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Cover: Immunofluorescence micrograph of a section of a rat dorsal root ganglion. Purple represents immunostaining of 200 kD neurofilament (using the antibody RT 97), and yellow is staining for the voltage-gated sodium channel $Na_v1.8$. The picture was modified in Adobe Photoshop.

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To my family

ABSTRACT

Chronic neuropathic pain is a burdensome clinical condition that causes major suffering for countless of individuals worldwide. It is characterized by a hyperreactive nervous system with hyperexcitable neurons that fire in the absence of stimuli or at mild stimuli, causing pain. Although the processes that lead to neuropathic pain are not yet fully understood, the insight into putative causative mechanisms has increased vastly in recent years. Much of the research in the field has focused on the voltage-gated sodium channels (VGSC). These are ion channels that activate in response to membrane depolarizations and initiate and propagate electrical impulses. Thus, regardless of what other mechanisms that may contribute to the changed neuronal phenotype after injury, in the end, it is the VGSC that carry out the electrical nerve impulses. This makes them attractive candidate targets for pain alleviating drug development. Our data on the expression of VGSC in the dorsal root ganglion (DRG) of a damaged nerve implies that these channels are indeed involved in pain development after nerve injury. In particular, we found a correlation of expression data on $Na_v1.6$ and $Na_v1.9$ with spontaneous pain in the neuroma model (autotomy). These two channels have distinct distribution pattern and electrophysiological properties and thus may contribute in separate ways to the formation of hyperexcitable neurons. Some of our data also suggest that Contactin, a molecule involved in trafficking of the sodium channels to the cell membrane, may contribute to a pain phenotype.

The majority of neuropathic pain conditions are seen at spinal level compared to the less frequent orofacial neuropathic pain states. For this reason, most experimental pain studies are carried out in the lumbar DRG region. Most probably, much data obtained from these studies are also relevant to studies of orofacial pain. However, it is now clear that the pathophysiology of the trigeminal nerve in many ways differ to that of spinal nerves. Thus, there is a need for pain models specifically adapted to the trigeminal system when studying neuropathic orofacial pain. Here, a novel model for trigeminal neuropathic pain was presented. The model involves a photochemically-induced ischemic injury to the infraorbital branch of the trigeminal nerve. VGSC were expressed and regulated in the affected trigeminal ganglion (TG) in a similar fashion as seen in the DRG. Thus, new effective pharmaceutical agents that target VGSC in chronic pain at spinal levels should be useful against orofacial neuropathic pain conditions as well.

In addition to VGSC, other molecules are likely to be involved in neuropathic pain. After peripheral nerve injury, a vast number of genes are regulated in the affected DRG leading to a changed composition of receptors, ion channels, neurotransmitters etc. in the neuron. It is yet to be found out which of these changes that are related to neuropathic pain. Against this background, we performed a genome-wide search for regulated genes in five inbred mouse strains with distinct pain phenotypes after nerve injury. Subsequently, expression data for each of the regulated genes was correlated against pain phenotypes. This yielded three gene lists, each associated with one of the phenotypes mechanical hypersensitivity, thermal hypersensitivity and spontaneous pain in the neuroma model. These lists need further refinement in order to find specific pain genes, but may, at this stage, serve as “look-up tables” for genes whose regulation likely contributes to pain variability.

LIST OF PUBLICATIONS

I. Eriksson J, Jablonski A, **Persson AK**, Hao JX, Kouya PF, Wiesenfeld-Hallin Z, Xu XJ, Fried K. Behavioral changes and trigeminal ganglion sodium channel regulation in an orofacial neuropathic pain model. Pain. 2005; 119: 82-94.

II. **Persson AK**, Gebauer M, Jordan S, Metz-Weidmann C, Schulte AM, Schneider HC, Ding-Pfennigdorff D, Thun J, Xu XJ, Wiesenfeld-Hallin Z, Darvasi A, Fried K, Devor M. Correlational analysis for identifying genes whose regulation contributes to chronic neuropathic pain. Molecular Pain. 2009; 5:7.

III. **Persson AK**, Xu XJ, Wiesenfeld-Hallin Z, Devor M, Fried K. Expression of pain-related DRG molecules after nerve injury – a comparative study among five inbred mouse strains with contrasting pain phenotypes. Manuscript.

IV. **Persson AK**, Thun J, Xu XJ, Wiesenfeld-Hallin Z, Ström M, Devor M, Lidman O, Fried K. Autotomy behavior correlates with the expression of sodium channels in inbred mouse strains. Brain Research, accepted for publication pending minor revision.

CONTENTS

LIST OF ABBREVIATIONS

AKR	AKR/J
ANOVA	analysis of variance
ATF-3	activating transcription factor 3
ATP	adenosintriphosphate
B6	C57BL/6J
CAM	cell adhesion molecules
CBA	CBA/J
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
COMT	catechol-O-methyltransferase
C3H	C3H/HeJ
C58	C58/J
DRG	dorsal root ganglion/ganglia
GDNF	glial cell line-derived neurotrophic factor
IB4	isolectin B4
i.p.	intraperitoneal
i.v.	intravenous
IASP	International Association for the Study of Pain
IoN	infraorbital nerve
L4/L5/L6	4^{th} / 5^{th} / 6^{th} lumbar
NGF	nerve growth factor
PLSD	protected least significant difference
p11	annexin II light chain
QTL	quantitative trait loci
r_P	Pearson correlation coefficient
r_S	Spearman correlation coefficient
RT-PCR	real-time PCR
S/N	signal/noise
SNL	spinal nerve ligation
SNP	single-nucleotide polymorphism
SSC	standard saline citrate
TdT	terminal deoxyribonucleotidyl transferase
TG	trigeminal ganglion/ganglia
TTX	tetrodotoxin
TTX-r	tetrodotoxin resistant
TTX-s	tetrodotoxin sensitive
VGSC	voltage-gated sodium channel/channels

1 INTRODUCTION

1.1 PAIN

Pain is one of the most important functions of the nervous system. It is essential in the learning process of recognition and avoidance of potential sources of injury. The importance of pain is cruelly reflected in individuals with congenital mutations causing inability to sense pain. These individuals are frequently subjected to serious injuries and they rarely reach adult age (Nagasako et al., 2003). Pain is not one uniform condition. It can be classified into different categories based on underlying causes: nociceptive pain, inflammatory pain, neuropathic pain and idiopathic pain (when no certain cause is known). Nociceptive pain is the normal pain response to acute trauma. Pain receptors recognize and react upon noxious pressure, chemicals and extreme temperatures. Nociceptive pain is usually temporary. Inflammatory pain is associated with inflammatory processes and may arise in conditions such as infections or chronic inflammatory diseases. Neuropathic pain is caused by a trauma to, or a dysfunction of, the nervous system. This type of pain is often chronic and can be extremely severe and crippling for the individual. The mechanisms behind neuropathic pain are not well understood and current treatments are often ineffective. Thus, although the evolutionary purpose of pain is to protect us from danger, ongoing pathological pain is a major problem which causes great suffering for countless of individuals (Breivik et al., 2006).

1.1.1 Pain processing

Pain processing involves the detection of noxious stimuli, pain signal transmission along the peripheral nerves and the spinal cord, and subsequently integration and perception in the brain. Thus, peripheral, spinal- and supraspinal brain regions all have specific roles in the generation of painful sensations.

Initiation of the pain signal occurs in the primary sensory neuron

The pain signal is normally initiated at the peripheral end of the primary sensory neuron (this neuron is also called the primary afferent neuron). Various types of noxious stimuli act on distinct receptors in the skin. Activation of the receptors results in opening of ion channels with a subsequent influx of ions to the nerve cell. Given that the stimulus is strong enough, an action potential is generated and subsequently spread along the axon. In recent years, great progress has been made in the molecular characterization of receptors and ion channels that are activated in response to various nociceptive stimuli:

P2X channels are purinergic receptors activated by adenosintriphosphate (ATP), which can be released by neuronal and non-neuronal cells and regulate sensory neuron activity (Burnstock, 2001). Especially interesting in the context of pain is the P2X3 receptor since it is selectively expressed at high levels in pain mediating neurons (Burnstock and Wood, 1996).

TRP channels constitute a group of thermoreceptors which are activated at distinct temperatures and by various chemical agents (Talavera et al., 2008). The most studied TRP channel, TRPV1 is activated by capsaicin and heat (temperatures above ~43°C), and is expressed in pain mediating neurons (Caterina and Julius, 2001). In a lower part of the thermal spectrum, TRPM8 is activated at ~26–31°C and by menthol, whereas TRPA1 is activated at ~17°C and by chemical compounds such as mustard oil. Especially TRPA1 has been suggested to underlie cold pain, but this modality likely involves additional thermoreceptors (Reid, 2005). TRPA1 has also been reported to have mechanosensitive properties (Damann et al., 2008).

The primary afferents are pseudounipolar neurons. Their axons project peripherally to the targets of innervation and centrally to the spinal cord (Fig. 1). The cell bodies are clustered in the dorsal root ganglion (DRG) located just lateral to the spinal cord. There are different types of primary afferent neurons. Large neurons mediate innocuous stimuli such as touch. These neurons produce Aβ-fibers, which are thick, myelinated, and fast-conducting. Medium-sized neurons produce thinly myelinated Aδ-fibers and are mostly associated with thermal stimuli and pain. C-fibers are thin, unmyelinated, slow-conducting fibers produced by small neurons, and are mainly pain-mediating fibers. C-fiber neurons can be further divided into subgroups depending on phenotype. Usually they are categorized as peptidergic or non-peptidergic, isolectin B4 (IB4) positive. The peptidergic neurons are regulated by nerve growth factor (NGF) and contain neuropeptides such as calcitonin gene-related peptide (CGRP) and Substance P (Averill et al., 1995; Molliver et al., 1995). The IB4+ neurons are mainly regulated by glial cell line-derived neurotrophic factor (GDNF) (Bennett et al., 1998; McMahon et al., 1994). Parallel pathways in pain processing have been suggested for the two subtypes (Braz et al., 2005).

Sensory neurons terminate and synapse in the spinal cord

Axons from the primary afferent neurons enter the spinal cord via the dorsal roots and terminate in the dorsal horn (Fig. 1). Here, they connect with projection neurons (2nd order neurons), either direct or via interneurons. The dorsal horn is organized in

different lamina. The pain-transducing fibers mainly terminate in superficial lamina (I-II). The peptidergic C-fiber neurons usually project to lamina I and the outer lamina II, whereas the IB4+ population projects to neurons in the inner part of lamina II. Aδ fibers terminate in lamina II and larger Aβ fibers, mediating innocuous stimuli, usually terminate in deeper lamina of the dorsal horn (Fig. 1). The 2nd order neurons project centrally via distinct tracts in the spinal cord. The classical pathway associated with pain and temperature processing is the spinothalamic tract. Before entering the brain, sensory signals are integrated in distinct nuclei in the ventral posterior nuclei of the thalamus (Blomqvist et al., 2000; Davidson et al., 2008).

