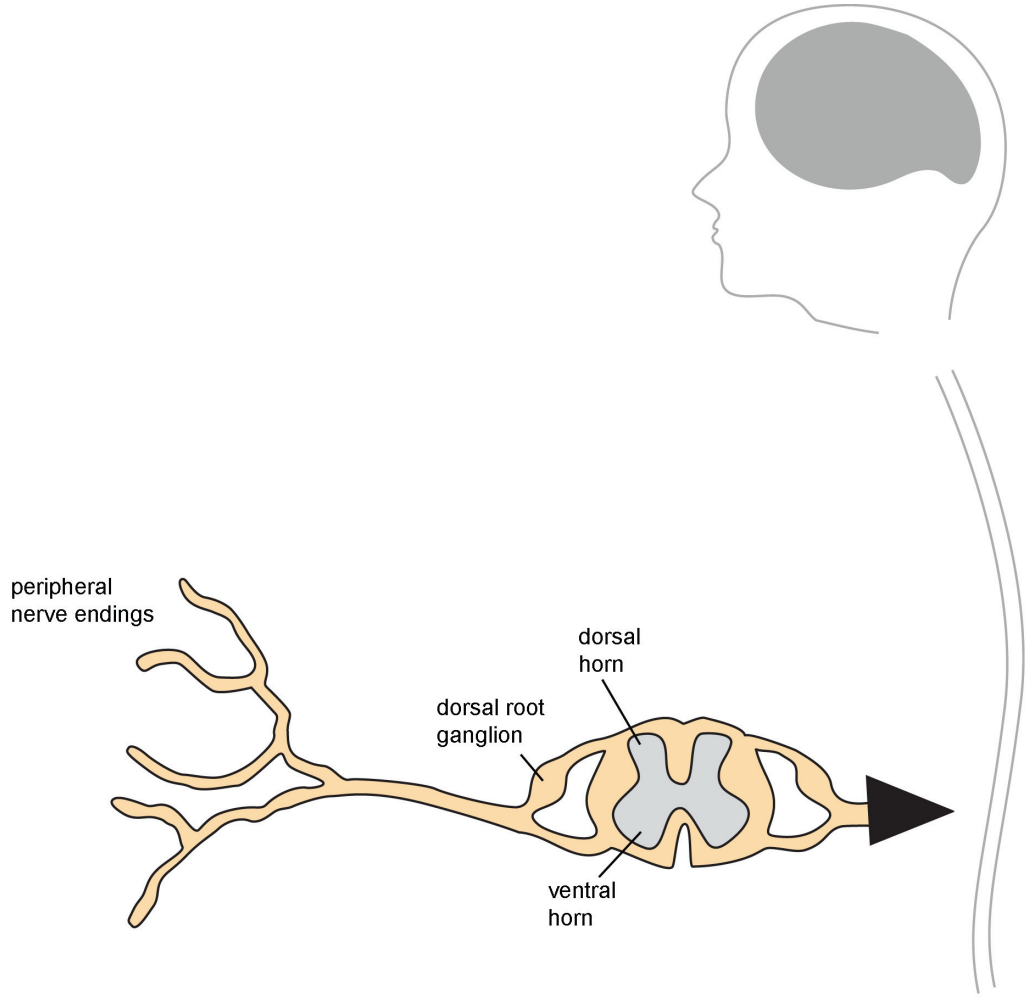

Figure 1. Schematic illustration of the spinal cord. The peripheral nerve endings detect sensory stimuli and transmit the information via sensory afferents to the dorsal horn in the spinal cord and furtheron in ascending pathways to the brain. The cell bodies of the afferents are collected in the dorsal root ganglia. The efferent signalling occurs in descending pathways from the brain to the ventral horn in the spinal cord from where peripheral motor neurons extend efferents which provide instructions for muscles and glands to act upon the original sensory stimuli.

1.1.1 The somatic nervous system

The somatic nervous system detects changes in the external environment and governs the functions of vision, hearing, somatic sensation (touch), taste and olfaction (smell). The somatic system also coordinates conscious movements of the body parts using motor neurons controlling the skeletal muscles. The somatic motor efferents have their cell bodies within the CNS in the ventral spinal cord or in the brain stem. The cell bodies of somatic sensory neurons reside in the cranial and spinal ganglia that extend nerve afferents to the periphery of the rest of the body. The spinal ganglia, the DRG, innervates the gut, the lower limb muscles and the skin via the sciatic nerve and can be divided into four sections, the cervical, the thoracic, the lumbar and the sacral ganglia presented from neck to tail. The sensory afferents reach the CNS in the dorsal spinal cord (Bear et al., 1996).
The cranial ganglia are spatially segregated reflecting their different developmental origins (see section 1.1.3). Somatic sensory neurons are collected in the "root" ganglia (close to the brain) and the general visceral and special visceral (gustatory) sensory neurons are situated in the "trunk" ganglia (more distal to the brain). The sensory afferents of the vagus nerve have their cell bodies in the nodose and jugular ganglia, situated in the neck region and innervate the nucleus tractus solitarus (NTS) in the brain stem. Also here the neurons locate in separate ganglia i.e. NG contains general and special visceral sensory neurons (placode-derived) and jugular ganglion contains somatic sensory neurons (neural crest-derived) (Undem and Weinreich, 2005).

1.1.2 The autonomic nervous system

The autonomic nervous system (ANS) controls the homeostasis of the body and is responsible for the unconscious actions throughout the body. For executing these tasks the hypothalamus instructs preganglionic motor neurons in the brain stem and ventral spinal cord to signal to the postganglionic neurons outside the CNS, with its cell bodies clustered in autonomic ganglia. The ANS can be further subdivided into the sympathetic and parasympathetic nervous system, which are responsible for responses related to arousal and energy generation ("fight-or-flight") or more calming, normal activity states like digestion ("rest-and-digest"), respectively. The sympathetic division has its preganglionic neurons in the thoracic and the lumbar spinal cord while the parasympathetic central axons arise from the brain stem and the sacral spinal cord. The sympathetic neurons send signals via the ventral roots to the postsynaptic ganglia in the sympathetic chain, a neuronal structure residing next to the spinal column. The signals from the parasympathetic preganglionic neurons travels in the cranial and sacral nerves to end up in postganglionic ganglia residing next to or in the target organs (Bear et al., 1996).
The enteric nervous system, innervating the gastrointestinal organs, are also regarded as a division of the ANS and contains both sympathetic and parasymathetic nerves. The neurons are collected in two types of ganglia in the lining of the digestive organs, myenteric (Auerbach's) and submucosal (Meissner's) plexuses, located in the muscularis externa and submucosa, respectively. These structures contain both visceral sensory and motor neurons and control the processes involved in the transport and digestion of food (Bear et al., 1996).

1.1.3 Organisation and developmental origin

After fecundation in a process called gastrulation three distinct areas (germ layers) are formed in the embryo i.e. the endoderm, that gives rise to the digestive system, the mesoderm, developing into the inner organs, and the ectoderm that is the source of the skin and the nervous system. Thereafter the embryo elongates and a bulge is formed in the middle, called the neural plate, which after reaching its maximum thickness invaginates and forms a tube, the neural tube. Longitudinally along the neural tube, two structures are created called the neural crest, a process initiated by the inducing factors BMP (bone morphogenetic protein), Wnt (wingless/INT) and FGF (fibroblast growth factor). The cells constituting the neural tube develop into the CNS and the cells in the neural crest give rise to many cells of the PNS including all satellite cells and schwann cells plus sensory and autonomic neurons innervating the trunk. A part of the neural tube forms the spinal cord, to which the central axons from the DRG projects (Basch et al., 2004; Sadler, 2005). The sensory neurogenesis, where neural crest cells migrate to the forming DRG, occurs in three waves where large proprioceptive and mechanoreceptive neurons are born first followed by a second wave resulting in all neuronal subtypes. Finally, the third wave, consisting of small nociceptive neurons, arises from the boundary caps which are clusters of cells at the entry and exit points of the peripheral nerve roots. In mouse, the sensory neurogenesis starts around embryonic day (E) 8.5 and finish around E11 when the boundary cap cells have migrated and the DRG have been formed. Maturation of the final neuronal phenotypes continues through the rest of the embryonic development well into postnatal life in parallel with the formation of synaptic connections between the target tissue and the neuronal cell body (Marmigere and Ernfors, 2007).
Neurons of the nodose and most cranial ganglia have a different developmental origin than the DRG. Instead of arising from the neural crest these cells are placode-derived and has nerve afferents extending to the viscera. However, the satellite cells and schwann cells in these ganglia do arise from the neural crest. The NG receive sensory information via the vagus nerve from the cardiovascular, gastrointestinal and respiratory organs and transmit it to the nucleus tractus solitarius in the brain stem. The epidermal placodes, 9-10 pairs, arise from the neural tube as thickenings of the ectoderm. A subset of these, the epibranchial placodes, are the origins of the visceral afferent neurons of the vagus nerve (Schlosser, 2006; Zhuo et al., 1997).

1.1.3.1 Functional specificity

How the different neuronal subtypes are formed and how they make connections to their target tissues are just in the beginning of being unravelled. Very little is known about these mechanisms in the NG why the following discussion solely describes these events in the DRG neurons. In addition, although the setup of neuropeptides and neurotransmitters seems to comprise the same players as in DRG the coupling of neurochemical expression to cellular function is far from clear.
The differentiation of neural crest cells into different neuronal subtypes and the survival after specification is dependent on both transcriptional programs and signals from the target tissue which it innervates. A tight regulation of events in this way steers the

originally multipotent cells into different paths of development leading to the diversity in sensory neuronal functioning seen in the adult sensory nervous system (Marmigere and Ernfors, 2007).

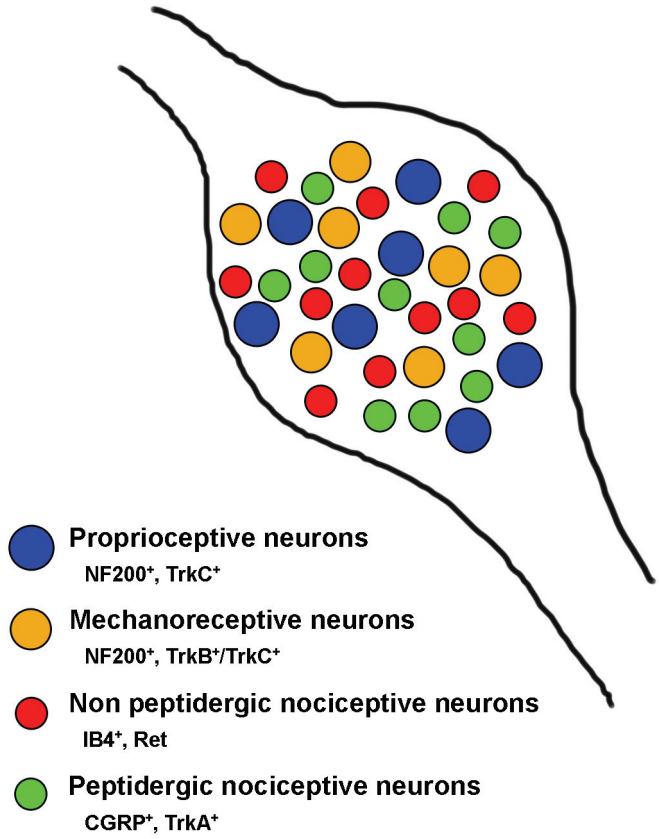

Figure 2. Schematic drawing of a DRG with the different neuronal subtypes and their specific expressions of neuronal markers and neurotrophin receptors.

1.1.3.1.1 Neurotrophins

Neurotrophins are growth factors, secreted by the target tissues, that promote survival of neurons and maintenance of neuronal phenotypes. Several different neurotrophins exist, supporting the survival of different neuronal subtypes by binding to the receptor tyrosine kinases (RTK) selectively expressed on the neurons. Nerve growth factor (NGF) binds to its receptor tropomyosin receptor kinase (Trk) A and is important for the survival of peptidergic neurons. Brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) bind to the TrkB receptor, maintaining the mechanosensory phenotype. Neurotrophin-3 (NT-3) binds primarily to TrkC, which is expressed by proprioceptors, but also to TrkA and TrkB. In addition, another neurotrophin receptor, p75, promotes cell death upon activation. p75 binds all neurotrophins and can also can interact with the RTKs and thereby fine-tune the signalling events (Bibel and Barde, 2000). During development 80% of the neurons in the forming DRG express TrkA. After the initiation of runt-related transcription factor *(Runx)* 1 expression in half of these neurons (see 1.1.3.1.2), TrkA is abolished postnatally giving rise to the non-peptidergic population of cells. These cells expresses another RTK, Ret (rearranged during transfection), are dependent on glial cell derived neurotrophic factor (GDNF) and bind to isolectin B4 (IB4) (Chen et al., 2006b; Molliver and Snider, 1997; Molliver

et al., 1997). The remaining TrkA expressing cells constitute the peptidergic population of cells which express neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (Averill et al., 1995). IB4, CGRP and substance P are frequently used as neuronal markers to identify the different subtypes *in vitro*. The TrkB and TrkC expressing cells that belongs to the mechanoreceptive and proprioceptive subtypes can be recognised by its ability to stain for neurofilament antibodies, e.g. neurofilament 200 (NF200) (Lawson et al., 1984).

