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PAIN-RELATED SODIUM CHANNELS IN SENSORY NEURONS: GENE EXPRESSION DURING DEVELOPMENT AND AFTER INJURY

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ABSTRACT

Pain that goes on over years as a result of nerve damage is an affliction which disables millions of people worldwide. Chronic injury-induced pain can become severely debilitating and extremely difficult to treat. The process of pain transmission in primary sensory neurons is extremely complex, and involves several sodium channels, which in many cases appear to be specific for pain processing. These channels could, if their roles were fully understood, become targets for new analgesics. Most work on pain mechanisms has been carried out in spinal nerve systems, and has provided great insight into the mechanisms of neuropathic pain. It is obvious that much of these data is relevant to studies of craniofacial pain. However, it is now clear that the pathophysiology of the craniofacial trigeminal nerve is in many ways different to that found in spinal nerves. Studies of trigeminal pain mechanisms require a thorough understanding of the regional neurobiological characteristics in combination with knowledge of the diverse and complex clinical problems which can arise. The aim of this thesis was to elucidate the regulation of pain-related sodium channels in developing and injured primary sensory neurons in spinal dorsal root ganglia as well as in trigeminal ganglia.

Investigation of the developing trigeminal ganglion using *in situ* hybridization showed that specific voltage gated sodium channels, some of which are specifically associated with pain-transmitting neurons, mature in waves that are particular for each channel. The results suggest that some craniofacial primary sensory neuron voltage gated sodium channels mature earlier than their counterparts at segmental levels during development. They also clearly indicate that different facial regions may differ in the ability to transmit sensory signals during early life.

The results also conclude that the down-regulation of the voltage gated sodium channel transcripts $Na_v1.8$ and $Na_v1.9$ mRNA that occurs in injured dorsal root ganglion cells is not influenced by interferon-γ. Thus, the reduced pain-related behavior seen in nerve-injured interferon-γ receptor knockout mice is not due to changes in the regulation of $Na_v1.8$ and $Na_v1.9$.

The evaluation of a photochemically-induced injury to the infraorbital nerve, a branch of the trigeminal nerve, revealed behavioral changes indicative of evoked and possible ongoing pain-like responses in the facial region, and a spread of mechanical hypersensitivity to the body. In the trigeminal ganglion, robust changes in the mRNA expression of voltage gated sodium channel transcripts were seen after the ischemic infraorbital nerve injury. It seems reasonable to assume that these changes are related to the development of pain-related behavior in the affected animals.

Using real-time PCR and *in situ* hybridization techniques, it was shown that the expression of specific voltage gated sodium channel isoforms in the dorsal root ganglion and the dorsal horn of the spinal cord correlate across mouse strains with pain behavior (autotomy) in the neuroma model of neuropathic pain. Nerve injury induced significantly altered levels of $Na_v1.3$, $Na_v1.5$ and $Na_v1.7$ in one strain only. However,

Contactin, a voltage gated sodium channel-associated protein, and $Na_v1.6$ were down-regulated in a majority of the strains to expression levels that tightly correlated to autotomy behavior. In the spinal cord, $Na_v1.7$ expression was strongly up-regulated following nerve injury to levels well correlated with pain-like behavior.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text with their Roman numerals:

I. Jonas Thun, Kaj Fried.
Differential expression of neuronal voltage-gated sodium channel mRNAs during the development of the rat trigeminal ganglion. Submitted.

II. Jonas Eriksson, Kaj Fried.
Expression of the sodium channel transcripts $Na_v1.8$ and $Na_v1.9$ in injured dorsal root ganglion neurons of interferon-gamma or interferon-gamma receptor deficient mice. Neuroscience Letters, 2003, 338, 242–246.

III. Jonas Eriksson, Aleksandra Jablonski, Anna-Karin Persson, Jing-Xia Hao, Poli Francois Kouya, Zsuzsanna Wiesenfeld-Hallin, Xiao-Jun Xu, Kaj Fried.
Behavioral changes and trigeminal ganglion sodium channel regulation in an orofacial neuropathic pain model. Pain, 2005,119, 82–94.

IV. Anna-Karin Persson, Jonas Thun, Xiao-Jun Xu, Zsuzsanna Wiesenfeld-Hallin, Marshall Devor, Olle Lidman, Kaj Fried.
Pain behavior induced by nerve injury correlates with the expression of specific sodium channels in mouse strains. Submitted.

CONTENTS

ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CCI	chronic constriction injury
CGRP	Calcitonin gene-related peptide
dpo	days post operation
DRG	dorsal root ganglion
GDNF	glial cell line-derived neurotrophic factor
GFRα1	GDNF Family Receptor α1
GFRα2	GDNF Family Receptor α2
IASP	International Association for the Study of Pain
IB4	isolectin B4
IB4-	primary sensory neurons that does not bind IB4
IB4+	primary sensory neurons that bind IB4
IFN-γ	interferon-γ
IoN	infra orbital nerve
KO	knock out
mRNA	messenger RNA
NCC	neural crest cells
NGF	nerve growth factor
NT3	neurotrophin-3
NT4/5	neurotrophin-4/5
SNL	spinal nerve ligation
SP	substance-P
TG	trigeminal ganglion
TNFα	tumor necrosis factor-α
TNFαR1	tumor necrosis factor-α receptor 1
TrkA	tropomyosin receptor kinase A
TrkB	tropomyosin receptor kinase B
TrkC	tropomyosin receptor kinase C
TTX	tetrodotoxin

INTRODUCTION

1. Pain: a definition

Pain is defined by IASP as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Clinical pain can be divided into nociceptive, neuropathic, psychological and idiopathic pain. Pain is experienced when activity from nociceptive thinly myelinated (Aδ) or C-fibers reaches the conscious brain. In normal physiological conditions, pain is classified as nociceptive by the activation of high threshold receptors expressed in the nerve terminals by tissue damage followed by the generation of action potentials and pain experience. Psychological pain is attributed to a psychological cause, e.g. depression with somatization, and idiopathic pain is pain without known cause.