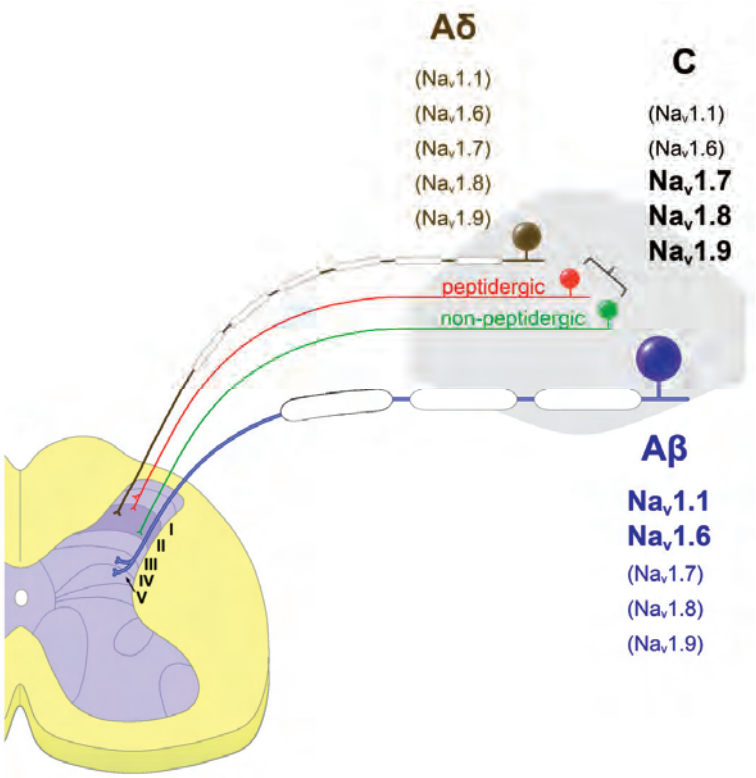

Figure 1. Schematic and simplified view of different primary afferent neuronal subtypes. Cell bodies are located in the DRG and the fibers connect to projection neurons in distinct lamina (denoted I-V on the picture) in the dorsal horn of the spinal cord. Different neuronal subpopulations (Aβ-, Aδ- and C- fiber producing neurons) have diverse compositions of voltage-gated sodium channel subunits. Brackets indicate documented or suggested expression of a specific α-subunit, and bold indicates well-documented expression of the α-subunit.

Pain perception in the brain

As mentioned, pain is a highly complex experience of multiple dimensions and can be influenced by factors such as mood, anxiety and attention (Tracey and Mantyh, 2007; Villemure and Bushnell, 2002). One sensory-discriminative and one affective-motivational part contribute to the pain experience (Albefessard et al., 1985; Treede et al., 1999). Various brain regions, sometimes collectively referred to as a "pain matrix", are involved in the perception (Tracey and Mantyh, 2007). Generally, the somatosensory cortex determines the localization and intensity of the pain whereas regions such as the cingulate gyrus and the limbic system, insula and the ventral/orbitofrontal cortex are involved in the emotional aspect. The work of this thesis deals mainly with peripheral mechanisms of neuropathic pain, and consequently the central mechanisms are only briefly described.

1.2 NEUROPATHIC PAIN

Neuropathic pain has been defined as "pain initiated or caused by a primary lesion or dysfunction in the nervous system" by the International Association for the Study of Pain (IASP). The lesion or dysfunction may arise from various types of clinical conditions such as amputation of a limb, diabetes mellitus, multiple sclerosis, spinal cord injury, cancer and cancer chemotherapy, trigeminal neuralgia or viral infections such as HIV and Herpes zoster. Neuropathic pain can involve either spontaneously arising pain, or reduced pain thresholds making normally innocuous stimuli painful (allodynia). Mechanical as well as thermal hypersensitivity are common symptoms of neuropathic pain (Backonja and Stacey, 2004).

1.2.1 Potential mechanisms

Neuropathic pain is the result of long-term changes in the nervous system, likely involving peripheral, spinal and supraspinal regions. After a peripheral nerve injury, several pathophysiological mechanisms occur in parallel in the damaged nerve and contribute to the generation of hyperexcitable neurons. Loss of target innervation causes disrupted neurotrophic support from the periphery. Wallerian degeneration, with concurrent Schwann cell proliferation, is initiated distal to the lesion in the axotomized nerve and causes a release of various mediators acting on the severed axons. Macrophages are recruited and an immune response is initiated (Mueller et al., 2001). Injured neurons show a marked phenotypic change, reflected in alterations in gene expression patterns and changes in morphological and functional properties. For example, there are dramatic changes in the expression of neuropeptides, ion channels, and receptors, probably reflecting adjustment by the neuron to survival and

regeneration (Hokfelt et al., 1994; Waxman et al., 1994; Xiao et al., 2002). Sprouts of nerve fibers, unsuccessfully trying to regenerate, can be trapped in a neural scar, or neuroma. Various sodium channels and receptors accumulate in the axons of the neuroma, which becomes an initiation site for spontaneous ectopic firing (Chen and Devor, 1998; Devor et al., 1993). Exaggerated firing of the primary afferent may produce an activity-dependent increase in spinal cord excitability, known as central sensitization. Central sensitization, in turn, leads to amplification of signals from the periphery, and is suggested to underlie the allodynia seen in patients with neuropathic pain (Decosterd et al., 2002).

1.2.2 Neuropathic orofacial pain

The orofacial region (including the face and teeth) is innervated by the trigeminal nerve. The trigeminal nerve is composed by three main divisions, the ophthalmic, the maxillary and the mandibular, each representing different cutaneous areas of innervation. The cell bodies are clustered in the trigeminal ganglion (TG) located on the inner surface of the skull, just lateral to the brain stem. Facial trauma or complications of surgical procedures within the orofacial region can cause injuries to trigeminal nerve branches, leading to the development of chronic neuropathic orofacial pain (Lobb et al., 1996; Polycarpou et al., 2005). Although neuropathic pain in the orofacial region is less common than chronic pain conditions in the spinal, segmental regions, orofacial neuropathic pain can be extremely painful and cause major suffering. It could be assumed that mechanisms behind the development of neuropathic orofacial pain are similar to those causing neuropathic pain elsewhere in the body. However, differences in the pathophysiology of the trigeminal nerve compared to spinal nerves (e.g. lack of sympathetic sprouting the former) indicate that unique mechanisms may underlie chronic pain in this region (Bongenhielm et al., 1999; Fried et al., 2001). This emphasizes the importance of pain models specifically adapted to the trigeminal system when studying neuropathic orofacial pain.

1.3 MOLECULES PARTICIPATING IN NERVE SIGNAL TRANSMISSION

All excitable cells in the body (including neurons and pacemaker cells in the heart) are able to generate electrical activity. The primary sensory neurons transduce external stimuli into electrical impulses and then transmit the electrical signal along the nerve. The initiation and transmission of the electrical impulse are carried out by the generation of action potentials. Ion channels, permeable for both sodium- and potassium ions are involved in the generation of an action potential. The initial,

depolarizing phase however, is dependent of the voltage-gated sodium channels (VGSC).

1.4 VOLTAGE-GATED SODIUM CHANNELS

In essence, the primary functional role of the VGSC is to generate the upstroke of the action potential (Caffrey et al., 1992; Catterall, 2000). VGSC are trans-membrane proteins constituting a pore that opens and closes in response to electrical changes across the cell membrane. At rest, the neuron is hyperpolarized and the VGSC are closed. Stimulation of the neuron causes an increase in positive charges on the inside of the cell, which in turn activates the VGSC. Activation of VGSC causes opening of the ion channels and selectively allows inflow of sodium ions into the cell. This results in an increased depolarization of the cell membrane. Rapidly after activation however, the channels inactivate (passage of ions through the pore is blocked by an inactivation gate) and remain in this state for a period of time. Subsequently, the channels recover from inactivation and return to their resting states, ready to be activated again.

1.4.1 α-subunits

VGSC are composed by one large, pore-forming α-subunit and one or two smaller β-subunits. The α-subunit contains the voltage-sensor and the channel pore of the VGSC. Nine different α-subunit subtypes are present within the nervous system. Of these, at least 7 have been detected in primary sensory neurons; $Na_v1.1$, $Na_v1.3$, $Na_v1.5$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ (Lai et al., 2003). All subtypes are formed by four homologous domains, I-IV, each of which is built up by six transmembrane-spanning units, S1-S6 (Fig. 2). The pore is made up by segment S5-S6 while S4 has voltage-sensing properties and thus regulates the opening of the channel. The intracellular loops contain many phosphorylation sites for both Protein Kinase A and Protein Kinase C. Phosphorylation can rapidly modify electrophysiological properties of the channel. Numerous sites on the intracellular loops are involved in inactivation, and in particular, the loop between domain III and IV comprises the inactivation gate. The different α-subunits are classified according to kinetic properties, voltage-dependence for activation and by pharmacological differences, including sensitivity to the neurotoxin tetrodotoxin (TTX) (Baker and Wood, 2001; Catterall, 2000).

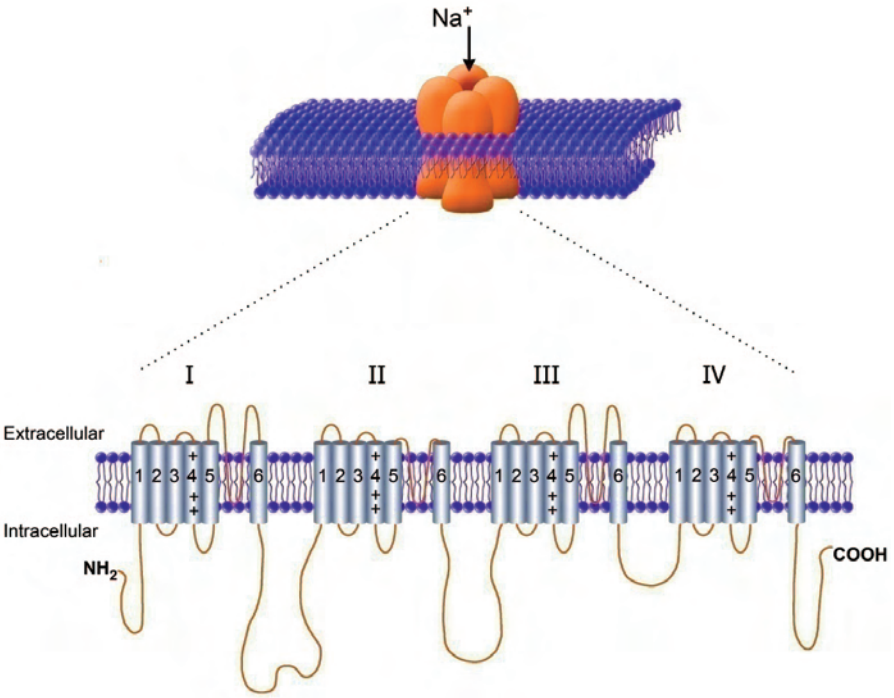

Figure 2. A schematic view of the structure of a VGSC α-subunit. The α-subunit constitutes the channel pore, through which sodium ions pass. The subunit is incorporated into the cell membrane and consists of four homologous domains, I-IV, each of which is built up by six transmembrane-spanning units. The pore is made up by segments S5-S6, while S4 functions as the voltage sensor. The intracellular loop between domain III and IV comprises the inactivation gate.

$Na_v1.1$

$Na_v1.1$ is a TTX-sensitive (TTX-s) sodium channel abundantly expressed in the brain and the primary sensory neurons. In the DRG, the expression appears to be restricted to A-fibers (Fukuoka et al., 2008). Several mutations on the $Na_v1.1$-coding gene (SCN1A) have been linked to epilepsy which makes it one of the most clinically relevant of all known epilepsy genes (Mulley et al., 2005). Homozygous null $Na_v1.1$ mice develop ataxia and die on postnatal day 15 and heterozygous null $Na_v1.1$ mice suffer from spontaneous seizures and sporadic deaths beginning after postnatal day 21 (Yu et al., 2006). These findings suggest important widespread central functions for $Na_v1.1$ which makes it a less attractive drug target for treatment of pain syndromes.