1.1.3.1.2 Transcriptional regulation

Transcription factor expression determines the cellular fate of the neural crest cells and induces migration. Neurogenin 1 (*Ngn1*) and neurogenin 2 (*Ngn2*) devote the cells to the sensory in favour over the autonomic lineage, where cells instead express the mammalian achaete-scute homologue 1 (*Mash1*) gene (Perez et al., 1999; Zirlinger et al., 2002). *Ngn2* induces the first wave of migration while *Ngn1* is responsible for the second wave, when the cells also start to express the forkhead transcription factor *Foxs1* and the POU homeodomain transcription factor *Brn3a* (Montelius et al., 2007). During the third wave *Krox20* expression is induced (Maro et al., 2004). Another important transcription factor is the high-mobility group transcription factor SRY (sex determining region Y) box 10 (*Sox10*) that acts to keep the cells in a multipotent state, allowing for a high proliferation rate during all stages of neurogenesis (Kim et al., 2003; Montelius et al., 2007). The final diversification into different neuronal subtypes is determined by the expression of *Runx1* and *Runx3*. During the first wave of neurogenesis *Runx3* expression drives the cells to become large sized proprioceptive neurons by suppressing the TrkB expression and maintaining the TrkC expression in the early TrkB/TrkC neuronal population. Neurons with maintained TrkB expression become mechanoreceptors and can co-express TrkC or Ret. Mechanoreceptors can also express Ret alone (Chen et al., 2006a; Kramer et al., 2006; Levanon et al., 2002). Later in embryonic and postnatal development, the nociceptive neurons are formed and subdivided into peptidergic or non-peptidergic phenotypes. Postnatally, RUNX1 is responsible for the downregulation of TrkA in half of the large TrkA-positive population observed before birth. RUNX1-positive neurons become non-peptidergic nociceptors and RUNX1-negative neurons develop into peptidergic nociceptors (Chen et al., 2006b; Kramer et al., 2006; Marmigere et al., 2006).

1.2 TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS

1.2.1 Structure and topology

The TRP channels were discovered in a *Drosophila melanogaster* mutant where the photoreceptors displayed a transient current in response to light instead of the sustained current seen in wild type. This mutant was named *trp* (transient receptor potential) (Cosens and Manning, 1969). The cloning of the ion channel occurred 20 years later and was the start for the discovery of a new family of ion channels (Montell and Rubin, 1989). TRP channels are channel forming proteins situated in the plasma or lysosomal membranes with diverse functions in a range of cell types. In mammals, 28 members of the TRP family have been discovered and divided into 6 subfamilies based on structural

homology, TRPC (canonical or classical), TRPM (melastatin), TRPV (vanilloid), TRPML (mucolipin), TRPP (polycystin) and TRPA (ankyrin) (Figure 3). Prediction suggests 6 transmembrane domains with the pore domain situated between the 5th and the 6th domain. C-terminal of the 6th transmembrane domain resides a sequence of 25 amino acids, called the TRP domain, which is conserved in many of the TRP channels. The TRP box constitutes a highly conserved, 6 amino acid long, proline rich segment within the TRP domain. Other common structural motifs are the coiled-coil and the calmodulin binding domains. Finally, some channels include ankyrin repeats of variable length (Montell, 2005; Ramsey et al., 2006).

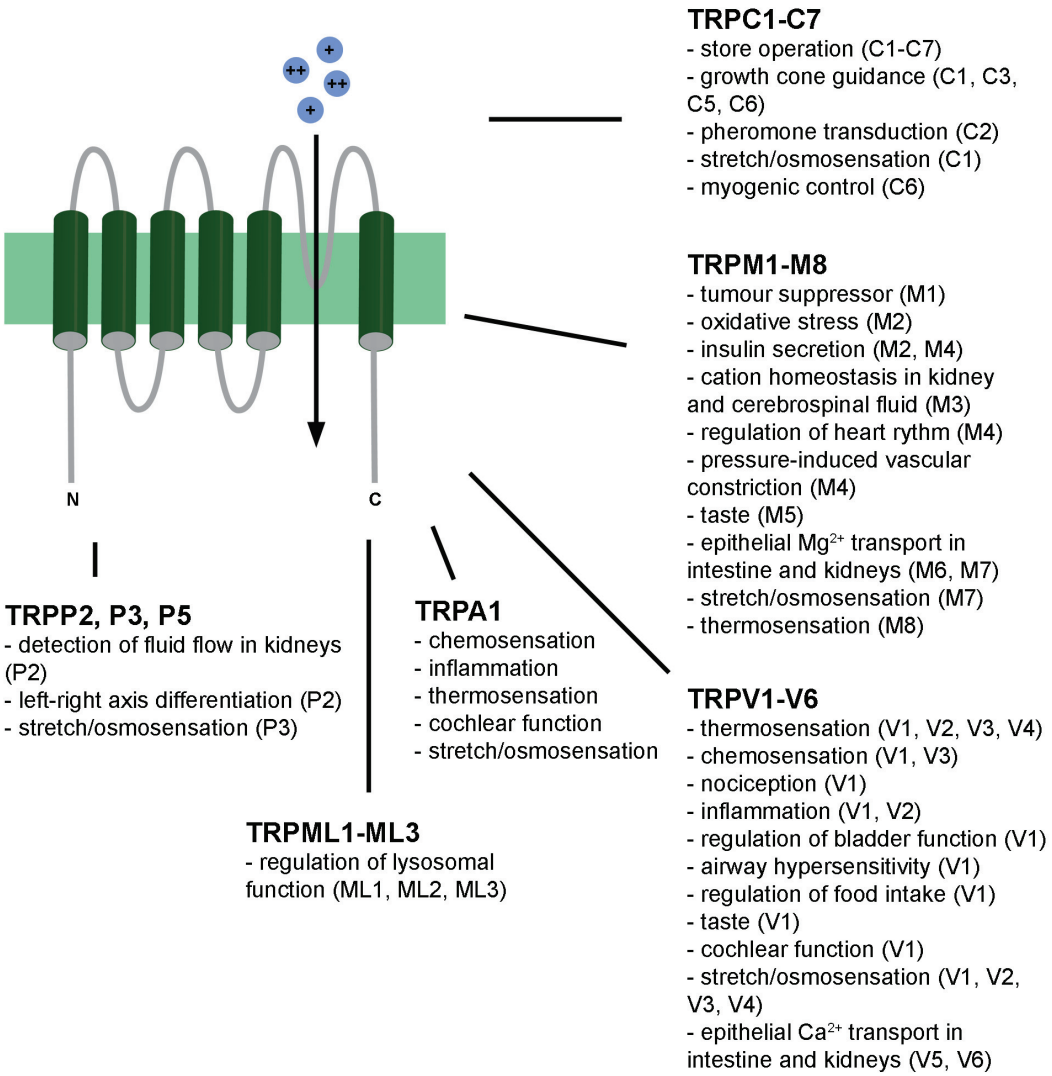

Figure 3. Topology and suggested functions of the TRP channels. All channels have 6 transmembrane domains with a cation-permeable pore loop between the 5th and 6th domain. The TRP superfamily consists of 28 members divided in 6 subfamilies based on sequence homology. Activation modes and functions have been found to be very diverse both within and between subfamilies. Proposed functions are listed for the members of each subfamily.

1.2.2 Activation

TRP channels are widely expressed and have many activation modes. They are permeable to cations, thereby regulating intracellular ion concentrations and can also affect membrane potential in both excitable and non-excitable cells. TRP channels can, by the influx of Ca^{2+} or Na^{+}, by itself depolarise the cell and drive the action potenial in neuronal cells or more indirectly regulate other voltage-gated ion channels in different cell types. Also, the influx of Ca^{2+} through the plasma membrane can be directly involved in cellular signalling by activating different effector proteins. Polymodal activation is common suggesting that the cellular context is decisive of the actual response to a given stimuli. TRP channels can be activated by endogenous and exogenous ligands, stimulation of receptors such as G protein coupled receptors (GPCRs) as well as directly with physical changes in the environment such as alterations in temperature, ionic strength, pH and possibly mechanical stimuli (Montell, 2005; Ramsey et al., 2006).

1.2.3 Properties of individual TRP channels

TRP channels are very heterogenous both regarding expression and function and often have different roles depending on the cellular context. Short comments around specific properties and known or speculated functions of the individual TRP channels are presented below and summarised in Figure 3. More details regarding the involvement of TRP channels in sensory biology are described in section 1.3.

1.2.3.1 The TRPC subfamily

The TRPC subfamily is divided into three groups, TRPC1/C4/C5, TRPC3/C6/C7 and TRPC2, based on function and sequence similarities (Clapham, 2003). Heteromultimers are formed between TRPC1 and TRPC4 and/or TRPC5 (Strubing et al., 2001) but also between mentioned channels and TRPC3 and TRPC6 (Strubing et al., 2003; Xu et al., 1997).

TRPC1 was the first identified mammalian TRP channel but its functional properties are still elusive (Wes et al., 1995). TRPC1 is widely distributed and has been assigned a range of functions. A number of studies suggest a role for TRPC1 as a store-operated channel (SOC), where depletion of internal Ca^{2+} stores is the trigger for activation. Despite the vast amount of data produced in this matter the function of TRPC1 as a SOC is still disputed (Beech, 2005). In fact, all other TRPC channels have been suggested to be involved in store-operated Ca^{2+} entry (SOCE). However, the results are conflicting and the importance of TRPC channels in SOCE remains controversial (Salido et al., 2009).

TRPC channels have several activation modes but all channels seem to use phospholipase C (PLC) pathways even though the activation mechanism is not clear (Montell, 2005). TRPC channels can be activated by diacylglycerol (DAG), its membrane permeable analogue 1-oleoyl-2-acetyl-glycerol (OAG) (Hofmann et al., 1999; Lintschinger et al., 2000) or the by interaction with the IP3 receptor (IP3R) itself

(Boulay et al., 1999; Kiselyov et al., 1998; Yuan et al., 2003). Activation can also be directly mediated by GPCR stimulation, independently of the IP3R or Ca^{2+} store depletion. It is suggested that second messengers such as G-proteins, IP3, DAG and Ca^{2+} directly activate TRP channels as exemplified by the TRPC6 involvements in receptor stimulated Ca^{2+} currents in aortic smooth muscle cells (Inoue et al., 2001; Jung et al., 2002) and the TRPC5 activation by muscarinic receptors in murine stomach (Lee et al., 2003b).

There have also been some physiological functions coupled to the TRPC channels. TRPC1, C3, C5 and C6 have been shown to be important for growth cone guidance and morphology (Greka et al., 2003; Li et al., 2005; Wang and Poo, 2005). In the vomeronasal organ of rodents TRPC2 is crucial for pheromone transduction resulting in loss of sex discrimination, sexual and social behaviours (Leypold et al., 2002; Lucas et al., 2003; Stowers et al., 2002). TRPC1 has been suggested to be stretch sensitive in oocytes (Maroto et al., 2005) and to be involved in cell volume regulation in liver cells (Chen and Barritt, 2003). TRPC6 is thought to control myogenic tone in vascular smooth muscle cells (Dietrich et al., 2005; Spassova et al., 2006; Welsh et al., 2002).

1.2.3.2 The TRPM subfamily

The TRPM subfamily is composed of 8 members (M1-M8) that are divided into 3 groups: TRPM1/M3, TRPM4/M5 and TRPM6/M7. TRPM2 and TRPM8 have low sequence homology to the rest of the TRPM channels. TRPM1, the founding member of the subfamily, was discovered as a tumour suppressor in melanoma tumours where it has been shown to be downregulated in highly metastatic tumours (Duncan et al., 1998). The other members of the TRPM family have been assigned diverse functions.

TRPM2 has been reported to be expressed in the brain and at lower levels in other tissues and is thought to have a role in oxidative stress. The C-terminal of this protein contains an ADP-ribose hydrolase domain which is activated by ADP-ribose (ADPR) and adenine dinucleotide (NAD) (Hara et al., 2002; Heiner et al., 2003; Inamura et al., 2003; Perraud et al., 2001; Sano et al., 2001). In line with this also H_2O_2, which induces oxidative stress and can activate the ADPR-forming enzyme poly (ADP-ribose) polymerase (PARP), generates TRPM2 currents (Wehage et al., 2002). TRPM2 has been linked to insulin secretion in pancreatic β-cells by regulating Ca^{2+} entry via cyclic ADPR-related molecules at body temperature (Togashi et al., 2006).

TRPM3 is constitutively active when heterologously expressed and is inhibited by intracellular Mg^{2+} as well as extracellular Na^{+} and K^{+} (Grimm et al., 2003; Lee et al., 2003a; Oberwinkler et al., 2005). Interestingly, different splice variants vary in ion selectivity, a property that may be of great functional importance. TRPM3 is sensitive to hypotonic solutions when expressed in HEK293 cells and was proposed to be involved in renal Ca^{2+} homeostasis due to its high expression in renal tubules (Grimm et al., 2003). TRPM3 is also expressed in the choroid plexus of the CNS. This combined with its responsiveness to extracellular cations led to speculations of a function in cation homeostasis of the cerebrospinal fluid (Oberwinkler et al., 2005).

TRPM4 is a Ca^{2+} impermeable channel which is activated by elevated cytosolic Ca^{2+} levels and permeated by monovalent cations (Launay et al., 2002; Nilius et al., 2003). It is ubiquitously expressed with the full length TRPM4b splice variant being the most prominent while TRPM4a seems to act as a dominant negative form (Launay et al., 2002; Nilius et al., 2003; Xu et al., 2001). TRPM4 has been proposed to be responsible for depolarising the cell membrane at high intracellular Ca^{2+} concentrations and thereby drive the activation of voltage-dependent calcium channels. This mechanism is for example used by the β-cells during glucose-induced insulin secretion (Cheng et al., 2007) and in sino-atrial node cells in the regulation of heart rhythm (Demion et al., 2007). In addition, TRPM4 was suggested to have mechanosensory properties in vascular smooth muscle cells, mediating pressure-induced vascular constriction i.e. the Bayliss effect (Earley et al., 2004).