Pain serves as an important warning system which emerges during early development. If a nerve injury occurs, however, this system is disrupted. Spontaneous activity develops in the nociceptive system, and this may lead to an ongoing pathological pain which serves no practical purpose. Pathological pain, also termed chronic pain or neuropathic pain is an affliction which disables millions of people worldwide. Chronic injury-induced pain can become severely debilitating and extremely difficult to treat.

2. Transmittors of pain impulses: Primary sensory neurons of Dorsal Root and Trigeminal ganglia

Primary sensory neurons are located in dorsal root ganglia (DRG) at spinal segmental levels, and trigeminal (TG) and glossopharyngeal ganglia at cranial levels. DRG pairs are associated with each vertebrae and serve distinctive body dermatomes, while the TG, which subserves sensory functions from most of the craniofacial region, is situated on the cerebral surface of the petrous portion of the temporal bone just lateral to pons and the internal carotid artery (Brodal, 1981). The central branches of the TG primary sensory neurons enter at the level of pons and project to the principal sensory and spinal trigeminal nucleus. From these nuclei, impulses are conveyed to thalamus, and further towards the sensory cortex (Dodd and Kelly, 1991). TG neurons convey information through three main divisions, the ophthalmic, the maxillary and mandibular divisions.

2.1. Primary sensory neurons: different types, different functions

Primary sensory neuron can be divided into a number of subgroups based on their anatomical, histochemical and physiological properties (Lawson et al., 1993). Medium- and large sized neurons can be distinguished by the binding of the monoclonal anti-neurofilament antibody, RT97. They make up about 40% of the total neurons in the DRG. They have myelinated axons with high conduction velocities, are mechanosensitive and respond principally to low-threshold stimuli (Lawson and Waddell, 1991). Another group, making up an additional 40 % of the total neurons in the DRG, is characterized by expression of neuropeptides such as Substance P and CGRP. Most of them have smaller cell bodies and possess unmyelinated C-fibers with slow conductance velocities. They are associated with high threshold polymodal nociceptors and are thus involved in nociception and thermal signaling. Within this peptidergic group, a portion of neurons with medium-sized cell bodies and thinly myelinated axons are seen. Most of these cells are nociceptors of the high-threshold mechanoreceptor type (McCarthy and Lawson, 1990; Lawson et al., 1996). The final class of neurons, comprising about 20% of DRG cells, can be identified by their binding of the lectin *Griffonia simplicifolia* IB-4 (Streit et al., 1986). They have unmyelinated fibers and are involved in nociception (Silverman and Kruger, 1990; Alvarez et al., 1991).

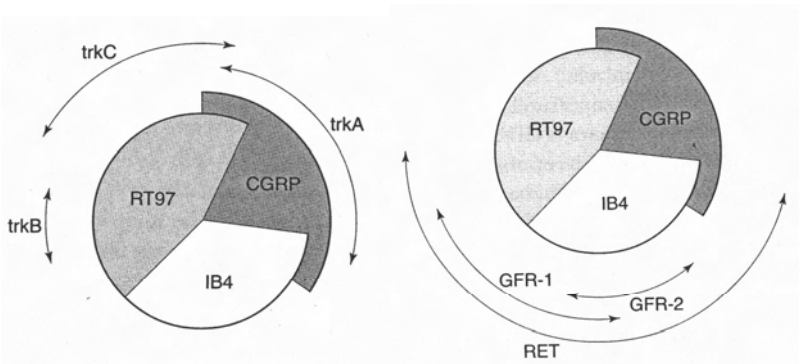


Fig. 1. Dependence on different neurotrophic factors and expression of cognate receptors for different groups of primary sensory neurons (From McMahon and Bennet, 1999).

The different subpopulations of primary sensory neurons are regulated by different neurotrophic factors. Consequently, each subpopulation expresses particular neurotrophic

factor receptors. The larger cells, which are RT97-positive, are regulated preferentially by Brain-Derived Neurotrophic factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4/5), and thus express the BDNF- and NT-4 receptor trkB and the NT-3-receptor trkC. CGRP-containing neurons are regulated by Nerve Growth Factor (NGF) and usually express the high-affinity trkA receptor (McMahon et al., 1994), while IB-4-binding neurons are regulated by Glial Cell line-Derived Neurotrophic Factor (GDNF) and express its receptors Ret, GFRα1 and GFRα2 (Bennet et al., 1996; Bennett et al., 1998). However, there is some overlap between the three groups (fig. 1) (for review see McMahon and Bennett, 1999).