$Na_v1.3$

$Na_v1.3$ is TTX-s and widely expressed in sensory neurons and brain during development but is absent in the adult nervous system (Waxman et al., 1994). During the last decade, $Na_v1.3$ has been a hot study object in the field of neuropathic pain for several reasons. Interestingly, $Na_v1.3$ has been reported to re-appear in adult DRG neurons after damage (Waxman et al., 1994), providing a possible explanation for the hyperactive neuronal state. Most other sodium channels present in the sensory neurons are down-regulated after damage and thus, association of these with increased sodium currents and hypersensitivity in the system is less obvious. Further, $Na_v1.3$ has been found to accumulate in axonal endings after peripheral nerve injury (Black et al., 1999; Black et al., 2008). The electrophysiological properties of $Na_v1.3$ support a possible contribution to hyperexcitability in neurons. The channel recovers relatively rapidly from inactivation, which makes it a candidate contributor to the high-frequency firing of injured neurons (Cummins et al., 2001). In fact, $Na_v1.3$ has been directly coupled to the ectopic action potential generation that occurs in injured neurons and it was at one point believed to be strongly involved in neuropathic pain (Boucher et al., 2000). However, more recent findings have questioned the role of $Na_v1.3$ in injury-induced pain. Thus, allodynia-like behavior as well as ectopic firing seem to be present in damaged nerves of mice even in the absence of $Na_v1.3$ (Nassar et al., 2006).

$Na_v1.5$

$Na_v1.5$ is TTX-resistant (TTX-r), and the main cardiac VGSC subtype (Gellens et al., 1992). Heart failure is a direct consequence of insufficient expression of $Na_v1.5$ (Hesse et al., 2007). In addition to heart muscle, $Na_v1.5$ has been detected in the brain (Hartmann et al., 1999) and in the embryonic DRG (Renganathan et al., 2002). The presence of $Na_v1.5$ in adult DRG is somewhat disputed, with some studies failing to detect the channel (Black et al., 1998), and others showing an expression although at low levels (Kerr et al., 2007; Renganathan et al., 2002). If $Na_v1.5$ would turn out to be a contributor to neuropathic pain in the peripheral nervous system it would be very difficult to target this channel with novel therapeutics due to risk of adverse side effects on heart activity.

$Na_v1.6$

$Na_v1.6$ is a TTX-s sodium channel and the main VGSC isoform at the nodes of Ranvier in myelinated axons (Krzemien et al., 2000). In a recent report, $Na_v1.6$ was found to be expressed selectively in Aβ-fiber neurons in the rat (Fukuoka et al., 2008). However, it has also been reported to be distributed in Aδ-fibers and along unmyelinated C-fibers (Black et al., 2002). $Na_v1.6$ exhibits particularly rapid recovery from inactivation, ~10-

fold faster than $Na_v1.7$ and 2-fold faster than $Na_v1.3$ (Herzog et al., 2003a). These are properties that enable high-frequency firing, making $Na_v1.6$ an interesting candidate channel in the pain field. Despite this, not much attention has been given $Na_v1.6$ as a participant in neuropathic pain development. However, it has been suggested to play a part in trigeminal pain states (Henry et al., 2007).

$Na_v1.7$

$Na_v1.7$ is a TTX-s VGSC that is abundantly expressed in nociceptive primary neurons within the DRG. It has biophysical properties that cause a response to small, slow depolarizations, and in this way it contributes to an action potential (Cummins et al., 1998). Several clinical syndromes in humans that involve abnormalities in pain perception have their origin in different mutations of the $Na_v1.7$ gene. One of the most frequently mentioned is inherited erythromelalgia which is characterized by a sensation of burning pain and redness of the extremities triggered by mild warmth (van Genderen et al., 1993). Erythromelalgia has been linked to different "gain of function"-point mutations of $Na_v1.7$, resulting in lowering of the activation threshold and thus a hyperexcitable neuron (Dib-Hajj et al., 2005). $Na_v1.7$ has also been implicated in congenital insensitivity to pain due to loss-of-function mutations (Cox et al., 2006) (see 1.6.1).

$Na_v1.8$

$Na_v1.8$ is TTX-r and was initially described as a C-fiber specific VGSC in the primary sensory neurons (Akopian et al., 1996). It is present both in the peptidergic and non-peptidergic subpopulations of C-fiber neurons (Fjell et al., 1999). However, although preferentially expressed by C-fiber neurons, $Na_v1.8$ has also been detected in Aδ fiber neurons and within a subpopulation of myelinated Aβ fiber neurons (Amaya et al., 2000). The biophysical properties of $Na_v1.8$ make it an interesting candidate molecule in the context of neuropathic pain: It inactivates slowly and recovers rapidly from inactivation (Cummins and Waxman, 1997; Renganathan, 2001). These characteristics make $Na_v1.8$ the largest contributor to the generation of an action potential. Further, these properties would be ideal for maintaining high-frequency firing of the neurons. Interestingly, $Na_v1.8$ also appears to be essential for pain perception at low temperatures. Whereas low temperatures enhance slow inactivation of TTX-s sodium channels, $Na_v1.8$ remains unaffected by cold (Zimmermann et al., 2007). Following nerve injury, the expression of $Na_v1.8$ is reduced in the cell bodies of DRG neurons (Sleeper et al., 2000). However, it has been shown to accumulate within human painful neuromas (Black et al., 2008). The role for $Na_v1.8$ in neuropathic pain has been extensively investigated, although with somewhat conflicting results. Antisense-

mediated knock down of $Na_v1.8$ reversed mechanical and thermal hypersensitivity induced by spinal nerve injury in rats (Lai et al., 2002). On the other hand, normal development of neuropathic pain behaviors after peripheral nerve injuries was noted in $Na_v1.8$ knockout mice (Kerr et al., 2001; Nassar et al., 2005). There is a possibility though, that compensatory mechanisms in the $Na_v1.8$ knockout mice affect the composition of VGSC.

$Na_v1.9$

$Na_v1.9$ gives rise to a TTX-r sodium current and is expressed in sensory neurons (Dib-Hajj et al., 1998b; Rugiero et al., 2003). In the DRG, $Na_v1.9$ is present mostly in the non-peptidergic population of C-fiber neurons (Fjell et al., 1999). The fact that $Na_v1.9$ is almost exclusively restricted to C-fiber neurons strongly indicates that it is important for nociception. $Na_v1.9$ has a voltage-dependence with a great overlap between activation and steady-state inactivation. In this way, it underlies a persistent current. Although not able to give rise to an action potential on its own, $Na_v1.9$ can amplify subthreshold depolarizations and works as a modulator for resting membrane potential (Dib-Hajj et al., 2002). Recently, it was shown that a soup of inflammatory mediators rapidly potentiate the $Na_v1.9$ activity (Maingret et al., 2008). In line with this, several studies have showed a contribution of $Na_v1.9$ to inflammatory pain (Amaya et al., 2006; Priest et al., 2005). However, there is no clearcut evidence for a role of $Na_v1.9$ in nerve injury-induced pain. For instance, antisense knock-down of $Na_v1.9$ produces no change in tactile and thermal hypersensitivity (Porreca et al., 1998).

1.4.2 β-subunits

While the α-subunits make up the actual pore of the VGSC, β-subunits act as modulators of channel function. In particular, they are important regulators of VGSC localization in the cell membrane by interacting with extracellular matrix proteins (Malhotra et al., 2000). Four different β-subunits, β1- β4, have so far been characterized (Isom, 2001; Yu et al., 2003). Within the DRG, β1 is mainly expressed in medium- and large diameter neurons (Oh et al., 1995). β2 have been detected at very low levels in DRG neurons of all sizes (Pertin et al., 2005) whereas β3 is mainly expressed by C-fiber neurons (Shah et al., 2000). Nerve injury does not appear to affect the expression of β1 in DRG neurons (Takahashi et al., 2003). Inconsistent findings from studies of β2 have shown either increased or unchanged levels of expression in peripheral nerve injury models (Pertin et al., 2005; Takahashi et al., 2003). All available evidence show that β3 is is upregulated in injured sensory neurons (Casula et al., 2004; Takahashi et

al., 2003). The increased expression of β3 and possibly β2 may reflect involvement of these auxiliary proteins in neuropathic pain.

1.4.3 Auxilliary proteins

Modulation of VGSC can have major impact on cell excitability in primary sensory neurons. A variety of molecules, e.g. ankyrinG, Calmodulin, p38 MAPK and TNFα, act as regulators of VGSC function using distinct mechanisms (Herzog et al., 2003b; Jin and Gereau, 2006; Scherer and Arroyo, 2002; Wittmack et al., 2005). Annexin II light chain (p11) and Contactin are examples of molecules involved in the insertion of VGSC into the axolemma. p11 is a Ca2+- and phospholipid-binding protein that attaches to the amino terminus of $Na_v1.8$ and promotes translocation to the cell membrane, producing functional channels. A deficit in p11 will thus cause a loss in membrane-associated $Na_v1.8$ (Foulkes et al., 2006; Okuse et al., 2002). Contactin is a neuronal cell surface glycoprotein belonging to the group of cell adhesion molecules (CAM). The protein regulates trafficking of various subtypes of VGSC α-subunits to the cell surface and thus increases channel density in the cell membrane (Kazarinova-Noyes et al., 2001; Liu et al., 2001; Shah et al., 2004). An up-regulation of Contactin at the protein level was demonstrated in axotomized DRG neurons and was suggested to contribute to the axotomy-induced expression of $Na_v1.3$ in the DRG (Shah et al., 2004).

Pharmaceutical agents acting on sodium channels

Sodium channel blockers are regularly used in the clinic to treat pain (Mao and Chen, 2000; Rice and Hill, 2006). Local anesthetics, such as Lidocain, act by binding to open and/or inactivated sodium channels and are effective in treatment for postherpetic neuropathic pain and painful diabetic neuropathy (Barbano et al., 2004; Meier et al., 2003). Anticonvulsants, in particular Carbamazepine and to a lesser extent Phenytoin, are used to treat trigeminal neuralgia and diabetic neuropathy. These compounds work by stabilizing sodium channels in their inactive state (Beydoun et al., 2007; Eisenberg et al., 2007). All currently available sodium channel blockers are however non-specific and target multiple sodium channels isoforms. Accordingly, the use of these drugs is limited due to cardiac and CNS side effects. Thus, there is an urge for the development of isoform-specific sodium channel blockers (Jarvis et al., 2007; Rajamani et al., 2008).

1.5 THE USE OF ANIMALS IN PAIN RESEARCH

To best mimic clinical pain conditions, experimental pain measures would ideally be performed on humans. However, ethical considerations as well as practical difficulties in collecting enough numbers of subjects suffering from appropriate neuropathies limit

the use of human experimental studies. Moreover, pain is a complex subjective experience influenced by a number of confounding factors such as mood, anxiety, attention etc. (Haefeli and Elfering, 2006; Van Damme et al., 2004). It is impossible to avoid psychological factors when measuring pain in humans. In animals however, the influence of at least some of these factors can be minimized and thereby more objective measures of behavioral responses to noxious stimulation can be made. Measuring pain in animals is however not trivial. Obviously, the inability to communicate with animals limits the chances of valid pain measurements. By studying a behavior, how can we be certain that it is actually pain that is studied? The usefulness of the currently available animal models in pain research is frequently being debated (Vierck et al., 2008). Ongoing development of new, better, animal models, as well as modifications and refinement of existing ones is of great importance for pain research.

1.5.1 Experimental pain models

Several nerve injury models have been developed for studies of neuropathic pain behavior in animals. Often, the target is the sciatic nerve and pain behavior is measured in the form of hypersensitivity in the sciatic nerve territory on the hindpaw.

The first established and perhaps most commonly used nerve injury model consists of ligation followed by complete transection of the sciatic and the saphenous nerves at mid-thigh level. The lesions cause the formation of neuromas in the proximal nerve stumps. A neuroma is mechanosensitive (Scadding, 1981) and constitutes an initiation site for spontaneous electrical activity (Wall and Gutnick, 1974). Due to complete transection of the nerves, sensation from the normal territory of innervation is lost, and evoked pain responses (in the form of mechanical or thermal hypersensitivity) cannot be measured. However, signs of spontaneous pain in the form of autotomy have been associated with this model (see 1.5.2).