TRPM5 was found in taste receptor cells and proved by knockout mice to be crucial for responding to sweet, amino acids (umami) and bitter taste (Perez et al., 2002; Zhang et al., 2003). Taste receptors are GPCRs which via PLC signalling pathways release Ca^{2+} from intracellular stores that activates TRPM5, which ultimately converts the biochemical signal to electrical currents (Liman, 2007; Liu and Liman, 2003; Zhang et al., 2007).

In contrast to TRPM7, which appears to be ubiquitously expressed (Nadler et al., 2001; Runnels et al., 2001), its closest analogue TRPM6 has been found to be mostly expressed in intestine and kidney (Schlingmann et al., 2002; Walder et al., 2002). Both channels are activated by decreased intracellular Mg^{2+} and are permeable to Ca^{2+} and Mg^{2+} (Voets et al., 2004b). TRPM6 has been suggested to be important for epithelial magnesium transport since it was discovered that mutations in the TRPM6 gene cause the rare disease hereditary hypomagnesaemia with secondary hypocalcaemia (HSH), where patients have defect magnesium absorption in the intestine (Walder et al., 2002). TRPM6 and TRPM7 form homo and heterotetramers and regulate transcellular transport of magnesium in the epithelium of the intestine and the kidney tubules (Chubanov et al., 2004; Schlingmann et al., 2007). TRPM6 and TRPM7 are the only TRP channels containing a kinase domain at the C-terminal end fused to the ion channel domain (Runnels et al., 2001). TRPM7 translocates to the plasma membrane in response to shear stress and osmotic swelling when recombinantly expressed. The suggestion is that TRPM7 functions in the pathological response to vessel wall injury in the vascular smooth muscle cells, which become exposed to shear stress after endothelial damage (Numata et al., 2007a; Numata et al., 2007b; Oancea et al., 2006).

TRPM8 was identified as a cold sensitive channel, permeable to both mono- and divalent cations, that was also activated by menthol, eucalyptol and icilin (McKemy et al., 2002; Peier et al., 2002a). The channel is expressed in sensory ganglia (McKemy et al., 2002; Nealen et al., 2003; Peier et al., 2002a; Zhang et al., 2004), gastric fundus (Mustafa and Oriowo, 2005), vascular smooth muscle cells (Yang et al., 2006), liver (Henshall et al., 2003), bladder and the male genital tract (Stein et al., 2004). The upregulation seen in prostate carcinomas has led to a proposal for TRPM8 to be used as a biomarker for prostate cancers (Henshall et al., 2003; Tsavaler et al., 2001).

1.2.3.3 The TRPV subfamily

The TRPV subfamily consists of 6 channels (TRPV1-V6) divided into 2 groups: TRPV1-V4 and TRPV5-V6. The founding member, TRPV1, was cloned from DRG and found to be activated by capsaicin and temperatures >43°C (Caterina et al., 1997b). It is predominantly expressed in small and medium sized peptidergic and non-peptidergic neurons in DRG as well as in trigeminal ganglia (TG) and NG. Apart from this it has a widespread distribution in the CNS, skin, bladder, smooth muscles, liver and blood cells (Tominaga and Tominaga, 2005).

TRPV1 is non-selective to cations with a preference for divalent over monovalent cations (Caterina et al., 1997b). It is also permeable to protons (Kress et al., 1996; Petersen and LaMotte, 1993; Tominaga et al., 1998), large polyvalent cations (e.g. gadolinium) (Tousova et al., 2005), polyamines (endogenous regulators of ion channels that are known to modulate inflammation and nociception) (Ahern et al., 2006), the styryl dye FM1-43 (Meyers et al., 2003) and the antibiotic gentamicin (Myrdal and Steyger, 2005). The function of TRPV1 has been studied in many parts of the body. In sensory physiology it is regarded as a detector of noxious external stimuli such as heat and chemicals. Due to the sensitisation by low pH, occurring during acidosis of inflamed tissue, and inflammatory mediators, including growth factors, neurotransmitters, small proteins or peptides, lipids, cytokines and chemokines, it is an important player in inflammation producing symptoms such as allodynia and hyperalgesia (Ma and Quirion, 2007). TRPV1 also has roles in regulation of bladder function (Birder et al., 2002), gastrointestinal motility (Geppetti and Trevisani, 2004), cochlear function (Zheng et al., 2003), airway hypersensitivity in respiratory diseases (Jia and Lee, 2007), reduction of food intake by activation of the fatty acid oleoylethanolamide (OEA) (Ahern, 2003; Wang et al., 2005) and detection of non-specific salt taste (Lyall et al., 2004).

TRPV2 is a weakly Ca^{2+} selective cation channel that has been found in the CNS (Wainwright et al., 2004), DRG, TG (Caterina et al., 1999b), myenteric plexus, NG (Kashiba et al., 2004), vascular smooth muscle cells (Muraki et al., 2003), trachea (Yamamoto et al., 2007) and mast cells (Stokes et al., 2004). Activation is accomplished with noxious heat (<52°C) (Caterina et al., 1999b), cell swelling or stretch (Muraki et al., 2003) and 2-aminoethoxydiphenyl borate (2-APB) which it has in common with TRPV1 and TRPV3 (Hu et al., 2004). TRPV2 is expressed in nociceptive medium and large sized Aδ- and C-fibers (Caterina et al., 1999b; Ma, 2001) and has been shown to be upregulated during peripheral inflammation being responsible for the heat hyperalgesia (Shimosato et al., 2005).

TRPV3 is sensitive to warm temperatures (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002), 2-APB (Hu et al., 2004) and camphor (Moqrich et al., 2005). It is abundantly expressed in brain, spinal cord, DRG, skin and testis while being less expressed in stomach, trachea, small intestine and placenta (Smith et al., 2002; Xu et al., 2002).

TRPV4 is a constitutively active non-selective cation channel that reacts to moderate heat (Guler et al., 2002; Watanabe et al., 2002), hyper- and hypoosmolarity (Liedtke et al., 2000; Nilius et al., 2001; Strotmann et al., 2000), mechanical stimulation (Suzuki et

al., 2003) and chemical stimuli e.g. endocannabinnoids, arachidonic acid and the phorbol ester 4α-PDD (Watanabe et al., 2003; Watanabe et al., 2002; Vriens et al., 2004). It has a widespread expression (heart, endothelium, brain, sensory ganglia, liver, placenta, lung, trachea and salivary gland) but is detected most frequently in kidney epithelia, especially in the distal tubule (Delany et al., 2001; Liedtke et al., 2000; Strotmann et al., 2000). The importance in systemic osmoregulation and response to noxious mechanical pressure and warm temperatures was confirmed in TRPV4$^{-/-}$ mice (Liedtke and Friedman, 2003).

TRPV5 and TRPV6 are highly Ca^{2+} selective and show constitutive activity (Vennekens et al., 2000). TRPV5 is primarily expressed in kidney, duodenum and jejunum and exists in lower abundances in pancreas, prostate, placenta, brain, colon, and rectum (Nijenhuis et al., 2003). TRPV5 and V6 are responsible for active transcellular transport of Ca^{2+} across the apical membrane in the kidney tubules and intestines respectively (Hoenderop et al., 2000) and their gene expression is influenced by 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), a product of vitamin D_3 hydroxylation that is important for Ca^{2+} homeostasis (Hoenderop et al., 2001; van Abel et al., 2003). In line with these findings the TRPV5$^{-/-}$ mouse showed increased Ca^{2+} loss via the urine accompanied with intestinal Ca^{2+} hyperabsorption due to increased 1,25-$(OH)_2D_3$ levels (Hoenderop et al., 2003; Renkema et al., 2005). TRPV6 has also been shown to be expressed in prostate tumours and the level of expression correlates with malignancy of the tumour (Fixemer et al., 2003).

1.2.3.4 The TRPML subfamily

The mucolipins have a function in regulation of membrane traffic and degradation of endosomes. Mutations in TRPML1 was identified as the main cause of the lysosomal storage disease mucolipidosis typ IV (MLIV) (Bach, 2001). MLIV is an autosomal recessive disease resulting in mental and psychomotor retardation, diminished muscle tone, achlorhydria and visual problems. The underlying cause seems to be defected pH regulation leading to accumulation of undigested material in the lysosomes resulting in aberrant cell function and autophagy (Puertollano and Kiselyov, 2009).

TRPML2 is also an endosomal protein but its function is largely unknown. Upon over expression in B-lymphocytes, where it is also normally expressed, accumulation of enlarged lysosomal structures occurs why speculations have been made on involvement in the immune response (Song et al., 2006).

TRPML3 is responsible for the varitint-waddler (Va) mouse phenotype by a gain-of-function mutation leading to constitutive activity. Expression is normally found in the lysosomes and plasma membrane of melanocytes and cochlear hair cells and Va mice exhibit dilute fur colour and deafness due to increased death of these cell types (Di Palma et al., 2002).

1.2.3.5 The TRPP subfamily

TRPP2, or polycystin-2, is encoded by *Pkd2*, a gene mutated in patients suffering from autosomal-dominant polycystic kidney disease (ADPKD) (Mochizuki et al., 1996). TRPP2 shows its highest expression levels in the kidney and interacts with polycystin-

1, which is the gene product of *Pkd1,* that is also linked to ADPKD (Reeders et al., 1985; Tsiokas et al., 1997), as well as to TRPC1 (Tsiokas et al., 1999). It functions as a non-selective cation channel for which no natural ligand has been identified. TRPP2 has been proposed to detect fluid flow in the kidney primary cilia epithelial cells possibly interacting with TRPV4 that would act as the actual mechanotransducer (Köttgen et al., 2008; Nauli et al., 2003). The *Pkd2* knockout mouse developed situs inversus which had already been linked to ADPKD. This gives TRPP2 a function in left-right axis differentiation which occurs very early in development (Pennekamp et al., 2002).

TRPP3 is a constitutively active, widely expressed, non-selective, calcium-modulated channel that is deleted in the *Krd* mutant mice that has renal and retinal defects (Chen et al., 1999; Nomura et al., 1998). It is sensitive to pH, voltage and cell swelling and is inhibited by large monovalent cations and by multivalent cations such as Mg^{2+}, Gd^{3+}, and La^{3+} (Shimizu et al., 2009).

No channel activity or functionality has so far not been proposed for TRPP5 (Ramsey et al., 2006).

1.2.3.6 TRPA1

TRPA1 seems to have a robust expression only in C-fiber nociceptors in the TG, DRG, NG and in the inner ear (Bautista et al., 2005; Kobayashi et al., 2005; Nagata et al., 2005). TRPA1 is activated by a range of pungent chemicals and environmental irritants such as allyl isothiocyanate (mustard oil), icilin, cinnamaldehyde (cinnamon), allicin (garlic), gingerol (ginger), acrolein (tear gas), Δ-9-tetrahydro-cannabinol (cannabis) (Garcia-Anoveros and Nagata, 2007). The proalgesic and proinflammatory agent bradykinin activates TRPA1 indirectly via the bradykinin receptor through the PLC pathway (Bandell et al., 2004; Jordt et al., 2004a). Accordingly, the $TRPA1^{-/-}$ mouse does not develop hyperalgesia in response to bradykinin administration (Bautista et al., 2006). It has also been speculated that noxious cold would activate TRPA1 but this issue is still unresolved (Reid, 2005). $TRPA1^{-/-}$ mice do not have defected responses to cold stimuli (Bautista et al., 2006). TRPA1 was proposed as a mechanotransducer in cochlear hair cells (Corey et al., 2004), a finding that was questioned later on due to normal hearing in $TRPA1^{-/-}$ mice (Bautista et al., 2006; Kwan et al., 2006). However, these mice did show deficits in sensing noxious punctuate cutaneous mechanical stimuli which suggested a role in mechanosensation (Bautista et al., 2006; Kwan et al., 2006). Recombinant expression of TRPA1 in HEK293 cells supported this suggested role by response to hyperosmolar solution (Zhang et al., 2008). Furthermore, the TRPA1 ortholog in *Drosophila melanogaster* and *Caenorhabditis elegans* is necessary for mechanosensation (Kindt et al., 2007; Tracey et al., 2003).

1.3 TRP CHANNELS IN SENSORY BIOLOGY

The sensory transduction of TRP channels acts to avoid potentially harmful stimuli in the form of extreme temperatures, noxious chemicals and vigorous stretch or pressure. More homeostatic features such as thermal homeostasis and osmoregulation are also

important tasks for this versatile group of ion channels. Sensory transduction of TRP channels is achieved by activation directly by ligand binding or physical stimuli or indirectly via receptors (e.g. GPCRs) or other players in intracellular transduction pathways (e.g. DAG or IP3R). Since TRP channels have polymodal activation ways the same channel can respond to e.g. both chemical and thermal stimuli and one stimuli can also attenuate or diminish the other (Damann et al., 2008).

1.3.1 Chemosensation

Many natural, pungent chemicals have been assigned as specific activators of individual TRP channels. Summarized in Table 1 are compounds known to activate the chemosensitive TRP channels identified.

Table 1. Chemosensory TRP channels: Activating compounds and natural origin.