2.2. Sensory neurons in DRG and TG have partly different developmental origins

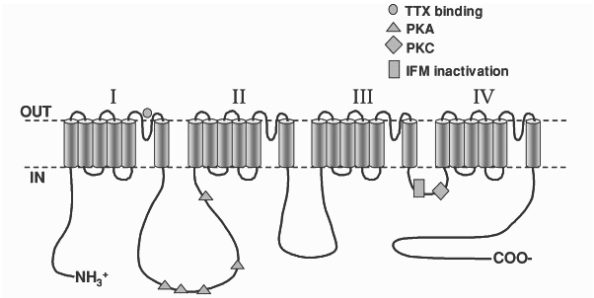
Sensory neurons of segmental DRG derive from neuroepithelial cells that form into neural crest cells (NCC). The NCC, which have stem cell-like properties, migrate from the neural tube to form the DRG (Kasemeier-Kulesa et al., 2005). Within the ganglion, under a general sensory neuron program directed by neurogenin genes, different subclasses of DRG neuronal progenitors leave the cell cycle to become primary sensory neurons at different developmental stages. Recent data indicate that members of the Runx family of genes subsequently play crucial roles in regulating development and survival of separate subpopulations of primary sensory neurons. In particular, Runx3 is considered the principal determinant for large proprioceptive neurons and Runx1 a promoter for small- and medium sized nociceptive nerve cells (Chen et al., 2006; Marmigére et al., 2006). In the rat, prospective large DRG neurons are born first, followed by smaller cells (Lawson et al., 1974; Kitao et al., 1996). The formation of different groups of cells at different time points is reflected in the pattern of peripheral target innervation, where A-fibers belonging to larger neurons reach their destination before small-sized neuronal C-fibers (Jackman and Fitzgerald., 2000). It can be assumed that the first wave of A-fibers that innervate the periphery later become myelinated and terminate as low-threshold mechanoreceptors in the skin (Fitzgerald, 1987). Other sensory functions such as nociception may mature slightly later (Jackman and Fitzgerald, 2000).

The TG and the segmental DRG are in many ways very similar with regard to functional and molecular/neurochemical characteristics. However, they have different developmental origins. Thus, TG is derived from both cranial neural crest and a specialized region of the surface ectoderm, the trigeminal placode (Graham and Begbie,

2000; Baker and Bronner-Fraser, 2001; Feldtsova et al., 2003), while DRG are formed entirely from spinal neural crest cells. The unique nature of the TG was recently underlined through studies showing that proper formation of the TG relies on reciprocal interactions between placode cells and NCC, with removal of either population resulting in severe defects (Shiau et al., 2008).

3. The carriers of impulses in primary sensory neurons: Voltage gated sodium channels

Nerve impulses in primary sensory neurons, as in all excitable cells, are mediated by ion channels. Here, the sodium channels constitute a fundamental family of proteins. Voltage-gated sodium channels (VGSC) are trans-membrane proteins that open and close in response to changes in the electrical field across the membrane. They mediate the influx of sodium ions into the cell in response to local membrane depolarization. VGSC are composed of a large pore-forming α subunit and one or two smaller β subunits. Nine different VGSC α-subunits are known to be present within the nervous system, of which 7 have been detected in primary sensory neurons. The α subunits are classified according to kinetic properties, voltage dependence for activation and by pharmacological differences, including sensitivity to tetrodotoxin (TTX) (fig. 2) (Baker and Wood, 2001; Catterall, 2000). In addition, the sodium channel α subtypes express different phosphorylation sites, which enable rapid modification of channel function (Murphy et al., 1993).



Subtype	Rat Orthologs	TTX Sensitivity (nM)	Activation Threshold	Inactivation Rate	Tissue Localization
$Na_v1.1$	Brain I	-	Low	Fast	CNS, also in DRG and motor neurons
$Na_v1.2$	Brain IIA	18	Low	Fast	CNS
$Na_v1.3$	Brain III	15	Low	Fast	Embryonic nervous system; adult CNS;
$Na_v1.4$	SkM1	5	Low	Fast	Skeletal muscle
$Na_v1.5$	H1	1,800	Low	Medium	Heart; embryonic DRG
$Na_v1.6$	SCP6, PN4	1	Low	Fast	DRG, motor neurons, also in CNS
$Na_v1.7$	PN1	2	Low	Fast	Mostly DRG, also in CNS
$Na_v1.8$	PN3, SNS	>100,000	High	Slow	DRG (80% small diameter; 20% large
$Na_v1.9$	PN5, NaN	39,000	Low	Medium	DRG (small diameter); also in CNS

Fig. 2. Structure and distribution of VGSC α subunits in primary sensory neurons (From Lai et al., 2004).

3.1. Distribution of VGSC α subunits in primary sensory neurons

The α subunits $Na_v1.1$, $Na_v1.8$ and $Na_v1.9$ are expressed in subpopulations of primary sensory neurons, while $Na_v1.6$ and $Na_v1.7$ show a more generalized expression pattern. $Na_v1.2$ is very modestly expressed in cells of different sizes in sensory ganglia, but most neurons lack this subunit (Black et al., 1996). The expression of $Na_v1.3$ and $Na_v1.5$ is normally seen during development only (Waxman et al., 1994; Renganathan et al., 2002). Several channel types, which give rise to different voltage dependencies and kinetics, can be expressed within single primary sensory neurons. Together, they will confer specific electric properties on the nerve cell in which they are expressed (Rush et al., 2007).

3.1.1. $Na_v1.1$

$Na_v1.1$, is expressed in the adult mammalian brain. Together $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$ comprise the major subtypes of VGSC in the adult mammalian brain. $Na_v1.1$ is also expressed in DRG, at higher levels in large neurons than small DRG neurons (Black et al., 1996). Several mutations of this channel are linked to epilepsy (Mulley et al., 2005). Homozygous null Scn1a–/– mice develop ataxia and die on postnatal day P15 and

heterozygous Scn1a+/– mice are associated with spontaneous seizures and sporadic deaths beginning after P21 (Yu et al., 2006), suggesting important central functions of this channel and consequently making it less attractive as a target in pain syndromes.