Since then, alternative pain models have been presented. In particular two of them are regularly used in pain research: the chronic constriction injury (CCI) model (Bennett and Xie, 1988), and the partial ligation model (Seltzer et al., 1990). In the CCI model, four ligatures are loosely tied around the sciatic nerve proximal to the sciatic trifurcation. This produces a gradual swelling and strangulation of the nerve under the ligatures, followed by an axonal Wallerian degeneration. The majority of Aβ and Aδ fibers lose their capability to conduct signals through the injury site, while the C-fibers mostly remain unaffected (Kajander and Bennett, 1992). Electrophysiological examination has revealed spontaneous discharges at the site of injury, mainly

originating from Aβ fibers (Kajander and Bennett, 1992). In addition, signs of mechanical, thermal and chemical hypersensitivity as well as spontaneous pain have been reported (Bennett and Xie, 1988). In the partial ligation model, about half of the sciatic nerve is ligated, leading to a mixed population of damaged and undamaged neurons. This model is associated with mechanical and thermal hypersensitivity and spontaneous pain, which appears to be dependent on the sympathetic outflow (Shir and Seltzer, 1991).

A common shortcoming of both these models is the difficulty to produce exactly the same lesion in each animal. Against this background, Kim and Chung (Kim and Chung, 1992) developed a model frequently called the Chung- or the spinal nerve ligation (SNL) model. In SNL, ligatures are tightly tied around the 5^{th} lumbar (L5) and 6^{th} lumbar (L6) (or the L5 alone) spinal nerves. The standardized procedure facilitates reproducible lesions and minimizes the risk of variation in behavioral outcome due to surgical discrepancies. Another key feature that makes the SNL model superior to other nerve injury models is that by damaging the L5 and L6 spinal nerves and leaving the 4^{th} lumbar (L4) spinal nerve intact, it is possible to separately study changes in affected and unaffected DRG. A complication of the SNL model is however the invasive surgery, which increases the risk of tissue and muscle damage. SNL produces signs of mechanical and thermal hypersensitivity as well as spontaneous pain in rodents (Dowdall et al., 2005; Kim and Chung, 1992). As with CCI, spontaneous activity has been recorded from injured Aβ fibers but not from C-fibers (Liu et al., 2000). Based on measurements from individual rats the ectopic activity has been positively correlated with mechanical hypersensitivity (Liu et al., 2000).

An alternative to these models is the spared nerve injury model (Decosterd 2000). This involves a lesion of two of the three terminal branches of the sciatic nerve, leaving the remaining branch intact. Pain behavior can be tested in the non-injured skin territories adjacent to the denervated areas, and this has shown robust changes up to 6 months after operation (Decosterd 2000).

The ischemic injury model was created in an attempt to decrease the variability seen in pain behavior following other experimental pain models. Here, a peripheral nerve (or the spinal cord when studying central pain) is exposed and after intravenous injection of a photosensitizing dye, erythrosin B, the nerve is irradiated with an argon laser (Gazelius et al., 1996). An ischemic injury is created as a result of vascular thrombosis involving the capillaries surrounding the nerve. The degree of injury is dependent on the duration of irradiation, and thus the injury can be graded and easily reproduced.

Another advantage is that neuropathy is produced through an ischemic mechanism which does not involve mechanical damage to the nerve.

1.5.2 Pain assessment in animals

Evoked pain

Pain evoked by mechanical and thermal stimuli are two frequently occurring symptoms of neuropathic pain (Backonja and Stacey, 2004). Behavioral signs of hypersensitivity to these different types of stimuli have been studied extensively in experimental pain models. Mechanical sensitivity is regularly assessed by applying von Frey filaments to the skin and recording the pressure needed to induce limb withdrawal. Hypersensitivity to thermal stimuli is often measured by recording the time latency for limb withdrawal from a heat source.

Spontaneous pain

Continuous spontaneous pain is a common and burdensome clinical symptom of neuropathic pain (Backonja and Stacey, 2004; Woolf and Mannion, 1999). In fact, this symptom is more frequent than mechanical and thermal hypersensitivity among neuropathic pain patients. Despite the high prevalence of spontaneous pain it is seldom in focus in experimental pain models, as compared to the much more extensively studied evoked pain responses (Mogil and Crager, 2004). This is probably due to difficulties in interpreting signs of spontaneous pain in animals. Stimulus-evoked pain can be easier appreciated from the withdrawal of a limb upon stimulation. Spontaneous pain, on the other hand, must be examined by an indirect interpretation of general behaviors.

To date, the behavioral assay that best reflects spontaneous pain in animals is possibly the autotomy model. It consists of scoring the animals' self-mutilation of a denervated limb after nerve transection and neuroma formation (Wall et al., 1979). It is debated, though, whether autotomy is a valid measure of pain and not just a result of a complete lack of sensation (Rodin and Kruger, 1984). There is however a large amount of evidence for an accurate correlation of this behavior with spontaneous pain (Devor, 2007). In particular, autotomy is reduced by drugs that relieve pain and reduce ectopia in patients, e.g. anticonvulsants, spinal cord stimulation and NMDA receptor antagonists (Coderre et al., 1986; Gao et al., 1996; Wiesenfeld-Hallin, 1984).

Other measures, such as spontaneous foot lifting, grooming and locomotor behaviors have also been interpreted as behavioral signs of neuropathic pain in animals (Choi et al., 1994; Mogil and Crager, 2004). There is, however, an urge for the development of new spontaneous-pain models with increased validity compared to the currently available ones.

1.6 GENETIC VARIATIONS IN PAIN PERCEPTION

1.6.1 In humans

Chronic pain associated with tissue injury is notably variable from patient to patient. For example, one individual may be experiencing agonizing pain after limb amputation while another is unaffected (Mogil, 1998). Earlier, these types of differences in pain experience were mainly ascribed to ethnical, cultural or psychosocial factors. Likely, such factors do indeed influence discrepancies in pain experience among human individuals. However, it is now widely acknowledged that also genetic factors play important roles in inter-individual differences in pain perception. In the wake of the era of genetics breakthroughs and the consequent exploration of the human genome, several clinical pain conditions have been linked to polymorphism of certain genes (LaCroix-Fralish and Mogil, 2009).

Possibly one of the most intriguing inheritable pain conditions known is the channelopathy-associated inability to sense pain, which demonstrates a link between gene mutations and pain phenotype (Cox et al., 2006). Individuals from three related families in the northern Pakistan were found with this unusual lack of pain appreciation. All had injuries to their lips and tongue (from biting themselves) and were covered with bruises and cuts. Three distinct mutations of the gene coding for the VGSC α-subunit $Na_v1.7$ were discovered to underlie the trait. Deficits were restricted to pain perception, whereas overall other sensory functions appeared normal. Interestingly, gain-of-function mutations in the same sodium channel, resulting in lower thresholds for activation, cause the painful chronic inflammatory condition primary erythromelalgia (Dib-Hajj et al., 2005). These clinical conditions emphasize both the importance for VGSC in pain as well as the vast impact single gene mutations may have on pain phenotype.

Studies on human populations, such as twin studies and large association studies have demonstrated a heritability component of various pain traits (Nielsen et al., 2008; Norbury et al., 2007). Further, quantitative trait locus (QTL) mapping and linkage

analysis have lead to the identification of a number of single-nucleotide polymorphisms (SNPs) associated with different pain phenotypes. The perhaps best characterized SNPs are those in the gene coding for catechol-O-methyltransferase (COMT). COMT is involved in the inactivation of catecholamine neurotransmitters, such as dopamine, adrenaline and noradrenaline. An impaired function of COMT leads to increased pain sensitivity (Zubieta et al., 2003). Other SNPs affecting pain sensitivity have been found in the gene GCH1, which is involved in NO synthesis (Tegeder et al., 2006) and in MC1R, which codes for the melanocortin-1-receptor (Mogil et al., 2005).

A growing body of evidence suggests that sex differences exist in pain sensitivity as well as in the susceptibility to certain clinical pain conditions (Berkley, 1997; Wiesenfeld-Hallin, 2005). Chronic pain conditions, in particular pain associated with temporomandibular joint disorder and fibromyalgia, are more common in women than in men (Greenspan et al., 2007). In experimental pain models, female rodents are often associated with lower pain thresholds than males (Aloisi et al., 1994; Barrett et al., 2002). These phenotypic differences may involve different levels of circulating gonadal hormones, e.g. estrogen and progesterone. They may also be the result of other sex dependent mechanisms, such as endogenous opioid system activity (Kuba and Quinones-Jenab, 2005; Zubieta et al., 2002).

1.6.2 In experimental animal models

Inter-individual differences in pain response can also be observed in laboratory animals. Several studies on rat and mouse strains initially demonstrated a contribution of a genetic component in pain susceptibility in experimental nerve injury models (Inbal et al., 1980; Mogil et al., 1999a; Shir et al., 2001; Wiesenfeld and Hallin, 1981). During the last decade, the awareness of the genetic contribution to pain in experimental models has greatly increased. In particular, Mogil and colleagues have performed extensive comparative pain studies on 11 inbred mouse strains that differ in their genomes (Mogil et al., 1999a). Twelve different types of nociceptive- and neuropathic pain behaviors were examined and strain differences were found for each of them. Further, different constellations of nociceptive assays with apparent common genetic factors were demonstrated (Lariviere et al., 2002; Mogil et al., 1999b). Among the 11 inbred mouse strains investigated by Mogil and colleagues are the ones used in this thesis: AKR/J (AKR), CBA/J (CBA), C3H/HeJ (C3H), C57BL/6J (B6) and C58/J (C58).

Efforts to identify genes that contribute to strain-differences in pain among animals have been approached using a number of strategies. One of the first genetic studies on experimental pain models involved selective breeding and the establishment of two rat strains with high (HA) and low (LA) susceptibility to autotomy behavior after nerve injury. At the time, the trait was suggested to be inherited through a single gene (Devor and Raber, 1990). Later, a QTL associated with autotomy in the HA and LA strains was identified on rat chromosome 2 (Nissenbaum et al., 2008). The QTL, denoted pain 2, covers several genes and it is yet to be revealed which of them that may contribute to autotomy. Furthermore, a similar strategy searching for autotomy-affecting genes in mouse strains has previously identified a QTL on another chromosome (Devor et al., 2005; Seltzer et al., 2001). This raises the possibility of a complex involvement of genes that influence autotomy behavior.

Transgenic and knockout mice have been widely used in a large number of studies in order to identify molecules that are important for pain phenotypes (Kieffer, 1999; Lacroix-Fralish et al., 2007; Mogil and Grisel, 1998). A pitfall in studies using genetic manipulations is however the risk of compensatory mechanisms in animals lacking a certain gene. Creation of conditional knockouts, i.e. reduction of gene expression in the adult animal with e.g. antisense oligodeoxynucleotides, or already existing inbred mouse strains, can be used to reduce these confounds (Kim and Rossi, 2007).

2 AIMS

The specific aims of this thesis were:

I To establish and evaluate a novel experimental rat model for trigeminal neuropathic pain by producing a photochemically-induced ischemic injury to the infraorbital nerve (IoN). Further, to study the nerve-injury induced regulation of specific VGSC subunits in this model and compare the data with what is known on the nerve-injury induced regulation of such subunits at spinal segmental regions.

II To examine the effect of nerve injury on DRG gene regulation in five inbred mouse strains with contrasting pain phenotype using the following approaches:

- a detailed morphological examination of the expression and nerve injury-induced regulation of a set of pain candidate genes, including VGSC within the DRG
- a broad whole-genome search for expressed DRG genes and genes that are regulated after nerve injury

III To evaluate the contribution of regulated genes and VGSC to the variability in pain-phenotypes among the five inbred mouse strains after nerve injury.