TRP channel	Pungent chemical	Natural origin
TRPM8	Menthol (McKemy et al., 2002; Peier et al., 2002a)	Peppermint
	Icilin (McKemy et al., 2002)	Synthetic
	Eucalyptol (McKemy et al., 2002)	Eucalyptus oil
TRPV1	Capsaicin (Caterina et al., 1997b)	Chili pepper
	Piperine (McNamara et al., 2005)	Black pepper
	Allicin (Macpherson et al., 2005)	Garlic
	Camphor (Xu et al., 2005)	Camphor tree
TRPV3	Carvacrol, eugenol, thymol (Xu et al., 2006)	Oregano, savory, clove and thyme
	Vanillin (Xu et al., 2006)	Vanilla bean
	Menthol (Macpherson et al., 2006)	Peppermint
	Cinnamaldehyde (Macpherson et al., 2006)	Cinnamon
	Camphor (Moqrich et al., 2005)	Camphor tree
TRPA1	Allicin, thiosulfinate (Bautista et al., 2005; Macpherson et al., 2005)	Garlic
	Allyl isothiocyanates (Bandell et al., 2004; Jordt et al., 2004b)	Mustard oil, wasabi, horseradish
	Carvacrol (Lee et al., 2008; Xu et al., 2006)	Oils from thyme, oregano and bergamot
	Acrolein (Bautista et al., 2006)	Tear gas, vehicle exhaust, cigerette smoke
	Menthol (Karashima et al., 2007; Macpherson et al., 2006)	Peppermint
	Cinnamaldehyde (Bandell et al., 2004)	Cinnamon
	Eugenol (Bandell et al., 2004)	Clove oil, nutmeg, cinnamon and bay leaf
	Gingerol (Bandell et al., 2004)	Ginger
	Methyl salicylate (Bandell et al., 2004)	Wintergreen oil

1.3.2 Thermosensation

The ability to distinguish different temperatures is a fine-tuned interplay between several TRP channels called the "thermoTRPs". TRPA1 responds to noxious cold (<17 ºC) (Story et al., 2003), TRPM8 to innocuous cool (8-28 ºC) (McKemy et al., 2002; Peier et al., 2002a), TRPV3 and TRPV4 to mild heat (>25-39 ºC) (Guler et al., 2002; Peier et al., 2002b; Smith et al., 2002; Watanabe et al., 2002; Xu et al., 2002), TRPV1 to heat (>43 ºC) (Caterina et al., 1997a) and TRPV2 to noxious heat (>52 ºC) (Caterina et al., 1999a; b). These channels are embedded in the free nerve endings in the skin, expressed by peripheral sensory neurons or keratinocytes and are directly activated by temperature changes. Activation is believed to occur through a temperature-dependent shift of the voltage-dependent activation curves so that activation occurs at more physiological membrane potentials (Voets et al., 2004a). There has also been evidence presented for other TRP channels to act as thermosensors in specific organs. TRPM5, which is highly expressed in the taste buds in the tongue and is important for taste transduction, was shown to be activated by warm temperatures. This heat activation is believed to be responsible for the altered sensitivity to tastes at different temperatures (Talavera et al., 2005). The same research group reported a similar temperature dependence of the close relative TRPM4, a more ubiquitously expressed channel. Warm temperatures has also been shown to activate TRPM2 in pancreatic β-cells and greatly potentiate the activation by cyclic ADP-ribose, which is important for the insulin secretion in these cells (Togashi et al., 2006).

1.3.3 Mechanosensation

TRP channels have been associated with different aspects of mechanosensation such as hearing, touch, osmoregulation and renal function. Even so, no candidate remains undisputed as a mechanotransducer and it is debated whether the TRP channels function as actual mechanotransducers or if they have more indirect roles (Christensen and Corey, 2007). There are several possibilities to distinguish between direct and indirect mechanical activation. The latency for the current upon stimulation has to be less than 5 ms to exclude involvement of second messenger systems. Also, the kinetics of the electrophysiological response should be connected to the strength of the stimuli. In addition, channel opening must be possible to induce by a mechanical movement of some part of the channel. Since above features sometimes are difficult to prove for a candidate mechanosensor, arguments can be based on more straightforward grounds. First of all the channel must be expressed in the cell of interest, at the site of mechanical transduction, during the developmental stage when mechanosensitivity arises. A true mechanosensor should also maintain the mechanosensing property when expressed heterologously. However, this is compromised if there are structural proteins or other cofactors missing in the cells used for the expression system. In addition, mutations in the pore region should alter the electrophysiological properties of the channel. Finally, downregulation by using, for example, small interfering RNA (siRNA) should abolish or reduce mechanotransduction and mice lacking the target gene should have defected mechanosensory physiology.

Several TRP channels have been implicated to be involved in mechanosensation in both sensory neurons and in non-neural systems. TRPC3, C6, V2, M4 and M7 have been associated with mechanosensory functions in smooth muscle cells (Dietrich et al., 2005; Earley et al., 2004; Muraki et al., 2003; Numata et al., 2007a; Numata et al., 2007b; Oancea et al., 2006; Welsh et al., 2002) and TRPP2 seem to be stretch sensitive in the primary cilia of kidney epithelial cells (Nauli et al., 2003). TRPV4 is thought to have a role in the systemic response to osmolarity changes in the body (Liedtke and Friedman, 2003). In the sensory nervous system in vertebrates hearing is the most well studied mechanosensory function. The hair cells of the inner ear contain stereocilias that are deflected in response to sound waves, resulting in channel opening of a mechanoreceptor that has not yet been identified. This channel should have non-selective cation permeability and large conductance why the TRP channels are attractive candidates (Christensen and Corey, 2007). In mammalians, TRPA1 is the most well studied candidate for the hearing transduction channel. It is expressed in the distal stereocilia of the hair cells, appears around E17, at the onset of mechano-transduction, and knock down of TRPA1 using siRNA decreases transduction currents in mouse hair cells (Corey et al., 2004). However, this study was followed up by the generation of $TRPA1^{-/-}$ mice, which neither showed hearing abnormalities nor impaired hair cell transduction (Bautista et al., 2006; Kwan et al., 2006). It is thus unlikely that TRPA1 forms the hair cell transduction channel. Another suggested candidate for mechanotransduction is TRPML3 which when mutated in the Va mouse results in deafness and loss of cochlear hair cells. The main subcellular location for this channel is cytoplasmic but it can also be found in the plasma membrane (Di Palma et al., 2002). The reason for the hearing defects seems to be disruption of lysosomal waste elimination resulting in developmental extinction of the hair cells why it is questionable if TRPML3 is the transduction channel (van Aken et al., 2008). TRPV4 is expressed in sensory hair cells and is the closest vertebrate homologue to *Inactive* and *Nanchung,* TRP genes essential for hearing in *Drosophila melanogaster* and to the invertebrate mechanoreceptor OSM-9 (Cuajungco et al., 2007). As mentioned before, TRPV4 is known to react to hypotonicity but a direct role as a mechanotransducer has not been proved.

Touch is the sensation of pressure or stretch to the skin. Detection occurs by free nerve endings of mechanosensitive neurons, with their cell bodies in the TG or DRG. There are both slowly adapting and rapidly adapting neurons which can have high or low threshold to mechanical stimuli. Reduced sensitivity to painful mechanical stimuli was observed in $TRPA1^{-/-}$ and $TRPV4^{-/-}$ mice. TRPA1 is primarily expressed in small sized nociceptive neurons but also in some neurons of larger size. TRPV4 seems to be strongly connected to noxious osmotic and mechanical sensation by measuring pain-related behaviour in $TRPV4^{-/-}$ mice (Alessandri-Haber et al., 2005). In studies on knockout mice, TRPV4 was found to be necessary for the reaction to noxious mechanical stimulation and for the development of inflammation induced mechanical hyperalgesia. TRPC1 was suggested to be stretch-sensitive in liposomes from oocytes. Recombinant expression of human TRPC1 in oocytes generated similar currents after mechanical stimuli. These currents were reduced after injection with antisense RNA directed towards TRPC1 (Maroto et al., 2005). However, further studies conducted on heterologously expressed TRPC1 failed to confirm these findings why the ability of TRPC1 to be a direct mechanotransducer was questioned (Gottlieb et al., 2008).

In conclusion, several TRP channels have been suggested to be mechanotransducers but none has so far been supported with convincing evidence. Further studies need to be conducted on the TRP channels and other channels to elucidate the molecular players in sensory mechanotransduction.

1.3.4 Pain

Nociception is the detection of noxious thermal, mechanical or chemical stimuli. The ability to react to painful stimuli is crucial for the prevention of extensive tissue injury. Detection occurs in the peripheral tissues by nociceptive transducers (e.g. TRPV1, TRPM8, P2X3 etc.) in the free nerve endings of fast myelinated Aδ-fibers or slow unmyelinated C-fibers (Foulkes and Wood, 2008). A depolarising current is generated and, if the stimuli is above a certain threshold, action potentials are initiated that are transmitted to the spinal cord and further on to the thalamus and cortex resulting in physiological and psychological responses to the elicited pain due to the release of neurotransmitters (Hunt and Mantyh, 2001). This acute pain is transient and although it can be unpleasant it has an important protective function. Pain can also become chronic as is the case in neuropathic, caused by injury or dysfunction in the peripheral nervous system, and inflammatory pain, a result of peripheral tissue damage or inflammation. Common symptoms are hyperalgesia, which is increased pain response to normally painful stimuli, and allodynia, a state where normally innocuous stimuli become painful (Campbell and Meyer, 2006). Both these forms of hypersensitivity are results of the plasticity of neurons i.e. the ability to change structure, function and expression profile.

1.3.4.1 Peripheral mechanisms

Signals from the primary afferents innervating the lesioned area have been shown to be of great importance in neuropathic pain. Activation, blockage or knockdown of several receptors and ion channels (e.g. cannabinoid receptor 2, Ret, $Na_v1.8$), which are exclusively expressed on peripheral neurons have effects on neuropathic pain symptoms. Also, local anaesthetics are pain relieving in some patients. There are multiple causes for primary afferents to induce and maintain neuropathic pain and the effects are mediated in different ways by both the injured and the neighbouring intact afferents (Campbell and Meyer, 2006).

1.3.4.1.1 Sensitisation

Spontaneous action potential generation is a common phenomena that is the result of sensitisation of the neurons (Campbell and Meyer, 2006). The excitability of the primary sensory neurons can be altered after nerve injury by different activation mechanisms. Glutamate, the main excitatory transmitter in the body, is generally important for the initiation of nociceptive signalling in peripheral afferents. Action potentials cause release of glutamate from vesicles in the presynaptic neuron that bind to the postsynaptic glutamate receptors, AMPA (α -amino-3-hydroxy-5-methyl-4-isoxalepropionate), Kainate, NMDA (N-methyl-D-aspartic acid) and mGlu (metabotropic glutamate). Members from these receptor families are expressed on DRG neurons and the upregulation and/or re-distribution of some of them in combination

with enhanced glutamate release after nerve injury result in increased excitatory signalling (Bleakman et al., 2006).
Voltage-gated Na^+ channels are generally important in action potential generation and conduction and four of these channels ($Na_v1.3$, 1.7, 1.8 and 1.9) are of special interest in neuropathic pain due to their expression on DRG nociceptors. The increased expression or re-distribution of voltage-gated Na^+ channels decreases the threshold for activation and/or increases the amplitude of the generated currents resulting in more frequent elicitation of action potentials (Cummins et al., 2007).
TRPV1 functions as a sensitiser of primary afferents under influence of a range of different agents commonly present at the site of injury, e.g. NGF, H^+, bradykinin, prostaglandins, leukotriene B_4, ATP etc. These inflammatory mediators phosphorylate the channel and thereby alter its probability for depolarisation (Szallasi et al., 2007; Woolf and Salter, 2000).

1.3.4.1.2 Phenotypic shifts

Neurotrophins are continuously provided from the target tissues, bind to the Trk receptors expressed on the nerve terminals and are crucial for survival and function of sensory neurons by regulating gene expression. After axotomisation of peripheral afferents the neuronal contact with the target tissues are interrupted and the cells are therefore deprived from this supply. The absence of neurotrophins leads to altered molecular make-up of the neurons, often resulting in loss of their subtype characteristics, and they thereby undergo phenotypic shifts (Campbell and Meyer, 2006). To add to the complexity, most cases of nerve lesions only involves a fraction of the afferents in a nerve bundle meaning that intact fibers exist in close contact with the injured ones. These intact fibers are subjected to a changed environment due to damage-related activities in the surroundings. When target tissues are denervated they increase their expression of neurotrophins resulting in higher exposure of growth factors for the intact neurons (Campbell and Meyer, 2006). This often has as an effect that genes that are negatively regulated in injured neurons are positively regulated in intact neurons and vice versa, as exemplified by TRPV1, TRPA1 and CGRP (Fukuoka et al., 1998; Fukuoka et al., 2002; Hudson et al., 2001; Katsura et al., 2006). Hence, phenotypic shifts also occur in the uninjured neurons. NGF is a key growth factor for nociceptors in the regulation of gene expression of a range of proteins in the pain pathway in adult life, e.g. CGRP, substance P, BDNF, TRPV1, P2X3, bradykinin receptor, μ opiate receptor and voltage-gated Na^+, K^+ and Ca^{2+} channels. Altered NGF supply therefore have dramatic and different effects in both injured and intact neurons (Pezet and McMahon, 2006). Another source of growth factors is the schwann cells which undergo wallerian degeneration (degradation of axon and myelin sheath accompanied with macrophage invasion, leaving only the neurolemma intact for guidance during nerve regeneration) after nerve injury, dedifferentiate and start to produce neurotrophins instead of myelin (Reynolds and Woolf, 1993). Moreover, growth factor synthesis can occur in the neurons in the DRG and the spinal dorsal horn, as is the case with BDNF. BDNF is produced in small and medium sized peptidergic nociceptors and is anterogradelly transported to the spinal dorsal horn where the peripheral afferents make central terminals. The regulation of this growth factor in neuropathic pain models varies depending on the type of lesion the animals is subjected to. This may be due to the degree of inflammation and nerve damage the different

models comprise but does also implicate that BDNF is involved in different neuropathic pain mechanisms (Pezet and McMahon, 2006).