3.1.2. $Na_v1.3$

$Na_v1.3$ is a TTX sensitive channel with fast activation/inactivation and repriming. This VGSC has been reported to become upregulated beginning a few days following axotomy (Waxman et al., 1994.). The upregulation could be responsible for the net increase in sodium current generated in axotomized DRG neurons (Rizzo et al., 1995) and for the accelerated kinetics which contribute to the overall electrical hyperexcitability of these neurons (Cummins and Waxman, 1997). Treatment with TTX has been shown to reduce neuropathic pain behavior (Lyu et al., 2000) and treatment with GDNF reverses both the expression of $Na_v1.3$ and neuropathic pain behavior following nerve injury (Boucher et al., 2000) suggesting a role for $Na_v1.3$ in neuropathic pain. However, the impact of $Na_v1.3$ in neuropathic pain has recently been questioned in a study by Nassar et al., 2006, where $Na_v1.3$ null mutant mice displayed nerve injury-induced allodynia and ectopic discharges at similar levels as wildtype mice.

3.1.3. $Na_v1.5$

$Na_v1.5$ is TTX-resistant and exhibits biophysical properties that results in fast activation and inactivation of the channel (Renganathan et al., 2002). It is the main cardiac subtype of VGSC (Gellens et al., 1992), with heart failure as a direct consequence of insufficient expression (Hesse et al., 2007). Besides in heart muscle, $Na_v1.5$ has been detected at much lower levels in brain, embryonic DRG and embryonic skeletal muscle (Donahue et al., 2000; Kallen et al., 1990; 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). A scenario with $Na_v1.5$ as one of the main contributors to neuropathic pain in the peripheral nervous system would meet great difficulties in treatment. Since $Na_v1.5$ is crucial in heart activity, blocking of this channel would have obvious adverse side effects.

3.1.4. $Na_v1.6$

$Na_v1.6$ is a TTX-sensitive channel that gives rise to a fast activating and inactivating current. (Dietrich et al., 1998). It is the main VGSC isoform at the nodes of Ranvier in myelinated axons (Caldwell et al., 2000) and is also distributed along the axons of unmyelinated C-fibers (Black et al., 2002). Only little attention has been given $Na_v1.6$ as a participant in neuropathic pain development, but it has been suggested to play a part in trigeminal pain states (Henry et al., 2007).

3.1.5. $Na_v1.7$

Nav1.7 is a TTX-s sodium channel preferentially localized to axon terminals (Toledo-Aral et al., 1997). Animals with a selective knock-out of the sodium channel $Na_v1.7$ in small nociceptive neurons do not differ compared to wildtype controls in the development of neuropathic behavior following spinal nerve ligation (SNL) (Nassar et al., 2005). However, mutations of $Na_v1.7$ are involved in Erythromelalgia, an autosomal dominant disease associated with episodic erythema and severe burning pain (Waxman and Dib-Hajj, 2005). This highlights the importance of this channel subtype for some types of pain.

3.1.6. $Na_v1.8$

$Na_v1.8$ is expressed exclusively in small and medium-sized primary sensory neurons, suggesting a role in pain-transmitting systems. It is with minor exceptions absent in the CNS (Akopian et al., 1996). Expression is localized to the cell body (Ritter et al., 1992), and to peripheral (Brock et al., 1998) and central terminals (Jeftinija, 1994). This sodium channel produces a slowly activating and inactivating TTX-resistant current which has relatively depolarized voltage dependence (Akopian et al., 1996). Following axotomy, there is a down-regulation of $Na_v1.8$ in injured neurons (Sleeper et al., 2000). $Na_v1.8$ is expressed in approximately one half of both IB4+ and IB4- neurons, suggesting that the expression is affected by both NGF and GDNF (Fjell et al., 1999a; 1999b). The role for $Na_v1.8$ in pain transmission has been investigated thoroughly, with somewhat conflicting results. In support of $Na_v1.8$ as a mediator of pain signaling after nerve injury, antisense-mediated knockdown of $Na_v1.8$ has been found to reverse injury induced mechanical and thermal hypersensitivity (Lai et al., 2002). Another study identified a critical role for $Na_v1.8$ in chronic post-infectious visceral hyperexcitability (Hillsley et al., 2006), and Roza et al., 2003 reported that the ectopic discharge in the

neuroma model (see below) was strongly reduced in mice lacking $Na_v1.8$ as compared to wildtype mice. However, Kerr et al. (2001) found no difference in the development of neuropathic pain behavior after nerve injury between $Na_v1.8$ knock out and wildtype mice.

3.1.7. $Na_v1.9$

$Na_v1.9$ is expressed preferentially in small IB4-positive primary sensory neurons (Fjell et al., 2000). This sodium channel subunit produces a persistent TTX- resistant current believed to contribute to the cell´s resting potential (Dib-Hajj et al., 1998; 2002). The fact that $Na_v1.9$ is restricted to DRG and TG neurons of the C-type strongly indicates that it is important for nociception. The $Na_v1.9$ knock-out (KO) mouse described by Priest et al., 2005 shows an analgesic phenotype, primarily with respect to the second phase of pain behavior during the formalin test, but also in a reduction of hyperalgesia brought about by subdermal application of PGE_2. Another $Na_v1.9$ knock out also exhibits an inflammatory phenotype and has deficits in the response to intraplantar UTP, a P2Y agonist (Amaya et al., 2006). Recent findings demonstrate that $Na_v1.9$ underlies the G-protein pathway-regulated TTX-resistant persistent Na^+ current in small diameter sensory neurons that may drive spontaneous discharge in nociceptive nerve fibres during inflammation. However, as of yet there is no clearcut evidence for its role in ectopic signaling after traumatic nerve injury (Ostman et al., 2008). As an example, antisense knock-down of $Na_v1.9$ protein produces no change in tactile and thermal hyperalgesia (Porreca et al., 1999).