3 MATERIALS AND METHODS

3.1 ANIMALS

All experiments conducted within the scope of this thesis were carried out according to the Ethical Guidelines of the International Association for the Study of Pain, and were approved by the local animal research ethic committee. Upon arrival to the animal department, the animals were acclimatized to the new environment for at least a week before experiments. All animals were housed under standard laboratory conditions, with a light:dark cycle 12:12, lights on at 06:00 AM. They were maintained in groups in transparent plastic cages bedded with wood shavings. Water and food pellets were available *ad libitum*.

In paper I, we used outbred adult male Sprague-Dawley rats (Möllegård, Denmark). All rats weighed 200-230 g at the beginning of the experiments. In paper II, III and IV we used inbred mouse strains from the Jackson Laboratory (Bar Harbor, USA). The animals were young adult males (8-19 weeks for Study II and III, and 10-20 weeks for Study IV) of the strains AKR, CBA, C3H, B6 and C58.

3.2 SURGICAL PROCEDURES

3.2.1 Anesthesia

In all papers chloral hydrate at a dose of 350 mg/kg intraperitoneally (i.p.) was used to anesthetize the animals. Before making any surgical incisions, proper anesthesia was verified by pinching the tail of the animals. If needed, an additional dose of chloral hydrate was injected i.p.

3.2.2 Photochemically induced ischemic injury

A 1 cm long incision was made in the *regio masseterica* and the IoN was separated from the surrounding tissue. Aluminium foil was placed under the nerve to isolate the surrounding tissue and to reflect light. Paraffin oil was applied on the nerve to prevent it from drying out during irradiation. The exposed nerve was irradiated with an argon ion laser beam of a 514 nm wavelength and an average power of 0.17 W (Innova Model 70, Coherent Laser Products Division, Palo Alto, CA, USA). Just before irradiation, erythrosin B (Red No. 3, Aldrich, 32.5 mg/kg dissolved in 0.9% saline) was injected intravenous (i.v.) through the tail vein. *(Paper I)*

3.2.3 SNL model

On the left side, an incision was made through the skin and the paraspinal muscles were separated from the spinous processes at the L5-L6 levels. The L6 transverse process was removed and the L5 spinal nerve was exposed and transected distal to the ganglion. A segment of 3-4 mm was removed from the distal nerve stump to inhibit regeneration. A small amount of carbon powder was applied on the proximal cut nerve end to facilitate identification of the corresponding DRG at post mortem dissection. The skin was closed in layers using stainless steel clips (Stoelting, Wood Dale, Illinois, US). *(Paper II and III)*

3.2.4 Sciatic and saphenous nerve transection

The left sciatic nerve was exposed at mid-thigh, ligated with 6-0 silk and transected approximately 1 mm distal to the ligation. A few millimeters of the distal nerve stump was removed to avoid regeneration. Subsequently, the left saphenous nerve was exposed near the knee and similarly ligated and transected. The skin was closed in layers using stainless steel clips (Stoelting, Wood Dale, IL, US). *(Paper IV)*

3.3 PAIN BEHAVIOR ASSESSMENT

3.3.1 Mechanical hypersensitivity

On the face

Mechanical hypersensitivity of the face was determined using a graded series of von Frey filaments which produced bending forces of 0.60, 0.91, 1.90, 3.47, 5.82, 7.95, 11.1, 18.4 and 40.14 g. During testing, the rat was gently held by the experimenter and the von Frey filaments were applied in ascending order within the IoN territory on the hairy skin of the vibrissal pad. First the nerve-injured, and then the contralateral side, were stimulated with each filament four times at 1 s intervals. The response threshold was taken as the force at which the rat presented any of the following aversive responses: withdrawal, struggle/escape or attack (Vos et al., 1994). The cut-off value was set to 40.14 g.

The rats were handled and habituated to the testing procedure and then tested once a day for 3 days. The median value from these 3 test occasions was taken as the pre-injury withdrawal threshold. Nerve injured and sham operated rats were tested on postoperative days 3, 7, 11, 14 and then once per week. *(Paper I)*

On the body

The mechanical hypersensitivity beyond the facial region was assessed by examining the vocalization thresholds to graded mechanical touch/pressure applied with von Frey hairs. During testing, the rats were gently restrained in a standing position and the von Frey hair was pushed onto the skin until the filament became bent. The frequency of the stimulation was about 1/s and for each intensity 5-10 stimuli were applied. The intensity of stimulation which induced consistent vocalization (>75% response rate) was considered as the pain threshold. *(Paper I)*

3.3.2 Heat hypersensitivity

The response to heat was tested with Hargreaves' test using a radiant heat source (IITC, Woodland Hills, CA, USA). The hairy skin of the lateral snout (the vibrissal pad) was stimulated. Rat head withdrawal latencies were automatically provided by the testing apparatus. The intensity of stimulation was adjusted so that the baseline withdrawal latency was 4–6 s.

After 3 days of handling and habituation to the testing procedure, withdrawal latencies were measured twice on each side with 15-min intervals between tests, and the mean value was taken as the pre-injury withdrawal response. After the nerve injury, response to heat was tested at the same days as the response to mechanical stimulation. *(Paper I)*

3.3.3 Spontaneous pain

Signs of spontaneous pain were assessed by autotomy scoring. The scoring was made according to the protocol of Wall and colleagues (Wall et al., 1979). Briefly, one point was given for loss of one or more toe nails with an additional point for injury to the proximal or distal half of each digit for a total possible score of 11. Scoring was made weekly for 5 weeks post op or at a single time point 7 days post op in a group of animals processed for mRNA analysis. *(Paper IV)*

3.4 IN SITU HYBRIDIZATION

3.4.1 Tissue preparation

Deeply anaesthetized rats of mice were perfused transcardially with Tyrode's solution. The tissue to be examined (DRG, TG or spinal cord) was dissected out, snap frozen on dry ice and kept at –70^{o}C. It was cryosectioned at 12 or 14 µm and thaw-mounted onto Super Frost/Plus slides (Menzel GmbH & co KG, Braunschweig, Germany). *(Paper I, II, III and IV)*

When comparing the expression of certain genes in different strains it is of particular importance to use an analysis model where small differences can be detected. To do so, confounding factors must be avoided as much as possible. We therefore aimed to process tissue from the 5 mouse strains simultaneously. Thus, tissue from all strains were embedded together in blocks formed using OCT compound (Tissue-Tek™, Sakura, Zoeterwounde, NL) and sectioned simultaneously (Fig. 3). *(Paper II and III)*

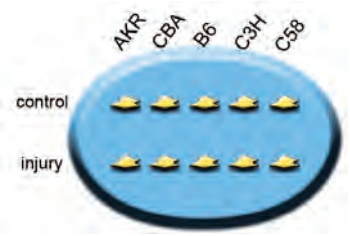

Figure 3. For simultaneous processing, control and affected DRG from the five mouse strains were embedded together in single blocks as shown in this schematic picture.

3.4.2 Hybridization protocol

For a detailed description of the hybridization protocol, see Material and Method sections for individual papers. *(Paper I, II, III, IV)*

Briefly, oligonucleotide probes were designed using the Software program Oligo 6.0. The lengths of the probes were between 42 and 52 bases. Specific criteria were used to select appropriate probe sequences: a GC content between 50-60 %, no loops or dimerizations stronger than 10 kcal/mol. All probes were synthesized by CyberGene, (Huddinge, Sweden). Probes were 3'-end labelled with (^{33}P)-dATP using terminal deoxyribonucleotidyl transferase (TdT). Tissue sections were hybridized with the labelled probe for 16-20 hours at 42 °C in a humidified chamber. Slides were then rinsed for 5× 15 minutes at 60 °C in 1× standard saline citrate (SSC) and the last rinse was allowed to reach room temperature. Slides were dipped in distilled water, dehydrated through graded series of ethanol (70%, 90% and 99.5%), air-dried, dipped in photographic emulsion (Kodak NTB2, diluted 1:1 in distilled water) and exposed at 4 °C for 2-4 weeks. Subsequently, the slides were developed, fixed and mounted.

3.4.3 Computerized digital analysis

For a detailed description of the quantification techniques, see Material and Method sections for individual papers. *(Paper I, II, III)*

Briefly, we used a Nikon E600 microscope equipped with a darkfield condenser and a Nikon DXM 1200 digital camera. Images were captured under darkfield and brightfield illumination to facilitate identification of cell borders. Using the Easy Image 3000 software (EI3000, Tekno Optik AB, Huddinge, Sweden) the darkfield image was thresholded based on pixel intensity. The mask thus created was overlaid on the brightfield image. The circumference of neuronal profiles in the field of view was outlined with an on-screen cursor and EI3000 extracted the area. The intensity of cellular mRNA expression was calculated as the ratio between the intensity in neuronal cell somata and the intensity in background, i.e. signal/noise (S/N) ratio.

3.5 IMMUNOHISTOCHEMISTRY

The distribution of damaged neurons within the TG was determined with immunolabelling of activating transcription factor 3 protein (ATF-3), a member of the ATF/CREB family of transcription factors and a widely used marker of injured primary sensory neurons (Tsuzuki et al., 2001). TG were removed as for in situ hybridization. The sections were post-fixed in 4% paraformaldehyde and then incubated with an anti-ATF-3 polyclonal antibody (Santa Cruz Biotechnology, CA, USA, 1:200). After rinsing, the sections were incubated with peroxidase-conjugated goat anti-rabbit antiserum (1:200; DAKO, Denmark) and subsequently reacted with diaminobenzidine tetrahydrochloride (DAB, DAKO, Denmark). Countings of the percentage of ATF-3-immunoreactive neurons were performed directly in the microscope. *(Paper I)*

3.6 REAL-TIME SYBR-GREEN PCR

3.6.1 RNA extraction and cDNA synthesis

The animals were deeply anesthetized and perfused transcardially with Tyrode's solution. Tissue was taken out similar as prior to in situ hybridization. DRG and segments of spinal cords were mechanically homogenized using the FastPrep system (Qbiogene, Irvine, CA; 4 m/sec speed in 30 seconds). In order to obtain total RNA the homogenate was subsequently processed using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Treatment with RNase-Free DNAse (Qiagen) was included in the procedure to promote degradation of genomic DNA. Total RNA was eluted in 35µl RNase free water and the samples were further processed by reverse transcription to acquire cDNA; using 10µl of total RNA, Random Hexamer Primers (Invitrogen, Carlsbad, CA), nucleotides (GE Healthcare) and Superscript Reverse Transcriptase (Invitrogen). *(Paper IV)*

3.6.2 PCR protocol

Real-time PCR (RT-PCR) amplification was performed on an iQ™5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with a three step PCR protocol. iQ™ SYBR® Green Supermix (Bio-Rad) was used in the reactions in combination with water, cDNA (2µl/well), and the specific primers (5µM). All primers were designed with the Primer Express software (Perkin Elmer, Waltham, MA). PCR products were examined on Ethidium Bromide gels in order to verify that they generated a single band of the expected size. In addition, primer specificity was also assessed by analyzing melting curves in each sample. All samples were analyzed in duplicates and a reference sample was included in order to perform inter-run calibration between different PCR plates containing the same target. For each target gene, relative amounts of transcript levels were calculated using the normalized expression method implemented in the iQ5 optical system software (Bio-Rad) with the value 1 representing the highest expression. The final values were subsequently expressed as ratios between the relative amount of the specific target and of the two corresponding endogenous controls Gapdh and Hprt. *(Paper IV)*