1.3.4.1.3 Sympathetic sprouting

After nerve injury, basket-like formations of sympathetic axons appear around the DRG of both injured and intact neurons of primarily large sized non-nociceptive neurons. Increased NGF presence is suggested as an inducer for sprouting by reaction with TrkA receptors expressed on the sympathetic postganglionic neurons. Also an increased cytokine production occurs in schwann cells undergoing wallerian degeneration. Cytokine leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) have both been shown to stimulate sympathetic sprouting. The close association between sympathetic fibers and sensory neurons allows for synaptic connections between the two, giving rise to abnormal excitation of the DRG neurons resulting in increased ectopic discharges. This phenomena could be responsible for the touch evoked pain, mediated by large sized myelinated Aβ-fibers, seen in the clinic (Bridges et al., 2001; Ramer et al., 1999).

1.3.4.2 Central mechanisms

1.3.4.2.1 Central sprouting

Normally, the primary afferents from the different neuronal subtypes terminate in different regions of the dorsal horn, i.e. myelinated Aδ-fibers and unmyelinated C-fibers (nociceptors) terminate in laminae I and II while myelinated Aβ-fibers (mechanoreceptors) terminate in laminae III and IV. After nerve injury the Aβ-fibers sprout into lamina II leading to interpretation defects in the spinal cord since the low threshold input from the mechanoreceptors are perceived as high threshold signals from nociceptors. Again, NGF seems to be an important player by being able to prevent this dysfunction upon intrathecal administration (Bridges et al., 2001).

1.3.4.2.2 Central sensitisation

Dorsal horn neurons can become sensitised by an increased response to repeated C-fiber stimulation in a process called “wind-up”. This can be due to either increased release of excitatory neurotransmitter or enhanced synaptic efficacy. Increased release of glutamate presynaptically seems to be important for sensitisation. One cause is downregulation of inhibitory GPCRs such as the μ-opoid receptor after nerve injury (Kohno et al., 2005). Moreover, upregulation of the α-2-δ subunit on voltage-gated calcium channels has been documented in DRG and spinal cord, leading to increased Ca^{2+} levels and increased glutamate release (Li et al., 2004). The importance of the α-2-δ subunit is further strengthened by the therapeutic action of gabapentin and pregabalin on neuropathic pain since these anticonvulsant drugs block this subunit. Phenotypic shifts of the Aβ-fibers resulting in their expression of substance P is also suggested as an additional sensitisation mechanism (Campbell and Meyer, 2006).
Postsynaptically, different activities on the glutamate receptors, AMPA, NMDA and mGlu, are important for development of chronic pain. AMPA has been shown to be upregulated after nerve injury and AMPA/Kainate receptor antagonists (NBQX and CNQX) block hyperalgesia and allodynia in several models (Bleakman et al., 2006). NMDA is important for long-term potentiaton (LTP), a phenomenon where increased

sensitivity to neurotransmitters enhance synaptic transmission over long time. LTP occurs both in the spinal dorsal horn and in cortical areas, especially the anterior cingulate cortex (ACC), a forebrain structure important for pain-related perception (Zhuo, 2007). Long-term depression (LTD) is the reciprocal process to LTP, where synaptic transmission is weakened, probably for auto-regulatory purposes. After nerve injury, loss of LTD has been observed in ACC slices leading to increased pain (Zhuo, 2007). There are several other activities that reduce inhibitory control (disinhibition), leading to increased spontaneous firing. In the dorsal horn, postsynaptic GABA receptors are downregulated and GABA levels are reduced. Also, inhibitory interneurons in lamina II die after nerve injury (Woolf and Mannion, 1999).

1.3.4.3 Immune responses

1.3.4.3.1 Peripheral immune reactions

Nerve injury is accompanied with a fast immune response by resident and invading macrophages. Chemokine-activated macrophages and denervated schwann cells release matrix metalloproteases that disrupt the blood-nerve barrier. Swelling and increased blood flow is initiated by the release of CGRP, substance P, bradykinin and nitric oxide (NO) from the injured nerve fibers. Schwann cells and macrophages release tumor necrosis factor α (TNFα) within the first hours after nerve injury. TNFα induces the release of proinflammatory cytokines (IL-1 and IL-6) from macrophages and schwann cells along the entire nerve undergoing wallerian degeneration. IL-1β promotes NGF release from the schwann cells leading to sensitisation of the adjacent intact nerve fibers and alters nociceptor gene expression. The function of these early events is to promote axonal growth and survival but they have also been shown to be crucial for the development of neuropathic pain (McMahon et al., 2005; Scholz and Woolf, 2007). In addition, other cell types such as neutrophils and mast cells infiltrate the tissue from the blood releasing inflammatory mediators e.g. NO, LIF, interferon-γ, cytokines and chemokines (CCL2 and CCL3) (Marchand et al., 2005; Scholz and Woolf, 2007). The distal immune response at the lesion site is discontinued when the process of wallerian degeneration has ended and the myelin debris is removed. However, in the DRG, the immunoreactivity remains for months. The density of macrophages around the injured neurons increases and phagocytosis of the injured neurons starts. Released IL-1, IL-6 and TNFα modulate neuronal activity and elicit ectopic discharges by increasing the density of voltage-gated sodium channels. Moreover, phenotypic shifts are induced by cytokines (e.g. LIF and IL-6) by altering the synthesis of neuropeptides. Finally, IL-6 has been shown to trigger the sprouting of sympathetic nerve fibers around the DRG neurons (Scholz and Woolf, 2007).

1.3.4.3.2 Central immune reactions

After nerve injury, microglia are activated and clusters around the injured motor neurons in the ventral horn and around the injured sensory nerve fibers in the dorsal horn of the spinal cord. Activation occurs by fractalkine, CCL2 and Toll-like receptors (TLR), which have all been shown to have influence on neuropathic pain symptoms. The microglia release cytokines (IL-1β, IL-6, IL-10 and TNFα), analogous to the macrophages at the peripheral lesion site, and might affect the dorsal horn neurons in ways promoting neuropathic pain symptoms. Studies suggest that the released

cytokines influence neurons both by increased excitability and release of inflammatory agents such as NO and prostaglandin E_2 (PGE_2). Astrocytes in the spinal cord also respond to peripheral injury by activation and proliferation but with unknown mechanisms of initiation (Marchand et al., 2005; Scholz and Woolf, 2007).

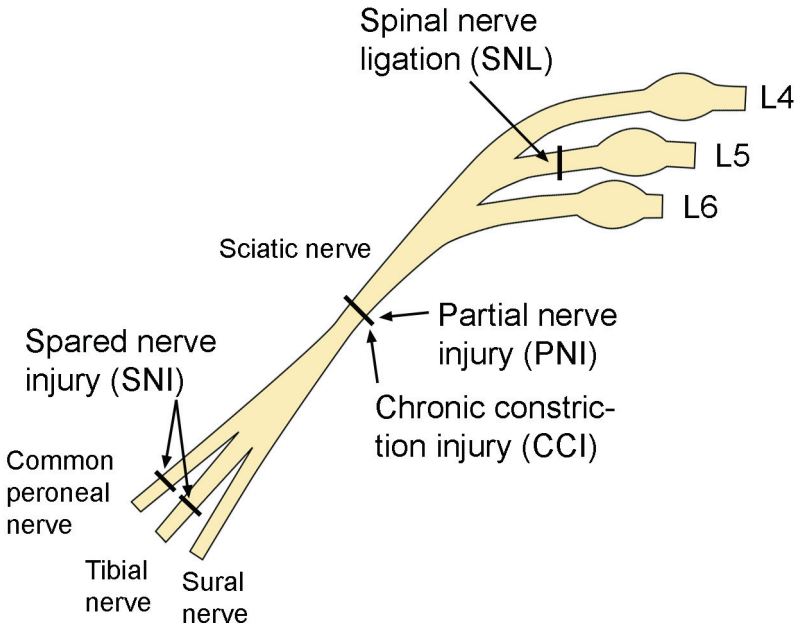

Figure 4. Schematic drawing of the most common nerve injury models. Ligation or transection of the sciatic nerve or its branches produces symptoms of neuropathic pain. In SNL the spinal nerve branching to L5 is cut. In PNI and CCI tight and loose ligations are made respectively around the sciatic nerve. In SNI two out of three of the distal branches are cut (common peroneal and tibial nerves).

1.3.4.4 Animal models of peripheral nerve injury

Nerve injury models performed in rodents have been crucial for the mechanistic studies of neuropathic pain since experiments on humans would be too invasive. Peripheral nerve injury is inflicted by cutting or ligating the sciatic nerve at different positions and to varying degrees (Figure 4). The nerve tissue on the ipsilateral side of the injury is then compared to the tissue from the contralateral side or from sham-operated or naive controls. The first model used frequently was axotomy where total transection of the sciatic nerve was conducted. Since this method often results in autotomy of the damaged foot, which is an unwanted phenomenon, it is not extensively used nowadays. In chronic constriction injury (CCI) loose ligations of the sciatic nerve are applied at mid-thigh level (Bennett and Xie, 1988). This method is accompanied with a substantial inflammatory response and mostly myelinated large and medium sized A-fibers are lost. Another popular method is partial nerve injury (PNI), also called partial nerve ligation (PNL), where a tight ligation is made around 1/3-1/2 of the sciatic nerve, also at mid-thigh level (Seltzer et al., 1990). Both CCI and PNI result in a mix of damaged and undamaged nerves in L4-L6 DRG. This is not the case for spinal nerve ligation (SNL), performed by transection or tight ligation of the spinal nerves branching to L5 and L6 DRG or L5 alone (Kim and Chung, 1992). Since the L4 nerve is left intact all neurons in the L4 DRG are uninjured why the effects in damaged neurons can

be easily separated from the events in intact ones. The most recently developed method is spared nerve injury (SNI) where the common peroneal and tibial nerves are tightly ligated and cut leaving the sural nerve intact (Decosterd and Woolf, 2000). Again, the injured and intact nerves are mixed in L4-L6 DRG but the co-existence of distal intact axons with degenerating axons is restricted why distinct regions on the hind-paw, only innervated by either damaged or undamaged fibers, can be identified and tested. All methods mentioned here gives robust manifestations of neuropathic pain e.g. cold and mechanical allodynia (Bridges et al., 2001).

1.3.4.5 TRP channels in pain

Several of the TRP channels are expressed in nociceptive neurons and are thought to have a role in nociception and clinical pain. TRPV1 is the best studied and is known to be activated by heat, protons and capsaicin, agents which are all nociceptive. TRPV1 knockout mice have no responses to these stimuli and they also lack inflammatory-induced thermal hypersensitivity (Caterina et al., 2000; Davis et al., 2000). In addition, presence of inflammatory mediators and low pH are factors known to sensitise TRPV1 to capsaicin and heat (Tominaga and Tominaga, 2005). TRPV1 actions in inflammatory pain seem to be complex and vary between different stages of the progressing disease. As an example $TRPV1^{-/-}$ mice displayed acute reduction of thermal and mechanical hyperalgesia in models using mild heat injury as well as complete Freund's adjuvant (CFA). Mechanical hyperalgesia was unaltered in the acute phase but was then attenuated during the chronic phase of inflammation induced by streptozotocin (diabetic neuropathy) or cisplatin (toxic neuropathy) (Bölcskei et al., 2005; Caterina et al., 2000; Davis et al., 2000). Upon peripheral nerve injury, TRPV1 expression has been shown to decrease in damaged but increase in intact neurons (Facer et al., 2007; Fukuoka et al., 2002; Hudson et al., 2001; Michael and Priestley, 1999). Other channels with reduced expression in injured neurons and elevated or unaltered expression in uninjured neurons after nerve injury are TRPA1 and TRPM8. CFA and NGF treatment increase TRPA1 but not TRPM8 expression (Ji et al., 2008; Katsura et al., 2006; Obata et al., 2005). $TRPA1^{-/-}$ mice show deficits in pain response to inflammatory mediators and direct nociceptive stimuli (Patapoutian et al., 2009). TRPV3 and TRPV4 have also been associated with pain, primarily by studies in knockout mice. $TRPV3^{-/-}$ mice lack responses to innocuous and noxious heat (Moqrich et al., 2005) while $TRPV4^{-/-}$ mice show reduced mechanical and osmotic hyperalgesia induced by inflammation and impaired nociceptive response to osmotic, acidic and mechanical stimuli (Patapoutian et al., 2009).

2 AIMS OF THE THESIS

TRP channels act as cellular sensors and convey information about changes in the environment. Despite the high interest in sensory biology and intense studies of certain of these channels the expression and function in sensory neurons of many of the TRP family members are not well characterised. The overall aim of this thesis was to better define the expression and cellular localisation of TRP channels in sensory neurons with the ultimate goal to better understand the functional roles of this important class of proteins both in sensory neuronal physiology and pathophysiology.

Specific aims:

- ✓ Characterisation of the developmental expression and cellular subtype distribution of TRP channels in the DRG.
- ✓ Identify TRP channels with involvement in neuropathic pain.
- ✓ Elucidate the importance of TRPC1 for the mechanosensory function of sensory neurons.