3.2. Distribution of sodium channel β subunits in primary sensory neurons

The β subunits are associated to α subunits, and modify the channel function. Five β subunits have been characterized, namely β1, β1A, β2, β3 and β4 (Isom, 2001).
The β-subunits can interact with extracellular matrix molecules and also function as important regulators of sodium channel localization in the plasma membrane (Malhotra et al., 2000). In the adult rat DRG, β1 is expressed in large-diameter Aβ fibres but appears to be almost absent in small-diameter unmyelinated C-fibres (Oh et al., 1995). β2 has been reported to be present in a small proportion of adult DRG neurons, but is upregulated after injury (Pertin et al., 2005; Lopez-Santiago et al., 2006). β3 mRNA is found in small-diameter C-fiber neurons (Shah et al, 2000) and is upregulated following

nerve injury (Takahashi et al., 2003). The increased expression of the subunits β2 and β3 following nerve injury could indicate involvements in neuropathic pain for these auxiliary proteins.

3.3. Accessory VGSC proteins and modulators of sodium channel function

Modulation of VGSC can have a major impact on cell excitability in primary sensory neurons. First of all, VGSC are linked to the axonal cytoskeleton through ankyrinG and other proteins. Consequently, the access to these proteins will determine the functional capacity of the VGSC (Scherer and Arroyo, 2002). Other proteins are important for the insertion of VGSC into the axolemma. One such example is the annexin II light chain (p11), which acts as a regulatory factor that facilitates the expression of $Na_v1.8$. It binds directly to the amino terminus of $Na_v1.8$ and promotes the translocation of $Na_v1.8$ to the plasma membrane, producing functional channels (Okuse et al., 2002). A deficit in p11 will thus render $Na_v1.8$ non-functional. Similarly, Contactin/F3, a cell adhesion molecule, has been shown to interact with and enhance surface expression of sodium channels $Na_v1.2$ and $Na_v1.9$. It also co-localizes with $Na_v1.3$ and, similar to $Na_v1.3$, Contactin is upregulated in axotomized DRG neurons and accumulates within the neuroma of transected sciatic nerve. This has led to the suggestion that the upregulation of Contactin and its colocalization with $Na_v1.3$ in axotomized DRG neurons may contribute to the hyperexcitablity of injured neurons (Shah et al., 2004).

Additional proteins not directly related to the molecular architecture of the VCSCs at the axonal membrane may have profound influences on VGSC function. As an example, Calmodulin is important for functional expression of $Na_v1.4$ and $Na_v1.6$. Thus, this protein can regulate the properties of VGSC via calcium-dependent and calcium-independent mechanisms. This suggests that modulation of neuronal sodium channels may play a role in calcium-dependent neuronal plasticity (Herzog et al., 2003), and modulation of the sodium current produced by $Na_v1.6$ might significantly impact axonal conduction. Further, p38 MAPK (Mitogen-activated protein kinase) can phosphorylate and modulate VGSC in response to injury, which probably might significantly impact axonal conduction (Wittmack et al., 2005). Of particular interest in this context is the influence of inflammatory mediators. It is well known that inflammatory mediators, when introduced into peripheral tissues, can trigger pain. This appears to be due to, at least in part, depolarization and increased excitability of nociceptive DRG neurons (England et

al., 1996). Recent findings indicate that inflammatory mediators, acting via a G-protein-dependent mechanism increase the $Na_v1.9$ sodium current (Rush and Waxman, 2004). Further, G-protein-triggered up-regulation of the persistent $Na_v1.9$ current can produce changes in membrane excitability sufficient to cause spontaneous activity (Baker et al., 2003; Waxman and Estacion, 2008). Tumor necrosis factor-α (TNFα) is a proinflammatory cytokine involved in the development and maintenance of inflammatory and neuropathic pain conditions. It has been shown that acute application of TNFα rapidly enhances TTX-resistant Na+ currents in isolated DRG neurons. This potentiation of TTX-resistant currents by TNFα is dramatically reduced in DRG neurons from TNF receptor 1 (TNFR1) knock out mice and is blocked by a p38 mitogen-activated protein kinase inhibitor, which also blocks mechanical hypersensitivity induced by peripherally applied TNFα (Jin and Gereau, 2006). Less is known on the effects of other cytokines, such as e.g. interferon gamma, or chemokines, on the function of sodium channel subunits in primary sensory neurons.

4. Nerve injury and the development of neuropathic pain

Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system. The etiology of neuropathic pain is diverse: several mechanisms e.g. metabolic, infection, ischemic and mechanical trauma are known causes. However, despite different etiologies, neuropathic pain share clinical features, including sensory deficit, allodynia, hyperalgesia, and spontaneous pain. Mechanisms in both the peripheral and central nervous system are involved in neuropathic pain.

4.1. VGCS and the development of neuropathic pain: structural changes

Following nerve injury there is a subsequent loss of target innervation and neurotrophic support to the neuron. Injured neurons show a marked phenotypic change, a shift from signal transmitting to regeneration which is reflected in alteration in expression pattern and change in morphological and functional properties. Damaged, but not dying, neurons immediately try to regenerate. Several growth cones develop from the severed proximal ending slowly elongating the axon towards its original target organ. When regeneration is successful normal sensation is reestablished. (Ramon y Cajal, 1928; Sunderland, 1978).