3.7 TAQMAN AND MICROARRAY ASSAYS

3.7.1 RNA extraction and cDNA synthesis

Mice were sacrificed by CO_2 inhalation and the lumbar spine was divided longitudinally. Identification of the L5 spinal nerve and DRG was confirmed by dissection proximally along the sciatic nerve. L4 and L5 DRG were removed into PBS, frozen on dry-ice and stored at -70°C. After DRG from all groups were collected, total RNA was isolated from the individual ganglia using the PicoPure RNA Isolation Kit following the manufacturer's protocol (Arcturus Bioscience Inc, Mountain View, CA, USA). Average RNA yield was ~500 ng/DRG. The high quality of the RNA extracted was verified using the Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA). cDNA synthesis was performed using the Applera Ltd. (Norwalk, CT, USA) Reverse Transcriptase Kit (N8080234) and RNase Inhibitor (N8080119). cDNA synthesis was performed from 90 ng RNA samples using 2.2 µL 10× RT buffer, 4.84 µL MgCl2 (25 mM), 4.4 µL dNTPs (10 mM), 1.1 µL random hexamers (50 µM), 1.1 µL oligo(dT)16 (50 µM), 0.44 µL RNase Inhibitor (20 U/µL), 0.55 µL Multiscribe (50 U/µL) in a total volume of 22 µL (final cDNA concentration: 4 ng/µl). Samples were incubated at 25°C for 10 minutes and 42°C for 60 minutes. The reaction was stopped by heating to 95°C for 5 minutes. *(Paper II)*

3.7.2 TaqMan analysis

TaqMan reactions were performed using Applera TaqMan Universal PCR Master Mix (4305719) and TaqMan Rodent GAPDH Control Reagents (4308313). Oligonucleotides were synthesized by Operon Biotechnologies GmbH (Köln, Germany). PCR was performed using the ABI Prism 7900 (Applera) under the following PCR conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles with 95°C for 15 s and 1 minute at 60°C. PCR was set up as a multiplex PCR using 0.125 μL target probe (50 μM), 0.45 μL target forward primer (50 μM), 0.45 μL target reverse primer (50 μM), 12.5 μL TaqMan 2× PCR Master Mix, 0.25 μL each of primers and probes (TaqMan Rodent GAPDH Control Reagents), 2.5 μL or 1.25 μl cDNA sample in a total reaction volume of 25 μl. *(Paper II)*

3.7.3 Microarray expression profiling

First-strand cDNA synthesis was performed using 500 ng total RNA with a 100 pM T7-(dT)24oligomer (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGG CGG -dT24) according to Baugh et al., (2001), and SuperScript II reverse transcriptase following the manufacturer's instructions. Double-stranded cDNA was synthesized and then extracted using phenol-chloroform followed by an ethanol precipitation step. An in vitro transcription reaction was performed with the double stranded cDNA sample using the BioArray High Yield RNA Transcription Labeling kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Transcription reactions were incubated at 37°C for 16 h. cRNA was purified using the RNeasy Mini kit protocol for RNA cleanup (Qiagen GmbH, Hilden, Germany) and quantified spectrophotometrically. Mice were excluded if < 12 μg of cRNA was obtained. The biotin-labeled cRNA was fragmented using a RNA fragmentation buffer (200 mM Tris-acetate, 500 mM KOAc, 150 mM MgOAc, pH 8.1). Hybridization and staining on mouse MG430_2 GeneChipsTM (Affymetrix Inc., Santa Clara, CA, USA) was performed according to the manufacturer's instructions. The microarrays were scanned using a GeneChip 3000 Scanner. The scanned data were analyzed using Resolver v5.1 expression data analysis software (Rosetta Biosoftware, Seattle, WA, USA). *(Paper II)*

3.8 CORRELATION ANALYSIS

In papers II and IV, the contribution of individual genes to variability in pain phenotype among inbred mouse strains was evaluated. This was done using the approach of correlational analysis. Strain data on gene expression (in form change in percentage of expressing neurons after nerve injury, mRNA levels etc.) were plotted against strain

pain behavioral data. Parametric correlation analysis, using actual values, yielded a Pearson correlation coefficient (r_P). Non-parametric correlation analysis, with data converted to strain ranks (1-5) yielded a Spearman correlation coefficient (r_S). The closer the r-value gets to 1, the stronger the association of the two parameters. r=0 indicates complete lack of association.

3.9 STATISTICAL ANALYSIS

Different statistical methods were used in the four papers. In all analyses involving Mann-Whitney U-test (also called the Wilcoxon Signed ranks test), Student's unpaired T-test, analysis of variance (ANOVA), Kruskal Wallis test and correlation analysis, $p<0.05$ was considered significant unless stated otherwise.

Paper I

Data on mechanical stimulation were analyzed with the Wilcoxon Signed ranks test or Mann-Whitney U-test. The data of thermal stimulation were analyzed with ANOVA with repeated measures followed by Fisher's protected least significant difference (PLSD) test. Differences in ratios for $Na_v1.8$, $Na_v1.9$ and β3 between the irradiation group and the sham group were analyzed using Student's unpaired T-test, $p<0.01$ was considered significant.

Paper II

Differences in the percentages of labeled neuronal profiles in control and affected DRG were analyzed using Mann-Whitney U-test. Inter-strain differences in the percentage of labeled neuronal profiles were analyzed using one-way ANOVA.

In the microarray study, the criterion for significant expression of a gene was a p-value < 0.001 for signal intensity vs. noise level in at least 5 L5 DRG, considering all 5 mouse strains together. For each of the five strains, L5 SNL-induced change in expression for each gene (fold up- or down-regulated) in the L5 and the L4 DRG was calculated. The log of the ratio of SNL vs. sham expression, based on intensity values merged over all animals in each strain, was implemented by the corresponding algorithm in Resolver v5.1. Transcripts with fold regulation ≥ 1.5 in at least three strains, or ≥ 2 in at least one strain were included in the correlation analysis. Both r_P and r_S were calculated and evaluated using false discovery rates (FDR) analysis yielding a q-value. $q<0.05$ was considered significant.

Paper III

Multivariate analysis was performed using factor analysis with the extraction method principle component analysis with Varimax rotation.

Paper IV

Inter-strain differences in the 5 weeks post op-autotomy assay were analyzed using ANOVA with repeated measurements, based on the area under the curve. Rank data from the animals assessed 7 days post op was analyzed using the Kruskal Wallis test. Change in mRNA expression levels after injury were analyzed by two-way ANOVA followed by Newman Keuls test for all strains ($p<0.05$ was considered significant). Inter-strain differences in mRNA expression levels were analyzed using one-way ANOVA followed by Newman Keuls test for all strains ($p<0.05$ was considered significant).

4 RESULTS AND DISCUSSION

4.1 A NOVEL MODEL FOR TRIGEMINAL NEUROPATHIC PAIN (PAPER I)

Trigeminal neuropathic pain can be caused by surgery or other types of trauma to branches of the trigeminal nerve (Lewis et al., 2007). Though this condition is often extremely painful, relatively few studies have reported data from experimental pain models in this region. In paper I, we presented a novel trigeminal neuropathic pain model in rat. This model involved an ischemic injury to the IoN, produced by argon ion laser irradiation to the nerve, and a subsequent injection of a photosensitizing dye, erythrosin B. A similarly nerve injury was previously applied to the sciatic nerve of rats and mice (Hao et al., 2000; Kupers et al., 1998).

4.1.1 Morphological changes of the IoN after injury

Qualitative morphological examinations of transverse sections of the control IoN revealed normally looking perineurium-covered fascicles of various sizes as well as epineurial blood vessels and connective tissue (Fig. 4). However, 1.5 minutes of irradiation caused obvious signs of injury to the axons. In particular, myelinated nerve fibers in various stages of degeneration and Schwann cell proliferation in the form of Bands of Büngner were seen. There was also an extensive occlusion of blood vessels with deformed blood cells, resembling previous observations made in the rat sciatic nerve after a similar type of nerve injury (Kupers et al., 1998; Yu et al., 2000). The extent of injury was clearly dependent on the time of irradiation. At 6 minutes of irradiation, there were many endoneurial macrophages with remnants of myelin in the cytoplasm, masses of proliferating Schwann cells and what appeared to be unmyelinated sprouts. Similar to the observations made after 1.5 minutes of irradiation, blood vessels were usually sealed with masses of erythrocytes and other blood cells (Fig. 4).

Nerve damage was also reflected by a substantially induced expression of the nuclear injury marker ATF-3 in animals subjected to laser irradiation. This expression was restricted to neuronal cell nuclei in the maxillary region of the TG.

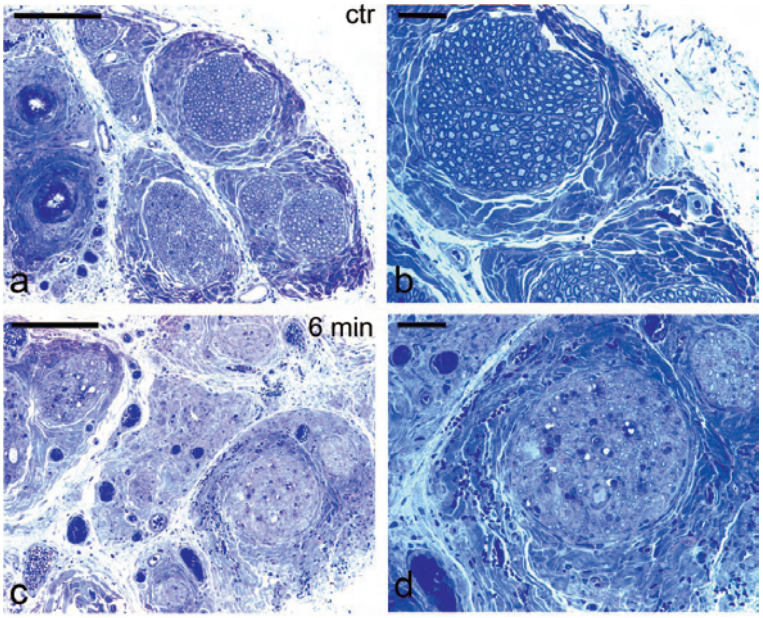

Figure 4. Semithin light microscopical sections of the IoN showing perineurium-covered fascicles of the IoN, blood vessels and connective tissue. (a and b), Control nerves displayed no substantial abnormalities. (c and d), 6 minutes of irradiation had marked effects on the nerve. Masses of proliferating Schwann cells, unmyelinated sprouts and small regenerating nerve fibers with thin myelin sheaths can be visualized. Blood vessels are sealed by masses of blood cells. Scale bar in a and c: 200 µm. Scale bar in b and d: 50 µm.

4.1.2 Pain behavior

The rats did not show any general behavioral abnormalities or stress reactions after injury. They also gained weight normally. In about 50 % of the animals however, we observed signs of increased grooming in the form of hair loss and/or facial injury on the ipsilateral IoN innervated territory. This likely reflects the reaction to some form of spontaneous sensations, possibly ongoing pain, within this region.

Mechanical hypersensitivity

Mechanical hypersensitivity on the ipsilateral side of the face was observed in rats irradiated for 3, 4.5 and 6 minutes, lasting for about 4-6 weeks. Notably, a shorter (1.5 minutes) or a longer (10 minutes) time of irradiation failed to induce mechanical hypersensitivity. A similar, bell-shaped relationship between duration of irradiation and effect on pain behavior was previously reported in mice and rats after sciatic nerve irradiation (Hao et al., 2000; Yu et al., 2000). Based on electron microscopic analysis, a

potential explanation for this pattern of duration-dependency was previously presented (and is applicable also to the data obtained here): The development of neuropathic pain behavior in this ischemic nerve injury model may require injury to unmyelinated C-fibers, which are relatively resistant to ischemic injury (Yu et al., 2000). Thus, 1.5 minutes of irradiation may not be sufficient to damage enough C-fibers to induce pain behavior. The lack of mechanical hypersensitivity in rats irradiated for 10 minutes may, in turn, be explained by lack of myelinated afferent input due to the high grade of injury.