3 RESULTS AND DISCUSSION

3.1 EXPRESSION AND SUBTYPE DISTRIBUTION OF TRP CHANNELS IN SENSORY NEURONS (PAPER I, II AND UNPUBLISHED RESULTS)

3.1.1 The connection of expression pattern and cellular distribution to function

TRP channels have so far mostly been studied individually regarding expression and function in various tissues. We took a broad approach analysing the expression profiles during development of whole subfamilies to find indications of new unassigned sensory functions. By quantifying the transcript levels for each channel in different developmental phases it is possible to get an indication of the potential roles for individual channels in sensory signalling. A high expression in early development followed by subsequently decreasing transcript levels indicates importance during the formation of the peripheral nervous system. In contrast, an increasing expression during development, reaching the highest levels after birth, speaks for a more specific sensory role in adult life. Important is also the time when expression can first be detected since that most likely correlates with the time of established sensory function. An increasing expression after birth would also be expected for channels with sensory functions considering the increased importance and utilisation of the sensory nervous system when the pup leaves the controlled environment in the womb and are exposed to the outer world. Furthermore, the cellular subtype distribution of a channel is adding valuable information regarding its potential functional role. Sensory neurons in the DRG have different phenotypes determined by its expression of specific sets of ion channels, receptors and neurotransmitters. The functional identity of DRG neurons can also be identified by size which correlates to function in a well defined manner (Goldstein et al., 1991; Lawson and Bisco, 1979; Molliver et al., 1995).

3.1.2 Developmental expression analysis and subtype distribution of individual TRP subfamilies

3.1.2.1 The TRPC subfamily (Paper I)

All TRPC channels were found to be expressed in adult DRG. TRPC1, C3 and C6 were the most prominently expressed while TRPC2, C4 and C5 had lower transcript levels and TRPC7 had very low levels. When studying developmental expression TRPC1, C3 and C6 was found to have increasing expressions from E12 to adult, even though small dips in the expression curves could be seen for TRPC1 and C3 in P4 DRG. TRPC4, C5 and C7 peaked in expression levels at E18 where after a decline was observed around birth, followed by increasing levels at adult stage. Finally, TRPC2, which is a pseudogene in humans, peaked in expression at E12-E14 where after a decline was evident. Based on these expression patterns one could speculate that several of the TRP channels might be important in sensory physiology since they show high expression

levels in the adult with increasing transcript levels after birth. In thoracic DRG and NG the expression patterns were largely similar with minor deviations.

A more extensive characterisation was done in lumbar DRG using *in situ* hybridisation and immunohistochemistry for one channel in each subgroup of the TRPC channels; TRPC1, C2 and C3. TRPC1 and TRPC2 were primarily found in NF200-positive cells while TRPC3 was exclusively expressed in IB4-positive cells. The NF200-positive cells are myelinated mechanosensitive and/or proprioceptive neurons. A role for TRPC1 in mechanosensation has been suggested but is not undisputed (Gottlieb et al., 2008; Maroto et al., 2005). Our findings that TRPC1 is expressed in the mechanosensitive population of DRG cells strengthen the possibility for a mechanosensory function. More functional evidence for a role of TRPC1 in mechanosensation is presented in Paper IV (see section 3.3).
The IB4-positive cells are non-peptidergic, i.e. does not express neuropeptides, unmyelinated C-fibers (Priestley et al., 2002). These cells appear to play an important role in pain pathophysiology since treatment with the GDNF family of trophic factors, which acts only on the non-peptidergic neurons, have shown therapeutic effects in neuropathic pain conditions (Boucher et al., 2000; Gardell et al., 2003; Malmberg et al., 1997). In addition, mice lacking *Runx1* fail to develop non-peptidergic neurons and do not develop mechanical allodynia (Chen et al., 2006b). Our results showing exclusive expression of TRPC3 in this subclass of neurons open for an involvement in nociception and/or neuropathic pain.

3.1.2.2 The TRPM subfamily (Paper II)

In the TRPM family, all channels except TRPM1 were found in mouse DRG. In adult lumbar DRG TRPM3 was the most frequently expressed channel followed by TRPM2, M4 and M7. Lower amounts were found of TRPM8 and very low levels existed for TRPM5 and M6. The expression of TRPM3, M5 and M7 peaked at E18 where after a decline was seen at P4. Thereafter TRPM3 and TRPM7 showed increased transcript levels again while TRPM5 expression continued to drop. TRPM2, M4, M6 and M8 also shared the expression pattern of increased mRNA levels from E12 to E18 or P0 when a decline was observed. However, after that age all these channels, except for TRPM8, showed increased expression reaching its highest levels in the adult. TRPM8 did also increase from P4 to P12 but after that the expression dropped somewhat in the adult. These expression patterns exclude a sensory role for TRPM1 in the DRG. Also for TRPM5 such a role in adult normal tissue is unlikely even though there is a possibility for a function during development. TRPM5 expression is reported to be largely restricted to taste receptor cells (Perez et al., 2002; Zhang et al., 2003), thus the lack of expression in adult DRG found in our study is not surprising. The rest of the TRPM channels show increasing expression levels from E12 and onwards with marked increases for most channels from P4 to adult, implying a role in sensory neurons. The temporary dip at P4 suggests two expression waves and might include reshaping of neurons postnatally. The patterns and levels of expression of the different TRPM channels had minor differences in thoracic DRG and NG compared to lumbar DRG.

TRPM8 has been studied rather extensively in sensory neurons due to its established role as a cold sensor (McKemy et al., 2002; Peier et al., 2002a). In line with previous findings, our studies using *in situ* hybridisation show that TRPM8 is expressed in small neurons in the postnatal and adult DRG. Staining with the neuronal markers NF200, IB4 and CGRP revealed no co-localisation with either marker, a result that confirms the findings of some but not all other research groups (Dhaka et al., 2007; Kobayashi et al., 2005; Okazawa et al., 2004; Peier et al., 2002a; Takashima et al., 2007). To further map this cell population a size frequency histogram was created illustrating that the TRPM8-positive neurons are very small and clearly out-group from other neuronal populations. TRPM8 expressions were also studied in P4 and P12 DRG. Also at these developmental stages very small neurons were stained for TRPM8 with no apparent co-localisation with the other markers. This further points out the exclusiveness of this subgroup since it does not seem to be recruited from other neuronal subtypes. In addition, the staining of P4 and P12 tissue showed that the number of TRPM8-positive neurons increased from P4 to adult, correlating with the expression curve, and also explaining this not to be due to upregulation of mRNA in individual cells.

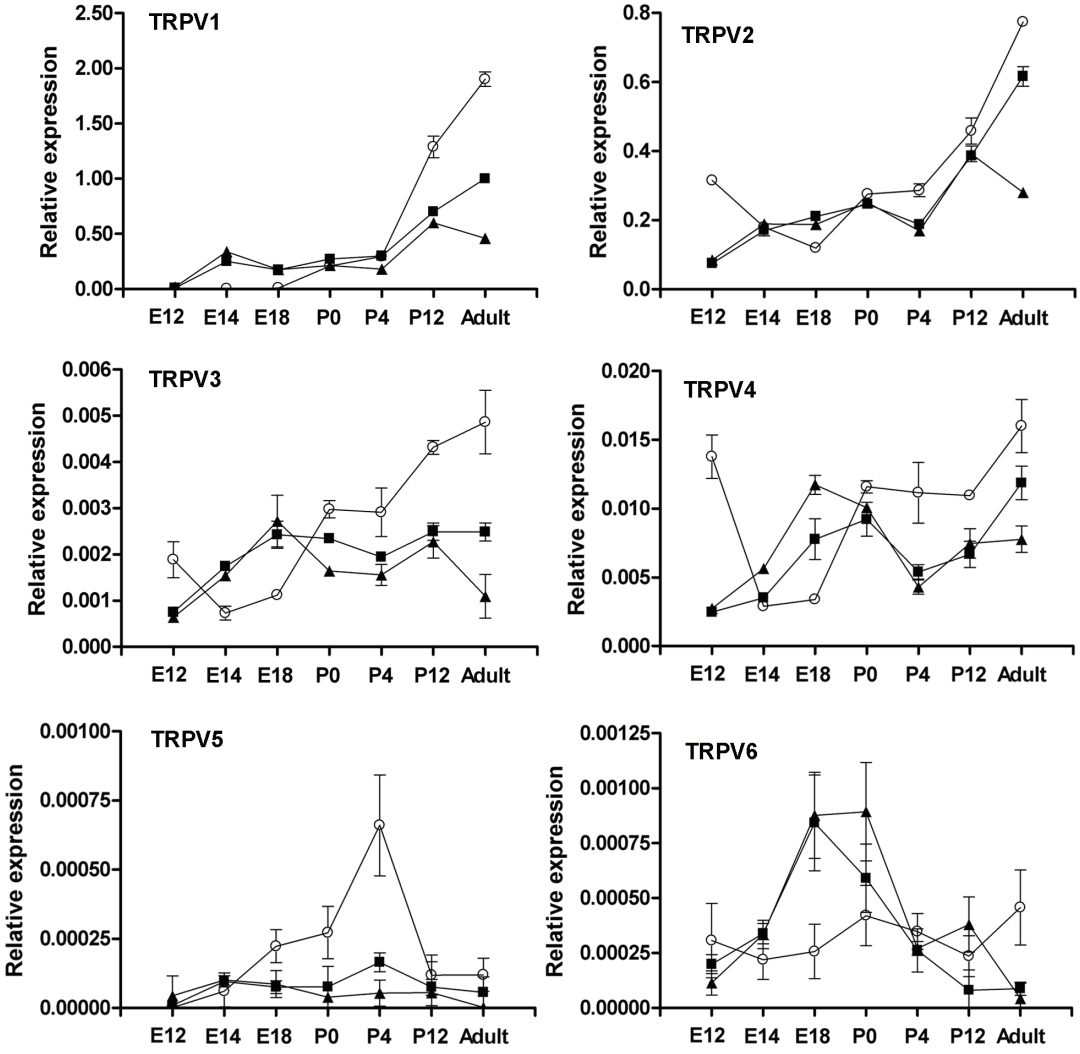

Figure 5. Developmental expression of TRPV channels in lumbar DRG (squares), thoracic DRG (triangles) and NG (open circles) measured by real-time PCR. Each data point is calibrated against TRPV1 expression in adult lumbar DRG using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). Error bars show standard deviation (n=3).

3.1.2.3 The TRPV, TRPA1, TRPP and TRPML3 subfamilies (unpublished)

Developmental expression from E12 to adult was also studied for the remaining TRP channel families (TRPV, TRPP and TRPA1). In the TRPV family, TRPV1 and V2 had the highest expression levels in the adult DRG followed by TRPV3 and V4. TRPV5 and V6 had very low levels (Figure 5). For TRPV1-V4, expression levels went from very low at E12 to more elevated during subsequent development stages. Similar to TRPC and TRPM family expressions there were reduced levels at P4 for TRPV2-V4 followed by higher levels in P12 and adult. For TRPV1 this dip was not seen but there was still a marked increase in expression after P4. The expression levels were very similar in lumbar DRG (squares), thoracic DRG (triangles) and NG (open circles) although some minor variations in the developmental expression pattern could be seen. Overall, NG had higher expression levels after birth and in the adult, followed by lumbar DRG and thoracic DRG. The opposite was seen before birth when the expression in NG was usually lower than in DRG.
The expression of TRPV1-V4 in DRG is no surprise since these channels have been assigned sensory roles as thermosensors in the warm to heat range (Schepers and Ringkamp, 2009). They have also been shown to have functions in pain and mechanosensation (see Introduction). For TRPV5 and V6 no sensory function has been assigned and the low expression levels in this study suggest a lack of importance for sensory functions in DRG and NG.

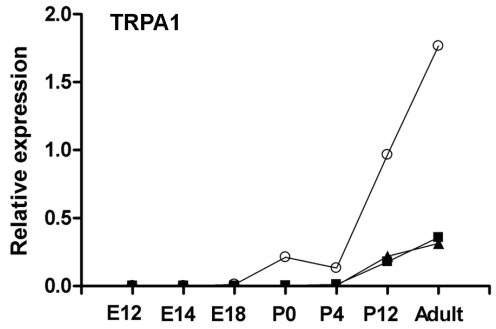

Figure 6. Developmental expression of TRPA1 in lumbar DRG (squares), thoracic DRG (triangles) and NG (open circles) measured by real-time PCR. Each data point is calibrated against TRPV1 expression in adult lumbar DRG using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Error bars show standard deviation (n=3).

TRPA1 had similar expression patterns in DRG and NG with very low expressions from E12 to P4 when suddenly steep increases in mRNA levels were seen in P12 and adult tissues (Figure 6). However, the expression reached much higher levels in NG (open circles) than in lumbar DRG (squares) and thoracic DRG (triangles). TRPA1 has been proposed to have roles in several types of sensory functions including mechano-, thermo- and chemosensation. Also, involvement in chronic pain has been suggested (see section 1.2.4.5). The developmental expression pattern with late onset and high levels in late development seen in this study do speak for a role in sensory physiology.

In the TRPP family, TRPP2 was the most frequently expressed channel at all stages in lumbar DRG while TRPP3 and TRPP5 had low or very low expression levels (squares in Figure 7). TRPP2 had a similar developmental pattern as many other TRP channels with low levels in early development, a decrease in mRNA levels at P4 and subsequent increasing levels in postnatal and adult tissue. A role in detection of fluid flow in the kidneys might open for a role in mechanosensation even though there are no studies in DRG that further investigate this (Köttgen et al., 2008; Nauli et al., 2003). TRPP3

mRNA was most frequent at E18 where after the levels quickly declined and TRPP5 had slowly increasing transcript levels throughout development. In thoracic DRG, the expression pattern and levels was similar to what was seen in lumbar DRG (triangles in Figure 7). In NG, TRPP2 expression had a dip at E18 instead of the increasing expression observed at that time in DRG (open circles in Figure 7). Furthermore, TRPP3 lacked the peak in NG seen at E18 in DRG and in TRPP5 a strong temporary increase could be seen at E18 in NG in contrast to the flat expression pattern in DRG (open circles in Figure 7).