Regeneration is unfortunately often impaired and the proximal part of the axon still connected to the cellbody forms a terminal swelling or end-bulb (Ramon y Cajal, 1928; Fried et al., 1991). Sodium channels accumulate in excess numbers in the axonal membrane at the neuroma end-bulb, (Lomber et al., 1985; Devor et al., 1989) (Fig. 3). The mechanism of the accumulation can have several explanations, e.g. loss of target area following axotomy, change in turnover time and change in expression (Brismar and Gilly, 1987; Fried et al., 1991). The accumulation of sodium channels renders the membrane hyperexcitable (Matzner and Devor, 1992), VGSC are thus believed to have a critical role in many types of chronic pain states (see above).

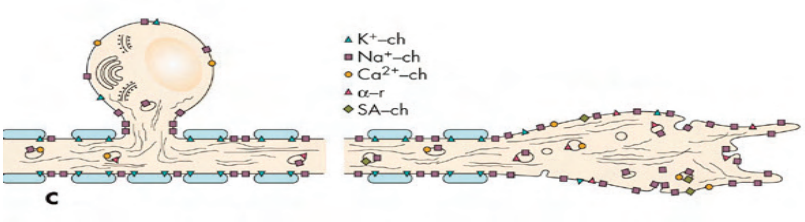


Fig. 3. Accumulation of various ion channels, transducer molecules and receptors in axon membrane of endbulbs and sprouts following nerve injury (From Devor and Seltzer, 1999).

In addition, there is clinical evidence that drugs that affect sodium channel function may be effective in relieving neuropathic pain, which further supports the notion that VGSC have a fundamental part in the etiology of neuropathic pain. The local anesthetic lidocain relives the symptoms of neuropathic pain in patients with postherpetic neuralgia, painful diabetes neuropathy and trigeminal neuralgia (Arnér et al., 1990; Rowbotham et al., 1996; Ferrante et al., 1996; Mao et al., 2000). The anticonvulsant Carbamazepin, which works by stabilizing sodium channels in an inactive state, is used for the treatment of trigeminal neuralgia. Other anticonvulsant drugs such as lamotrigine, phenytoin, also have some efficacy in treatment of neuropathic pain (Lunardi et al., 1997; McCleane, 1999; 2000).

5. Variability of neuropathic pain: the influence of genetic factors

The intensity of pain experienced as a result of a disease or injury to the nervous system varies considerably among individuals (Green et al., 2003). Individual variability in the burden of pain has traditionally been attributed to psychosocial factors. However, new data indicate that there is an important heritable predisposition to pain, particularly to the development of neuropathic pain. Likewise, pain behavior in inbred mouse strains shows distinctive strain-related differences, even when environmental factors are controlled (Mogil et al., 1999). Accumulating evidence highlights the relevance of genetic factors as important causes of these rodent strain variations in pain susceptibility (Inbal et al., 1980; Shir et al., 2001; Diatchenko et al., 2007). Any of a number of neurotransmitters, receptors and nociceptor ion channels which are involved in pain modulation could be a critical factor that determines genetic differences in pain and much effort is put into finding regulated genes of interest. Of particular importance in this context are the VGSC, since they are essential for the primary sensory neurons to fire and thus for pain processing from the periphery (see above). Pain susceptibility genes are intrinsically hard to detect in human lineages and populations. However, through the alternative approach of exploiting rodent models of neuropathy to uncover pain susceptibility loci and associated neurobiological processes (using inbred mouse strains that show high versus low pain phenotype), it may be possible to investigate if genetic differences in VGSC will affect pain phenotype after chronic nerve injuries.

6. Experimental models in studies of neuropathic pain

Different animal models have been developed to study the pathology of nerve injury and neuropathic pain (fig. 4). The sciatic nerve is often used in these experimental models due to its size and easy access, but other nerves, including major trigeminal nerve branches, have been used as well.

A commonly used *neuroma model* is the transection and ligation of the sciatic nerve in mid-thigh level. Regeneration is prevented by cutting the distal nerve stump and by ligation of the proximal part. At the tip of the nerve a neuroma will then form. Electrophysiological recordings have showed ectopic firing, spontaneous activity and mechanical hyperalgesia in the area of the neuroma (Wall and Gutnick, 1974a; 1974b,).

In this model, behavioral sensory testing is not possible since the sensory loss of the paw is almost complete. However, animals with this type of injury display a self-mutilation behavior, termed autotomy, which is considered as related to spontaneous pain. Autotomy can be quantified, and is regularly used as a measure of ongoing pain.

To be able to measure and correlate biochemical and physiological changes with behavior, additional partial injury models have been developed. *Chronic constriction injury* (CCI) is created by loose ligation of the whole sciatic nerve at mid-thigh level (Bennett and Xie, 1988). The operation produces a gradual swelling and strangulation of the nerve under the ligatures, followed by degeneration of all A-fibers and many but not all C-fibers (Basbaum et al., 1991). Electrophysiological examination of the CCI site reveals massive ectopic firing originating both at the nerve injury site and in the sensory neuron cell body (Xie and Xiao, 1990). CCI yields symptoms in the affected areas such as hypersensitivity to thermal stimuli and increased sensitivity to mechanical stimuli, all of which can be measured.

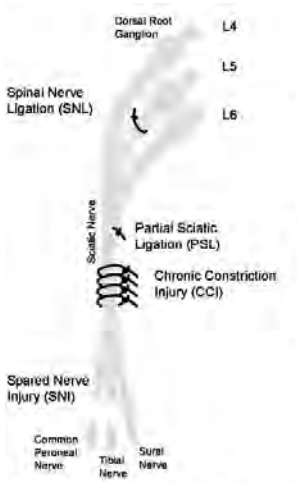


Fig.4. Models of neuropathic pain (from Campbell and Meyer, 2006).