The irradiation of the left IoN also had behavioral effects on the contralateral side of the face. Bilateral effects on pain behavior after ipsilateral injury to a peripheral nerve have been reported previously in rodent models of hindlimb nerve, IoN injury and inflammation (Benoliel et al., 2002; Hao et al., 2000; Koltzenburg et al., 1999; Seltzer et al., 1990; Vos et al., 1994). Although a well-known phenomenon, the mechanisms behind these bilateral effects are not yet established. There are, however, a number of proposed mechanisms, such as possible anatomical connections between the two sides of the spinal cord and the brain stem, a supraspinal loop involving bilateral descending facilitation as well as possible bilateral glial cell activation (Koltzenburg et al., 1999; Milligan et al., 2003; Porreca et al., 2002). Interestingly, in rat, the bilateral effects seem to be more consistent after IoN nerve injury than after injury to the sciatic nerve. This may be related to the well-documented projection to contralateral medullary dorsal horn by trigeminal primary afferents (Ellrich and Messlinger, 1999; Jacquin et al., 1990).

The rats also exhibited a reduced vocalization threshold when mechanically stimulated on the neck and upper back region. This increased mechanical hypersensitivity was limited to areas adjacent to the face. Previously, similar conditions of spread hypersensitivity have been attributed to a generalized reaction of stress (Vos et al., 1994). However, the fact that hypersensitivity is restricted to areas bordering to IoN innervated regions, as observed here, implies that it is indeed due to a somatosensory change in the respective areas.

Heat hypersensitivity

In contrast to the marked mechanical hypersensitivity, ischemic injury to the IoN did not induce consistent thermal hypersensitivity in the IoN-innervated facial region. There is not much literature available from other IoN injury models for comparison, since most previous studies only provide data on mechanical hypersensitivity. A few studies however, demonstrate robust heat hypersensitivity in rats subjected to CCI or

partial ligation of the IoN (Imamura et al., 1997; Shinoda et al., 2007). The inconsistency in the development of heat hypersensitivity may be due to differences in degree of injury to myelinated versus unmyelinated afferents in response to ischemia.

4.1.3 Changes in VGSC expression

Until recent years, there was not much literature available on the expression of VGSC in trigeminal ganglion neurons and their potential role in pain conditions in the orofacial region. Almost all data on VGSC in injured nerves was collected from animal models involving L4-L6 neurons. However, in recent years, an increased interest in this field has resulted in a number of illuminating studies (Davies et al., 2006; Henry et al., 2007; Morgan and Gebhart, 2008). Nevertheless, there is still a great lack of understanding concerning the role of VGSC in orofacial pain states. Here, the TG mRNA expression of a subset of VGSC subtypes was analyzed after sham operation and 14 days post 6 minutes of IoN-irradiation. The channels examined were: the α-subtypes $Na_v1.3$, $Na_v1.8$ and $Na_v1.9$; the β-subtype β3 as well as the $Na_v1.8$-associated regulatory factor p11.

As expected, the TTX-s sodium channel $Na_v1.3$ was not expressed by TG neurons in sham operated rats. However, ischemic injury to the IoN induced an upregulation of this VGSC subunit. This is in line with previous observations in the DRG after sciatic injury (Black et al., 1999; Waxman et al., 1994). Judging from qualitative examinations in situ hybridization darkfield images, the expression of Nav1.3 appeared to be induced mainly in medium and large-sized neuronal profiles. The two TTX-r subunits $Na_v1.8$ and $Na_v1.9$ were markedly down-regulated at 14 days post op as measured by the percentage of mRNA expressing neuronal profiles in the TG. Both were preferentially expressed by small neurons, although also detectable in a subset of medium sized and larger neurons. Ischemic nerve injury did not change this pattern for neither of these channels. β3, which has been suggested to contribute to the generation of ectopic impulses in damaged DRG cells (Takahashi et al., 2003), was upregulated in the TG after injury. The regulatory factor p11 promotes translocation of $Na_v1.8$ to the plasma membrane and by this facilitates the surface expression of this channel (Okuse et al., 2002). The TG expression of p11 was unaffected by ischemic injury to the IoN. Thus, our data indicate that reorganization of $Na_v1.8$ after nerve injury is not the result of a decreased expression of p11.

Overall, our findings suggest that VGSC are expressed and regulated after nerve injury in the TG in a similar fashion as in the more extensively studied lumbar DRG region.

Thus, similar mechanisms likely underlie neuropathic pain states in the orofacial and lumbar/spinal region and similar targets should be addressed when developing pharmaceutical agents against these clinical conditions.

4.2 CORRELATION ANALYSIS – A TOOL FOR IDENTIFYING GENES THAT CONTRIBUTE TO NEUROPATHIC PAIN (PAPER II AND III)

Damage to the axon of a peripheral nerve has dramatic effects on gene expression in the DRG neurons. Many hundreds of genes have been found to be regulated after neuronal damage (Costigan et al., 2002; Diatchenko et al., 2007; Xiao et al., 2002). However, a crucial question still remains: which of these genes contribute to hyperexcitability and pain? Many related or unrelated processes are initiated shortly after axotomy, e.g. nerve regeneration, tissue repair, apoptosis and inflammation. The regulated genes could be involved in any of these processes and do not necessarily contribute to a hyperexcitable pain state. Thus, strategies are needed for identifying genes that are regulated in a way that directly affects pain susceptibility. In paper II and III, we presented such an approach by the use of inbred mouse strain with divergent pain phenotypes (Mogil et al., 1999a). Generally, strain gene expression data were plotted against strain pain behavior data after nerve injury. The calculated correlation coefficient indicates the strength of the correlation and thus the association of the two parameters. The aim was to identify genes for which regulation after nerve injury reflects nerve injury-induced pain behavior across strains.

The L5 spinal nerve was transected in mice from the strains AKR, B6, CBA, C3H and C58. At 3 days post op, the mRNA expression in the DRG was analyzed. First we performed a detailed quantitative morphological analysis of the expression of 6 selected pain candidate genes. We then performed an extensive genome-wide search for regulated genes that contribute to pain phenotypes based on the total level of DRG mRNA. Strain gene expression data was subsequently correlated to strain behavioral pain data in order to evaluate the contribution of separate genes to pain phenotype. Three different pain phenotypes were considered and data on these were previously published; spontaneous pain in the neuroma model (Minert et al., 2007), mechanical hypersensitivity (Mogil et al., 1999a) and heat hypersensitivity (Mogil et al., 1999a).

4.2.1 Morphological analysis of a set of pain candidate genes

The expressions of the VGSC $Na_v1.8$ and $Na_v1.9$, the ATP receptor P2X3 and the TRP channels TRPV1, TRPA1 and TRPM8 were analyzed on DRG sections using in situ hybridization. Our interest in these targets was due to previous data suggesting roles in pain transmission and physiological properties making them potential contributors to electrical signal transmission.

The VGSC α-subunits $Na_v1.8$ and $Na_v1.9$

In all strains, both $Na_v1.8$ and $Na_v1.9$ was expressed in about half of all neuronal profiles (mostly small- and medium-sized) in the normal DRG. Both channels were downregulated after injury (Table 1), with $Na_v1.9$ in a strain-dependent manner which correlated with spontaneous pain in the neuroma model (oneway ANOVA, $p<0.05$; $r_S = -0.90$, $p<0.05$). Thus, our findings suggest that $Na_v1.9$ contributes to strain variability in spontaneous pain behavior after nerve injury. Hence, potentially this channel is involved in the axotomy-induced spontaneous firing which is thought to underlie spontaneous neuropathic pain. The regulation of $Na_v1.8$ did not significantly correlate with any of the pain parameters.

Table 1. The direction of nerve injury-induced gene regulation calculated from the percentage of expressing neuronal profiles within the DRG. ↓ downregulated, ↑ upregulated, – unchanged.

	AKR	B6	CBA	C3H	C58
$Na_v1.8$	↓	↓	↓	↓	↓
$Na_v1.9$	↓	↓	↓	↓	↓
P2X3	–	–	–	↑	↓
TRPV1	–	↓	↑	–	–
TRPA1	↓	↓	↓	↓	↓
TRPM8	↓	↓	↓	↓	↓

The purinergic receptor P2X3

Throughout the strains, P2X3 was expressed in 33-45 % of all neuronal profiles in the normal DRG. The mRNA was predominantly present in small- and medium-sized neuronal profiles. As a neuronal receptor for ATP (which is released by damaged tissue at the site of nerve injury), P2X3 is an obvious study target in the context of nerve-induced pain. In the past, it has been disputed whether nerve injury actually causes an up- or down-regulation of this receptor (Bradbury et al., 1998; Eriksson et al., 1998). Some years ago, a putative explanation for these contradictory results was provided: P2X3 was reported to be down-regulated in axotomized neurons, but up-regulated in neighboring neurons with uninjured fibers (Tsuzuki et al., 2001). Using the SNL model, we examined a homogenous population of damaged DRG neurons and found that the expression levels of P2X3 were unaffected by injury in three out of five strains while regulated in opposite directions in the two remaining strains (Table 1). Hence, we suggest that genetic factors influence the impact of nerve injury on P2X3 expression.

However, there was no correlation between the axotomy-induced regulation of P2X3 and pain behavior.

The thermally activated TRP channels

TRP channels constitute a large group of ion channels with the shared characteristics that they are cation-specific channels that open in response to specific stimuli. Many of these channels are activated by several different types of stimuli; e. g. thermal, chemical and mechanical, and some of them have been specially implicated in pain for a number of reasons (Patapoutian et al., 2009). In paper II and III, we focused on three different TRP channels; TRPV1, which is the receptor for capsaicin and heat, TRPA1, which is activated by noxious cold and various chemical irritants and TRPM8, the receptor for innocuous cool temperatures and menthol.

16-21 % of the neuronal profiles in the normal DRG expressed TRPV1 among the strains. In all strains, the majority of the TRPV1 positive profiles were small. Similar to P2X3, nerve injury altered the expression of TRPV1 in a strain dependent way. In three strains, the expression levels were unchanged. However, the proportion of TRPV1 positive neurons was increased in CBA and decreased in B6. Interestingly, the increased proportion of TRPV1-positive neuronal profiles in CBA seemingly occurred through the addition of a subpopulation of larger neurons, which normally do not express TRPV1. However, the SNL-induced gene regulation did not correlate to pain phenotype.

TRPA1 was expressed in about 20 % of all neuronal profiles in all strains which was similar to previous data from immunohistochemistry on rat DRG (Bautista et al., 2005). SNL caused a striking decrease in TRPA1 positive profiles resulting in almost undetectable percentages. The regulation of TRPA1 after injury did not correlate with pain phenotype.

TRPM8 was expressed in slightly varying proportions in normal animals, within the range 7-10 % of all neuronal profiles. As with TRPA1, TRPM8 was strongly down-regulated after injury. The across-strain magnitude of down-regulation was significantly correlated with heat hypersensitivity (r_S = -0.98, $p<0.01$). The greater the down-regulation of TRPM8, the less the heat hypersensitivity.

4.2.2 Genome-wide search for regulated genes that contribute to pain phenotypes

Regulation of DRG genes after SNL

A large number of genes were expressed in the normal DRG in all five strains. Averaging over the five mouse strains gave a total of 22,003 ± 1,784 genes in the L5 DRG and 22,555 ± 527 genes in the L4 DRG. Thus, there was no considerable difference between the number of genes expressed in these two ganglia in the normal state.

3 days after SNL, we found that on average 2,552 ± 477 genes were significantly regulated in the L5 DRG (including both up- and down-regulated genes). This represents about 12 % of all expressed genes in the DRG. Generally, it is a higher percentage than reported in prior studies with a similar study disposition (Costigan et al., 2002; Valder et al., 2003; Xiao et al., 2002). We believe that the relatively large number of regulated genes in our study is due to the proximal location of the lesion, and possible also to an improved protocol for detecting changes.