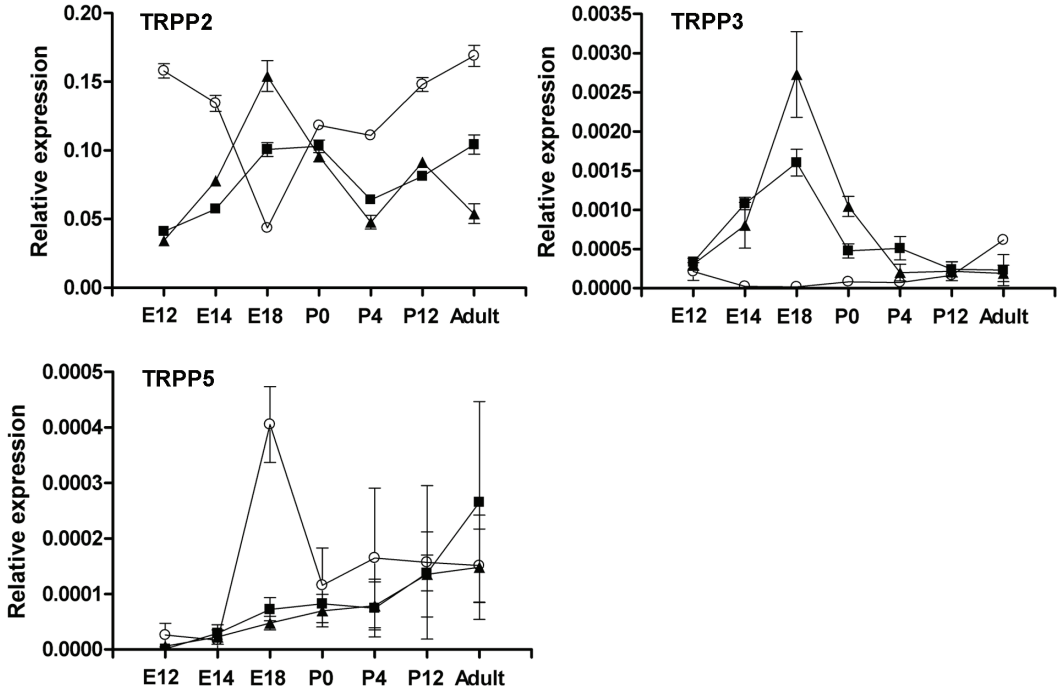

Figure 7. Developmental expression of TRPP channels in lumbar DRG (squares), thoracic DRG (triangles) and NG (open circles) measured by real-time PCR. Each data point is calibrated against TRPV1 expression in adult lumbar DRG using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). Error bars show standard deviation (n=3).

All TRPML channels were found in adult lumbar DRG (squares in Figure 8), thoracic DRG (triangles in Figure 8) and NG (open circles in Figure 8) although the expressions of TRPML2 and ML3 were very low. TRPML1 had increasing mRNA levels throughout development reaching high levels in the adult. However, in thoracic DRG a decrease in expression was seen in adult compared to P12. A small dip in expression was seen at P4 in all tissues, similar to what have been observed for a range of other TRP channels. TRPML2 had consistently low levels at all stages. For TRPML3 the highest mRNA levels were seen at E12 where after the transcript amounts progressively declined. This pattern was most pronounced in the NG, where the levels at E12 were substantially higher then in the DRG. The data concerning TRPML expression in lumbar DRG are also included in Paper III.

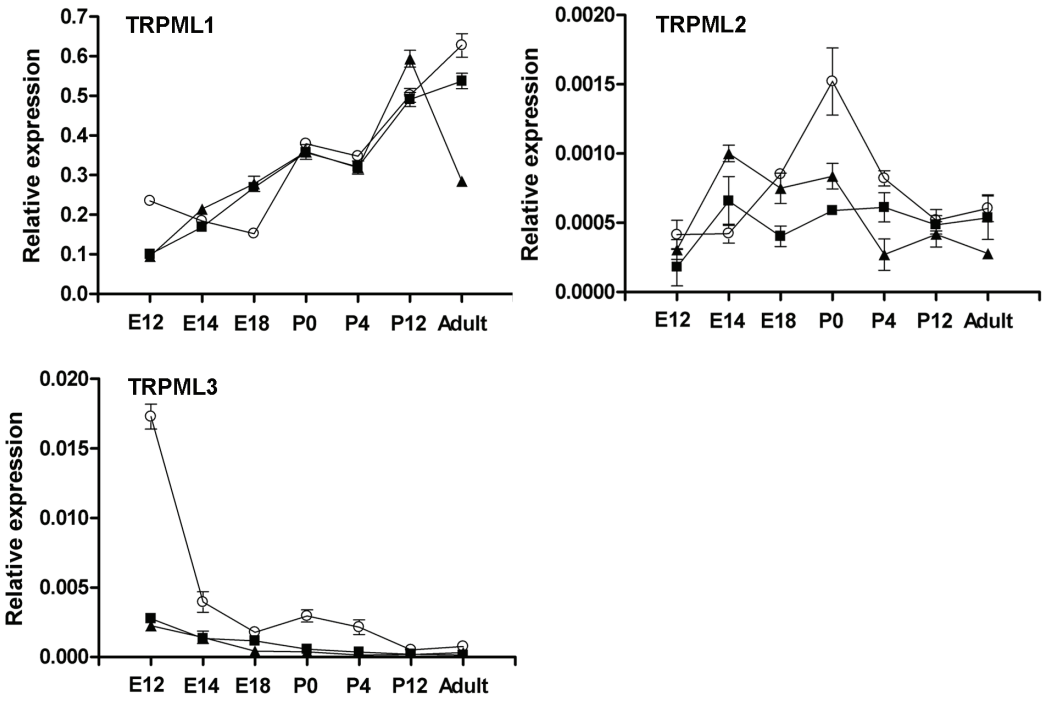

Figure 8. Developmental expression of TRML channels in lumbar DRG (squares), thoracic DRG (triangles) and NG (open circles) measured by real-time PCR. Each data point is calibrated against TRPV1 expression in adult lumbar DRG using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Error bars show standard deviation (n=3).

3.1.3 Reshaping of neurons continues in postnatal life

An outstanding point of these expression studies is that reshaping of neurons is still ongoing extensively after birth. This is obvious taking into account the large changes in expression seen for most channels from P0 to adult. A dip around P4 is frequent in many subfamilies indicating that the neurons are shifting expression patterns. In Paper II the percentage of cells staining for the neuronal markers was counted showing that the numbers of IB4-, NF200- and CGRP-positive neurons increased significantly from P4 to adult stage. This further emphasises the large changes in gene expression that occurs during the first few weeks after birth determining the different and specific functions of the cells. In fact, the non-peptidergic IB4-positive population is known to be recruited from the TrkA-positive population postnatally, leading to extinction of TrkA and a new dependence for GDNF (Molliver and Snider, 1997; Molliver et al., 1997). The remaining population is CGRP-positive in the adult (Averill et al., 1995).

3.1.4 Why are there so many TRP channels expressed in sensory ganglia?

After studying the developmental expression of all TRP channels it is clear that most channels are expressed in sensory ganglia. Furthermore, the expression levels of the majority of these channels are increasing in late development to reach its highest levels

in postnatal and adult life. This correlates with the expression patterns of the TRP channels that have defined sensory roles in the peripheral nervous system, e.g. TRPV1, TRPA1 and TRPM8. Hence, it is very likely that many more TRP channels than previously assigned have functions in sensory neurons. So why would so many proteins from the same family be necessary for executing the sensory functions? One answer might be that for a distinct sensory property each channel work in a very defined and precise manner as is seen in thermosensation where each channel cover a defined and very limited range of temperatures. In that way many channels are needed to cover the whole spectra of different temperatures that the skin can be subjected to and so far 6 TRP channels have been identified as responders to thermal stimuli in sensory neurons. If one speculates that other sensory functions such as nociception and mechanosensation works in a similar way, i.e. different channels responding to different strengths or character of the stimuli, it is not hard to imagine that the number of channels needed is quite high. TRP channels can of course also have other functions in DRG than being sensory transducers, for example maintain ion homeostasis, regulate intracellular trafficking, mediate apoptosis or alter neuronal excitability as has been proposed in different cell systems.

3.2 THE ROLE OF TRP CHANNELS IN NEUROPATHIC PAIN (PAPER III)

3.2.1 Several TRP channels are differentially regulated after nerve injury

The SNI model was used to study the differential regulation of TRP channels in neuropathic pain. Expression analysis of all channels using real-time PCR revealed up- or downregulation of several TRP channels from different subfamilies after nerve injury. Downregulation were observed for TRPM6, TRPM8, TRPV1, TRPA1, TRPC3, TRPC4 and TRPC5 while a large upregulation was seen for TRPML3. Downregulation of the thermoTRPs (TRPM8, TRPV1 and TRPA1) in damaged neurons have been observed in other studies using different nerve injury models (Facer et al., 2007; Hudson et al., 2001; Katsura et al., 2006; Michael and Priestley, 1999; Obata et al., 2005). An effect on these after nerve injury is not farfetched since cold allodynia and heat hyperalgesia are common symptoms of neuropathic pain patients (Campbell and Meyer, 2006). However, previous studies on TRPV1 imply a more complex involvement of this channel in the development and maintenance of neuropathic pain. In addition to thermal hyperalgesia TRPV1 also promotes mechanical hyperalgesia. Moreover, both pro-nociceptive and protective functions have been observed in different pain models. The sensitisation of TRPV1 by inflammatory mediators might explain some of this controversy (Levine and Alessandri-Haber, 2007). Another aspect is that in most nerve injury models not all nerves are damaged, resulting in coexistence of injured and uninjured fibers in the same nerve. It has been shown that both the remaining intact neurons and the axotomised or damaged ones have different transcriptional regulation and it is strongly believed that they contribute in different ways to the disease symptoms. Therefore, the degree of nerve injury might heavily influence the outcome of neuropathic symptoms (Campbell and Meyer, 2006). Although TRPV1 is the most well studied thermoTRP with regards to pain a similar

complexity might apply to the other channels constituting the exclusive group of thermosensitive channels.

The TRPC channels have not been associated with nerve injury or nociception before. Certainly the finding that the TRPC channels are expressed in DRG neurons of different subtypes (Paper I) indicates that there are sensory roles to be discovered for members of this subfamily. Specifically which functions these channels have in neuropathic pain can only be speculated and further studies need to be conducted to elucidate this. TRPC3, C4 and C5, which were all down regulated after SNI, are modulated by agonist-mediated exocytosis. Recombinantly expressed TRPC4 and TRPC5 are incorporated into the plasma membrane upon stimulation of the epidermal growth factor (EGF) receptor (Bezzerides et al., 2004; Odell et al., 2005). TRPC3 also increased its surface expression using exocytotic delivery upon agonist-stimulation in both neural and non-neural cells (Singh et al., 2004). In this way these TRPC channels might increase the duration and amplitude of local Ca^{2+} signalling in important cellular processes such as cell proliferation, differentiation and motility that are all stimulated by growth factors. In relation to this the explanation for the downregulation of TRPC3, C4 and C5 after nerve injury could be that usage of these channels are diminished since the retrograde growth factor delivery in axotomised neurons is interrupted. The neurons thereby lacks activation signals for recruitment of the TRPC channels to the plasma membrane and the need for synthesis of new channels decreases.

TRPM6 is expressed in the distal tubules of the kidney and in the intestines (Schlingmann et al., 2002; Walder et al., 2002; Voets et al., 2004b). It is permeable to divalent cations and has been proposed to actively reabsorb Mg^{2+} in the kidneys (Voets et al., 2004b). Mutations in TRPM6 result in hypomagnesaemia with secondary hypocalcaemia (HSH) as a result of impaired renal and/or intestinal Mg^{2+} reabsorption (Schlingmann et al., 2002; Walder et al., 2002). No previous observations have linked this channel to pain and more studies need to be executed to give information explaining the downregulation seen after nerve injury.

3.2.2 Both damaged and undamaged neurons undergo profound changes after nerve injury

Different cellular regulation of the same ion channel can occur within a ganglion after nerve injury since neurons with damaged and undamaged nerve fibers coexist. Loss of target-derived growth factor supply has a deep impact on the injured cells while the uninjured cells experience an increased exposure to the same factors. For TRPV1 a larger downregulation is seen after complete transection than after partial section of the nerve. This implies that TRPV1 is lost primarily in the damaged neurons. In line with this, other reports show decreased expression in injured DRG and increased in the adjacent uninjured ganglion (Fukuoka et al., 2002; Hudson et al., 2001). For TRPV1, the reduced expression in the damaged neurons is probably due to loss of NGF delivery from the peripheral tissues. It is known that DRG neurons lose their TRPV1 expression and capsaicin sensitivity when cultured without NGF (Bevan and Winter, 1995; Ogun-Muyiwa et al., 1999; Winter et al., 1988) and after sciatic nerve section *in vivo* (Hu-Tsai et al., 1996). The damaged nerves undergoes wallerian degeneration leading to

release of inflammatory mediators from schwann cells and macrophages as well as increased production of neurotrophins (Raff et al., 2002). The undamaged nerves, which exist in the near vicinity of the damaged ones, are exposed to these substances and react to them. As a result the expression of individual ion channels can increase or be unaltered in the undamaged neurons while decreasing in the damaged as in seen for TRPV1 but also for TRPM8 and TRPA1 (Katsura et al., 2006; Obata et al., 2005). At present it is unclear to what degree the changes in the damaged and undamaged nerves respectively contributes to developed neuropathic pain.