The spinal nerve ligation model consists of a tight ligature of one (L5) or two (L5 and L6) spinal nerves close to the DRG, leaving the L4 component of the sciatic nerve intact (Kim and Chung, 1992). The SNL-model is associated with spontaneous activity in afferent neurons with myelinated fibers but not in those with unmyelinated nerve fibres. A correlation between ectopic activity and allodynic behavior was found in this model (Liu et al., 2000), suggesting that the ectopic activity in SNL-lesioned afferent neurons might

be important for the maintenance of the neuropathic pain behavior. As with CCI, SNL produces behavioral signs of nerve injury which can be readily observed and quantified. *The ischemic nerve-injury model* was created in an attempt to decrease the variability seen in pain behavior following other animal models. Here, the nerve is exposed, and after an intravenous injection of a photosensitizing dye, erythrosin B, the nerve is irradiated with an argon laser (Gazelius et al., 1996). The advantages of this model are its high reproducibility and the fact that the nerve injury can be easily quantified and graded by using different irradiation times. The severity of the damage correlates with time of irradiation. Behavioral signs of neuropathic pain develop similarly as with CCI and SNL, and can be evaluated accordingly.

AIMS OF THE STUDY

As seen from the Introduction, VGSCs constitute a key family of proteins in primary sensory neurons of DRG and TG. A widened insight into the biology of these ion channels will further the understanding of how pain signals emerge and are transmitted, during development, in the normal mature system and, in particular, in the damaged nervous system. Against this background, the main objectives were as following.

- To examine the expression patterns of VGSC in TG during development.

Little information has been available on how voltage-gated sodium channels in sensory neurons are expressed during development in the craniofacial region, where sensitivity is fundamental during early stages of life, or if it is different due to functional demands on an early developed sensitivity.

- To study the DRG mRNA expression of $Na_v1.8$ and $Na_v1.9$ in IFN-γ - or IFN-γ receptor-deficient mice

The tissue trauma that accompanies peripheral nerve injury leads to a local inflammatory response, which is regulated by signaling molecules, including Interferon-γ (IFN-γ). IFN-γ receptor knockout mice display a reduced pain-related behavior after sciatic nerve injury. This could indicate that IFN-γ could be involved in the regulation of sodium channels in axotomized sensory neurons.

- To develop and evaluate a new experimental rat model of trigeminal neuropathic pain produced by a photochemically induced ischemic injury to the IoN.

Very little information has been available on how voltage-gated sodium channels of the trigeminal ganglion (TG) are affected by injury, despite the fact that sodium channel remodeling probably plays an important role in the development of orofacial pain-related behavior.

- To study the expression of VGSC in inbred mouse strains with contrasting pain phenotypes - correlations between pain behavior and expression of VGSCs.

Dramatic variability in pain behavior is observed across inbred strains of mice where it has been attributed to genetic polymorphisms. Identification of cellular correlates of pain variability, such as VSCGs, across strains can advance the understanding of underlying pain mechanisms.

GENERAL EXPERIMENTAL PROCEDURES

1. Ethical considerations

Animal numbers used in the studies included in this thesis were kept to a minimum. Animals were housed in a 12:12 hours light: dark cycle with food and water available *ad libitum*. All experiments 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 ethics committee.

2. Surgical procedures

2.1. Axotomy of the sciatic nerve in mice

As described by (Wall and Gutnick, 1974a), the left sciatic nerve was exposed in anesthetized mice at mid-thigh, ligated with 6-0 silk and cut approximately 1 mm distal to the ligation. A few millimetres of the distal nerve stump was removed to avoid regeneration. (Paper II)

In addition, the left saphenous nerve was exposed near the knee and similarly ligated and cut. (Paper IV)

2.2. Photochemically-induced injury of the infraorbital nerve (IoN) in the rat

The left IoN was exposed in anesthetized rats by a 1 cm long skin incision in the *regio masseterica* and carefully separated from the surrounding vasculature with a glass hook. The exposed nerve was irradiated with an argon ion laser (Innova Model 70, Coherent Laser Products Division, Palo Alto, CA) operating at a wavelength of 514 nm with an average power of 0.17 W. The irradiation was performed with a knife-edge beam across the nerve. Aluminum foil was placed under the nerve to isolate the surrounding tissue and to reflect light. Paraffin oil was applied on the nerve to prevent the nerve from drying out during irradiation. The following irradiation durations were used: 1.5 min, 3 min, 4.5 min, 6 min and 10 min. Just before irradiation, erythrosin B (Red No. 3, Aldrich, 32.5 mg/kg dissolved in 0.9% saline) was injected i.v. through the tail vein. The injection was

repeated after 5 min for the longer duration of irradiation. In the sham operated rats the left IoN was exposed without irradiation. After the irradiation the incision was closed in anatomical layers and the rats were returned to their cages for recovery. (Paper III)

3. Behavioral tests

3.1. Sensitivity to mechanical stimulation

Following photochemically induced injury of the IoN the mechanical sensitivity of the face was determined with a graded series of von Frey filaments which produced a bending force of 0.60 g, 0.91 g, 1.90 g, 3.47 g, 5.82 g, 7.95 g, 11.1 g, 18.4 g and 40.14g. During testing, the rat was gently held by experimenter and von Frey filaments were applied in ascending order within the IoN territory on the hairy skin of the vibrissal pad. The stimulation with each filament consisted of four consecutive placements (interval 1 s) on the nerve-injured, and then the contralateral side. The response threshold was taken as the force at which the rat presented any of the following aversive response; withdrawal, struggle/escape or attack (Vos et al., 1994). The cut-off value was set at 40.14 g. The rats were subjected to handling and habituation to the testing procedure and they were then tested for 3 days and the median value was taken as the pre-injury withdrawal threshold. Rats that sustained nerve injury, as well as the sham operated ones, were tested on d 3, 7, 11, 14 and then once per week. (Paper III)