Interestingly, we found that the expression of genes in the L4 DRG was rather unaffected by the lesion of the L5 spinal nerve. Only an average of 24 ± 28 genes was regulated in the L4 DRG in the five mouse strains. This is a good verification that gene regulation in the L5 DRG is in fact related to axotomy and not “side effects” of the incision. The lack of impact on gene regulation in the L4 DRG is also interesting and somewhat surprising from another aspect. Based on functional studies, it has been suggested that abnormal activity in intact neurons adjacent to injured ones could contribute to mechanical hypersensitivity (Ali et al., 1999). Perhaps this abnormal activity is the result of changes in sensory signaling processes not related to gene regulation in the intact neurons.

Correlation with pain behavior

Transcripts with a fold change ≥ 1.5 in at least three strains, or ≥ 2 in at least one strain after SNL were further processed for correlation analysis with pain behavior. These criteria were met for 3,927 transcripts. 144 of these were significantly correlated ($p \leq 0.05$) with mechanical hypersensitivity, 141 with heat hypersensitivity and 45 with spontaneous pain behavior in the neuroma model (autotomy). There was a little overlap among the groups of genes significantly correlated to the three pain assays (Fig. 5). This could indicate that diverse mechanisms underlie these three types of nerve-injury

induced pain behavior. Gene ontology analysis showed that pain-correlated genes fell into large categories roughly in proportion to their representation in the whole genome. However, in particular for genes correlated with mechanical hypersensitivity, there was a significant enrichment in categories related to immune and inflammatory processes and the regulation of cellular metabolism.

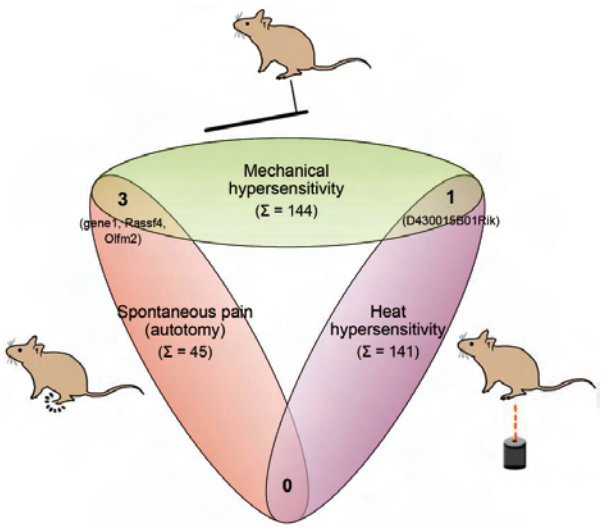

Figure 5. The number of genes for which the regulation in the L5 DRG at 3 days after L5 SNL was correlated across strains with each of the three pain phenotypes investigated. There was very little overlap in genes that correlated with the different pain phenotypes. Numbers in the center of each ellipse refer to genes significantly correlated to the pain phenotype indicated, mechanical hypersensitivity, heat hypersensitivity or spontaneous pain in the neuroma model. Numbers in the overlapping regions at the ends of each ellipse refer to genes shared by the two phenotypes, with gene names provided.

To summarize, our strategy of using correlation analysis to identify DRG genes whose regulation may contribute to neuropathic pain, yielded three lists that are enriched in genes with a potential impact on neuropathic pain. One could argue that we should have been more stringent when deciding criteria for a significant correlation. This would yield a shorter list of possible candidate genes to choose from for further studies. We believe however, that the risk of eliminating functionally significant transcripts exceeds the benefits with such a restriction. Even though the lists may contain too many genes to randomly pick candidate genes for further studies from, they may serve as a look-up table for screening candidates derived from other pain studies.

4.3 NERVE TRANSECTION, PAIN BEHAVIOR AND VGSC EXPRESSION IN 5 INBRED MOUSE STRAINS (PAPER IV)

The TTX-r VGSC $Na_v1.8$ and $Na_v1.9$ have been associated with the development of neuropathic pain. Thus, they have been the study targets in a number of publications on nerve-injury induced pain. In paper II and III, the gene expressions of these channels as well as their relations to pain behavior after SNL were examined. In particular, $Na_v1.9$ was linked to the development of spontaneous pain in the neuroma model. Additional VGSC may however also be of importance for the establishment of a hyperexcitable neuron and increased pain sensitivity after nerve injury. In paper IV, we continued the examination of VGSC by studying the remaining TTX-s DRG sodium channels $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$, the TTX-r sodium channel $Na_v1.5$ and the auxiliary molecule Contactin. The mRNA levels of each of these sodium channels were assessed with quantitative RT-PCR in naive mice and 7 days after sciatic and saphenous nerve transection. Subsequently, correlational analysis, as presented in paper II, was used to examine their relation to nerve injury-induced pain.

4.3.1 Pain behavior as assessed by autotomy scoring

Since autotomy is a measure with late endpoint, one group of animals (with mice originating from the strains AKR, B6, CBA, C3H and C58), was scored weekly for 5 weeks after sciatic and saphenous nerve transection. Autotomy developed differently among the strains (ANOVA with repeated measures, $p<0.01$), with AKR showing least signs of autotomy followed by B6, C58, CBA and C3H in an ascending order. In a second group of animals, the autotomy was assessed at 7 days post op before the animals were sacrificed and the tissue dissected and further processed for RT-PCR or in situ hybridization. The strain rank order based on autotomy scoring in these animals equaled the rank order obtained from the animals assessed at 5 weeks post op. Overall, the autotomy strain ranks obtained in this study resembles those from a previous report on the same strains (Minert et al., 2007). The agreement between the independently performed autotomy assessments can be considered a good verification of robust, reproducible strain differences. Thus, it justifies the use of the currently reported rank order in the correlation analysis of pain behavior versus gene expression.

4.3.2 Nerve injury-induced changes in mRNA expression of VGSC and Contactin

In the DRG

Somewhat surprisingly, we found no consistent up-regulation of $Na_v1.3$ in the damaged DRG. There were increased mRNA levels in one strain only (CBA, $p<0.05$) while levels were unchanged in the other strains. $Na_v1.3$ is an embryonic channel, more or less absent in the adult nervous system, but has been reported to re-appear in injured neurons (Waxman et al., 1994). This fact, along with the biophysical properties of $Na_v1.3$, makes it a candidate for being responsible for the ectopic action potential generation occurring in injured afferent neurons (Boucher et al., 2000). However, since we found no consistent upregulation of $Na_v1.3$ following axotomy we suggest that this sodium channel does not have a crucial role in pain behavior in the neuroma model in mice.

$Na_v1.6$ on the other hand, was down-regulated in two (B6, $p<0.05$ and C3H, $p<0.01$) out of five strains. Further, the residual mRNA levels of $Na_v1.6$ correlated tightly with autotomy behavior ($r_P=0.89$, $p<0.05$). The most pain-prone strains had the highest mRNA levels after injury. This data suggests that $Na_v1.6$ may make a particular contribution to ectopic firing in axotomized neurons.

Contactin was consistently affected by nerve injury, since the mRNA was down-regulated in all strains (AKR, C58, B6, CBA, $p<0.05$ and C3H, $p<0.001$). A suggestive correlation was seen between the mRNA levels of Contactin at 7 days post op and pain behavior ($r_P=0.80$, $p=0.20$). Although not statistically significant, this tendency implies a putative link between Contactin and the development of ectopic discharge and pain signalling in damaged sensory neurons. Presumably, this link would involve the mechanism of trafficking of VGSC to the cell membrane.

Residual levels of the remaining sodium channels did not correlate with pain behavior.

In the spinal cord

In the spinal cord, we found increased levels of $Na_v1.7$ mRNA in all analyzed strains ($p<0.001$, C58 was excluded due to an insufficient number of animals). On the in situ hybridization sections we could visualize an increased mRNA signal ipsilateral to the nerve injury in a subset of large neurons (presumably motor neurons) in the ventral horn (Fig. 6). Likely, the addition of $Na_v1.7$ expressing neurons detected with in situ

hybridization reflects the increased mRNA levels detected with RT-PCR. Interestingly, the spinal cord mRNA levels of $Na_v1.7$ after injury were also strongly correlated with pain behavior (r_P=0.91, $p<0.05$). However, since the mRNA increase appeared to occur in motor neurons, the coupling to pain, if any, is uncertain and needs to be further investigated.

None of the remaining sodium channels or Contactin were regulated by axotomy in the spinal cord. Nor were the mRNA levels after injury of any of them correlated with pain behavior.

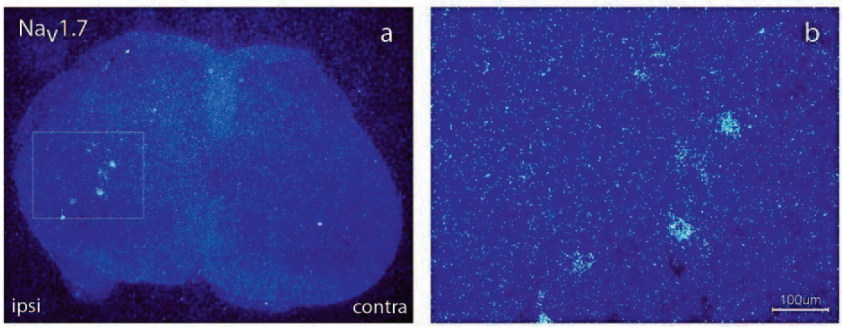

Figure 6. Dark-field micrographs from the affected segment of the spinal cord (within L4-L5) at 7 days post op. a) shows localization of $Na_v1.7$ mRNA to the ipsilateral side and b) the $Na_v1.7$-expressing neurons in the ventral horn at a higher magnification. Micrographs are from the CBA strain.

5 FINAL CONCLUSIONS

- A novel experimental rat model for trigeminal neuropathic pain was established by photochemically inducing an ischemic nerve injury to the IoN. The model caused clear morphological signs of nerve injury as well as pain behavior in the rats. The extent of morphological changes and pain behavior could be monitored by varying the time of laser irradiation to the nerve.

- Ischemic injury to the IoN induced robust changes in the mRNA expression of specific nociceptive neuron-associated VGSC subunits in the TG. The changes in mRNA expression of these VGSC resemble the changes occurring in the DRG following sciatic nerve injury. This suggests that spinal and craniofacial neuropathic pain may have similar underlying mechanisms.

- Following sciatic and saphenous nerve injury, different levels of spontaneous pain was seen among five inbred mouse strains. Post-injury levels of $Na_v1.6$ mRNA in the DRG were correlated with strain ranks for spontaneous pain. mRNA expression of $Na_v1.7$ was induced in the ventral horn of the spinal cord in what appeared to be motor neurons. The post-injury mRNA levels of this channel in the spinal cord were tightly correlated with spontaneous pain data, but the significance of this correlation in the context of neuropathic pain remains uncertain.

- The degree of down-regulation of $Na_v1.9$ in DRG after nerve injury was associated with spontaneous pain. The regulation of the TRP channel TRPM8 was associated with heat hypersensitivity following SNL.

- A large number of genes (22,003 ± 1784) were expressed in the L5 DRG in the five mouse strains. L5 SNL caused a regulation of, on average, ~12 % of these genes. The effect on the adjacent L4 DRG was however minor, resulting in regulation of, on average, ~0.1 % of the normally expressed genes.

- Genes whose regulation in the L5 DRG that was significantly correlated with a distinct pain phenotype (mechanical hypersensitivity, heat sensitivity or spontaneous pain) among the five inbred mouse strains where identified.

The thus obtained gene lists may serve as “look-up tables” of genes whose regulation likely contributes to pain variability.

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