3.2.3 The potential role of TRPML3 in neuropathic pain

The novel finding of TRPML3 upregulation in nerve injury models raises questions whether it has a role in pain transmission and/or the development of neuropathic pain. Previously, expression has been identified in hair cells of the inner ear and in melanocytes in mice due to the discovery that a mutated form of TRPML3 is responsible for the varitint-waddler (Va) phenotype exhibiting deafness, dilute fur colour and prenatal death (Di Palma et al., 2002). The channel appears to be localised both in the lysosomal and the plasma membranes and exhibits only small ionic currents at normal physiological conditions. Depletion of extracellular (or extracytosolic) cations induces a large inward current which can be inhibited by low pH. This type of weak voltage-dependence, where activation results in increased numbers of opened (shift towards more negative potentials) or closed (shift towards more positive potentials) channels at physiologically relevant membrane potentials, is a common feature of several TRP channels (Nilius et al., 2005). The physiological relevance of TRPML3 activation at low Na^+ concentration in *in vitro* systems is hard to interpret. Indeed, the concentration of particular ions can reach very low levels locally in the near vicinity of other signalling ion channels that depletes the immediate surroundings. For Na^+ to be an actual activator in real life TRPML3 must therefore be tightly associated with another ion channel which has inwardly rectified Na^+ currents, for example voltage-gated Na^+ channels. These channels are of known importance in pain physiology by altering the electrical excitability of neurons (Cummins et al., 2007). Another possibility is that there are unknown activators of TRPML3 that produce the same effects as depletion of Na^+ does in artificial systems.

The pH-block is a property shared with TRPML1, which is also expressed in lysosomes and is known to heteromultimerise with TRPML3. Mutations in TRPML1, responsible for MLIV, causes aberrant exocytosis of lysosomal contents probably due to defected pH regulation (Puertollano and Kiselyov, 2009). The involvement of TRPML3 in endocytotic intracellular trafficking has not been studied but a regulatory role in these events is not unlikely regarding the close functional and structural relationship with TRPML1.

Since the natural activating mechanism of TRPML3 is unknown the reason for the massive upregulation after nerve injury is hard to guess. Our data show that increased expression of TRPML3 occurs in both nociceptive (peptidergic and non-peptidergic) and mechanosensitive neurons, leaving possibilities open for direct involvement in pain transmission, in the development of neuropathic pain or in pain responses such as nerve

regeneration and repair or apoptosis. TRPML3 could also be a supporting actor facilitating the actions of other proteins by controlling ionic balance in the microenvironment and thereby altering the activation threshold of the neuron. The very low levels of TRPML3 mRNA present in normal adult DRG suggest a non-significant role in healthy neuronal tissue. Higher expression levels at early development do however open for a function in the initial formation of the peripheral nervous system. The upregulation of an embryonic channel after nerve injury is not unique to TRPML3. $Na_V1.3$ is another embryonically expressed channel induced after nerve injury that causes hypersensitivity of peripheral nerve endings due to spontaneous firing (Cummins and Waxman, 1997; Waxman et al., 1994).

3.3 TRPC1 INVOLVEMENT IN MECHANOSENSITIVITY (PAPER IV)

By using RNA interference the expression of TRPC1 was knocked down in DRG neurons. A lentiviral transduction system efficiently delivered an shRNA-expressing vector and resulted in a 75% reduction of TRPC1 mRNA in infected neurons compared to non-infected control cell. Primary neuronal cultures were infected with shRNA directed towards TRPC1 or an unrelated target and the intracellular calcium response was measured after exposure to hypoosmolar solution. Results showed that in cultures where TRPC1 had been knocked down only 21% of the cells responded to hypoosmolar solution compared to 49% in the non-target shRNA group and 59% in the non-infected cultures. Thus, a clear reduction in mechanosensory response was seen upon stretch of the plasma membrane when TRPC1 was downregulated.

TRPC1 has previously been associated with mechanotransduction in oocyte membranes (Maroto et al., 2005). The difficulties to measure mechanosensory currents in heterologous cell systems expressing TRPC1 led to questioning of TRPC1 as a mechanosensor (Gottlieb et al., 2008). Our results imply that TRPC1 do have a role in mechanosensitivity in native sensory neurons, either directly or indirectly. The calcium imaging technique used does not have the time resolution necessary to distinguish between those two possibilities. The fact that the intracellular Ca^{2+} response to hypoosmolar stimuli was slower than the response observed upon application of capsaicin and KCl shows that the measured Ca^{2+} release is an indirect readout of the stimuli, probably involving second messenger systems. Therefore, it is impossible to study mechanistic details using this method. The difficulties in measuring activated currents from TRPC1 using mechanical stimuli in heterologous systems can be due to lack of accessory proteins, need for heteromerisation with other channels to accomplish a mechanotransductory complex or a situation where other important components are missed in the expression system. It is also possible that TRPC1 is not a part of the actual mechanotransducing unit but is involved in downstream events necessary for the mechanosensory response. Another possible way for indirect involvement in the signalling is that TRPC1 might act in the close vicinity of the mechanotransducer, increasing local Ca^{2+} delivery and thereby lowering the activation threshold. This has been a proposed mechanism of action for TRPC6 in association with the large conductance calcium-activated potassium channels (BK_{Ca}), a group of channels that have been shown to be stretch-activated in several tissues (Dopico et al., 1994; Mallouk

and Allard, 2000; Mienville et al., 1996). BK_{Ca} has in itself a low affinity for Ca^{2+} and need Ca^{2+} concentrations way over the cytosolic for activation. However, by being expressed very close to Ca^{2+} permeable channels the Ca^{2+} concentrations can be transiently elevated and thereby the activation voltage-dependence can be shifted into physiological membrane potentials, speeding up the action potential repolarisation of BK_{Ca}. This way of functioning has been shown in the brain by interaction of BK_{Ca} with different types of voltage-activated Ca^{2+} channels (Berkefeld et al., 2006; Grunnet and Kaufmann, 2004; Loane et al., 2007; Zou et al., 2008). Interestingly, TRPC3 and TRPC6 were shown to be co-expressed with BK_{Ca} channels in the non-excitable podocytes, presumably having a similar function as voltage-activated Ca^{2+} channels have in excitable cells, reducing the threshold for activation by providing locally high Ca^{2+} concentrations. In addition, siRNA knockdown of TRPC6 resulted in a decreased BK_{Ca} current due to a reduction in surface expression of BK_{Ca} (Kim et al., 2009). Although a DRG neuron is very different to a podocyte and no conclusions around the mechanosensitivity of TRPC1 can be drawn upon these results the role of TRPC6 in BK_{Ca} signalling is an interesting example of one possible way of co-operation between different ion channels in a mechanotransduction complex.

4 CONCLUSIONS

The principal conclusions in this thesis are the following:

- ✓ The majority of all known vertebrate TRP channels are present in the adult DRG and the NG.
- ✓ The majority of the TRP channels show increased expression levels through embryonic and postnatal development.
- ✓ TRP channel family members are differentially expressed among neuronal subtypes.
- ✓ Several TRP channels are differentially regulated in a model of neuropathic pain. TRPC3, C4, C5, V1, M6, M8 and A1 were downregulated and TRPML3 was drastically upregulated after nerve injury.
- ✓ Downregulation of TRPC1 with shRNA reduces the mechanosensory response from cultured DRG neurons upon hypotonic stimulation. This strongly implies a role for TRPC1 in mechanosensation.

5 ACKNOWLEDGEMENTS

The contribution and support of many people have been important for the finalisation of this thesis and I want to extend my gratitude to you all.

I especially want to thank:

Patrik Ernfors, for taking me on board in your lab. For your always positive, enthusiastic and constructive approach and for fast and efficient help with small and large things. You have been a great inspiration with your intelligence and scientific brilliance. Thank you for bringing out the best in me.

Jan Mattsson, for giving me the opportunity and helping me realise this adventure. For having confidence in me and always being there, pushing me forward when I needed it and giving me a clap on the shoulder when I needed it even more. I have really appreciated your positive attitude, experience, dedication, helpfulness and great sense of humour during our time together.

AstraZeneca, for financial support and a great working environment. In particular **Jan Fryklund**, for the opportunity to accomplish this thesis and especially for the continuous support after the GI reduction.

Frédéric Marmigère, for being my solid support, especially during the first year. You have taught me almost everything about molecular biology (I think I needed to use every trick there is in the cloning of that lentiviral vector!). Thank you also for the fantastic french dinners and for sharing the ups and downs of research, life and love. I owe you big time!

The people at the Department of Disease Biology, AstraZeneca Södertälje, involved in the SNI paper. It was a pleasure to meet such enthusiasm and helpfulness when approaching you with the project idea! A special thanks to **Sandra Oerther** for excellently performing the nerve injury surgery, providing me with tissue and your assistance during the writing process.

The members of the Ernfors and Arena labs during my time at KI for making me feel at home straight away. For a challenging, highly ambitious environment and for all the fun in- and outside the lab. My roommates: **Karin Agerman**, **Jens Hjerling-Leffler** and **Anna Stenqvist Castelo-Branco** for all great discussions and big laughs in the office. Special thanks to Jens for taking extra care of me, sharing valuable skills like dissecting DRG neurons, enjoying Stockholm nightlife and all things in between. I have greatly appreciated your friendship and support. I am also grateful to Karin for introducing me to her volleyball team. **Dmitry Usoskin**, for being a generous and helpful person and for watching over and taking care of the TRPC1 mice. **Marina Franck**, for helping me out with the final TRPM experiments and for a generous and helpful attitude. Everyone I met on daily basis who made the lab a fun place to be in and the social events loud and crazy, **Michael Andäng, Kalle Lundberg, Jorge**

Aquino, Christel Baudet, Andreas Montelius, Tibor Harkany, Paul Berghuis, Orsolya Penz, Linda Edman, Nina Rawal, Anita Hall, Clare Parish, Kyle Sousa, Julianna Kele, Emma Andersson, Gunnar Schulte, Gonçalo Castelo-Branco, Carmen Salto and Ernest Arenas.

The former GI people on HB3, for a helpful, generous, easy-going, collaborative and tolerant work atmosphere. **Ingela Maxvall**, for your big smile and openness. **Johanna Husmark**, for your prestigeless and helpful style. **Stefan Pierrou**, for saving me from formamide exposure when pregnant and for great social skills. **Ulrika Lind,** for your persistence, effectiveness and dedication. **Bengt von Mentzer**, for your ability to always look at everything from the bright side and for the company and chats during the late evenings. **Karin Svensson**, for taking care of all social events and the great sense of humour. Good luck with your new career! **Ingela Ahlstedt**, for being so cool, competent and generous. **Helena Lindmark**, for your sharp sense of humour and efficiency. **Malin Enerbäck**, for your social skills, always caring and listening to everyone and constantly having a positive approach. **Agnes Leffler**, for having a strong personality and not being afraid to speak you mind. **Monica Bergström**, for being a fighter. **Mary Fyfe**, for determination and integrity. **Karin Gedda**, for openness, kindness and generousity. **Sofi Nielsen**, for having a spontaneous and sparkling personality, always sharing the good and bad stories of your life. **Anders Peterson**, for inviting us to your spectacular kräftskiva.

The Thrombosis and Haemostasis section, in particular the Haemostasis group, for making me feel welcome in my new belonging and for an open and honest atmosphere. **Kenny Hansson**, for visualising my future and supporting me even though you haven´t had much use of me yet! **Fredrik Westberg**, for company and nice chats at the lab bench as wells as advices on molecular biology issues. **Johanna Deinum**, for the greatest introduction into the world of research many years ago. Science is fun with you!

All other collegues at AstraZeneca who create a collaborative, stimulating and challenging work atmosphere. I have had the pleasure to interact closely with many people and the list of names is too long to be written here. I am very grateful for all the support and good times through the years. I do however want to extend special thanks to the following persons: **Sven Göpel**, for your never-ending enthusiasm on research in general and on ion channels in particular. Thank you also for all lunches, coffee breaks and a great trip to Toulouse. **Anders Lehmann**, for being a scientific role model and for your humoristic but always respectful approach. **Peter Brodin**, for guidance on molecular biology issues. **Kerstin Lundgren**, for being so helpful and always knowing where to find things and how to get things done in house.

All friends outside science for all the fun during the years and for love and support.

Annika, for being there all the way, for laughing and crying with me and for always listening and understanding. Our friendship means the world to me!

Stina, for all the good times. For your high energy and positive attitude. For your trust and confidence and your ability to always fix everything for everyone but most of all for your love and care. Good luck on your big day!

Morbror Anders, for generously sharing your home with me during my trips to Stockholm. I have greatly appreciated the fantastic dinners, the refreshing discussions and your positive and entertaining personality.

Barbro, Björn, Kristina and **Tomas** for welcoming me into the family and for wonderful and relaxing stays at Värmdö.

Mamma och pappa, for unconditional love and support. For always being there for me, taking the time to listen and helping out in all situations of life. I greatly appreciate your interest and support in every challenge I take on. You are the best!

My beloved sisters, **Marie**, **Elisabeth** and **Sara** for just being you. For making me relax by almost never having discussions around work and for making all the minor celebrations become large feasts! A special thanks to Sara for helping me with the illustrations in this thesis. I also thank **Martin** and **Daniel** for bringing your great personalities into the family and **Elliot** and **Philip** for being so adorable and full of life.

Johan, for sharing my life, my dreams and my future. For your continuous love and support even though there sometimes have been very little of my time and energy left for you. Love you!

Oliver, my sunshine, for being the wonderful, magnificent little boy you are. You are my everything!

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