3.2. Sensitivity to thermal stimulation

The response of the rat to heat was tested with a radiant heat source, which stimulates the hairy skin of the lateral snout (the vibrissal pad). Rat head withdrawal latencies were measured automatically. The intensity of stimulation was adjusted so that the baseline withdrawal latency was 4-6 s. After 3 days of handling and habituation to 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 III)

3.3. Body pain

The mechanical hypersensitivity was assessed by examining the vocalization thresholds to graded mechanical touch/pressure applied with von Frey hairs. During testing the rats was 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 at each intensity 5-10 stimuli were applied. The intensity of stimulation which induced consistent vocalization (>75% response rate) was considered as pain threshold. (Paper III)

3.4. Autotomy

Autotomy behaviour was assessed weekly for 5 weeks, 35 days post operation (dpo) after sectioning the sciatic and saphenous nerves. Scoring followed the protocol of (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. (Paper IV)

4. Immunohistochemistry

Immunolabelling of ATF-3 protein, a member of the ATF/CREB family of transcription factors which is a widely used marker of injured primary sensory neurons (Tsuzuki et al., 2001), was performed on sections from TGs of animals irradiated for 6 min as above (Paper III). The sections were post-fixed in 4% paraformaldehyde in 0.14 M phosphate buffer for 10 min. They were then incubated with an anti ATF-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted 1:200 in 0.01 M phosphate-buffer saline (PBS) (pH 7.4) with 5% bovine serum albumin, 3% normal goat serum (DAKO, Denmark) and 0.3% Triton. The sections were incubated for 12-16 h at 4 °C. After rinsing with PBS the sections were then incubated for 60 min. with peroxidase-conjugated goat anti-rabbit antiserum (1:200; DAKO) in a humid atmosphere at room temperature. Following rinsing with 0,01 M PBS, the sections were subsequently reacted in 0.05% diaminobenzodine tetrahydrochloride (DAB, DAKO) and 0.01% H_2O_2. (Paper III)

5. In situ hybridization (ISH)

Oligonucleotide probes were designed using Software program Oligo 6.0. The lengths were between 42 and 52 bases. Specific search criteria were used to select the appropriate probes: GC content 50-60%, loops and dimerizations stronger then 10 kcal/mol were exluded. The specificity of the probes was tested by blast search. Probes with more than 23/23 hits to other mRNA sources were excluded. The probes were synthesized by CyberGene, Huddinge, Sweden. Procedures for ISH were as previously described (Dagerlind et al., 1992), but with slight modifications. Briefly, oligonucleotides were 3´-end labeled with (^{33}P)-dATP using terminal deoxyribonucleotidyl transferase. Sections were hybridized for 16-20 hours at 42°C in a humidified chamber with 3.75 µl labeled probe ($2x10^5$ cpm/µl labeled probe) made to 100 µl for each slide in a mixture of 4 x SSC, 50% formamide, 1 x Denhardts´s solution , 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate, 500 mg/ml heat denaturated salmon sperm DNA and 200 mM dithiothreitol. Slides were then rinsed 5 x 15 min. at 58-60°C in 1 x SSC and the last SSC rinse was allowed to reach room temperature. Slides were dipped in distilled water, dehydrated through graded series of ethanol (70%, 95%, 99.5%), air-dried, dipped in photographic emulsion (Kodak NTB2, diluted 1:1 in distilled water) and exposed for 3 weeks. After developing, the slides were counterstained with cresyl violet and mounted in Entellan® (Merck, Darmstadt, Germany). (Paper I, II, III, IV)

6. *mRNA quantification using real-time PCR*

6.1. RNA extraction and cDNA synthesis

DRGs spinal cords sampled from the naïve mice and mice subjected to sciatic nerve section 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 manufacturers' 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)

6.2. Real-time SYBR-green PCR

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 (Table 1) 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 ddCT 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)

7. Analysis

Semi-quantitative analyses of the mRNA hybridization signals were performed using a Nikon E600 microscope equipped with a darkfield condensor and a Nikon DXM 1200 digital camera. The gray scale of the darkfield image was adjusted and segmented using the enhance contrast and density slicing feature of the Easy Image software (version 3000) so that the density of silver grains over the neurons in the TG/DRG could be assessed automatically. The intensity of cellular mRNA expression was calculated as the ratio between the intensity in neuronal cell somata and the intensity in background, S/N ratio. Cells having a hybridization signal five times the background level or higher were considered positive. Digital photographs were processed using Adobe Photoshop 7.0. All slides that were analyzed were coded, and the identities of the sections were thus not known to the examiner. (Paper I, II, III, IV)

8. Statistics

8.1. Paper I

Changes in expression levels in paper I were analyzed by Kruskal-Wallis, ANOVA by Ranks. Individual comparisons between different time periods were made by Mann-Whitney U Test. $P<0.05$ was set as the level for significance.

8.2. Paper II

Ratios for $Na_v1.8$ and $Na_v1.9$ positive cells/total number of cells were obtained. Data were compared using Student's unpaired *T*-test, level of significance at all survival periods ($p<0.01$). Differences in expression between the knockout- and wild type-mice were analyzed using Student's unpaired *T*-test, significance level $p<0.05$.

8.3. Paper III

The 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 Fishers's PLSD test, $p<0.05$ was considered to be statistically significant. To assess differences in ratios for $Na_v1.8$, $Na_v1.9$ and β3 between the irradiation group and the sham group, data were compared using Student's unpaired T-test. The results were considered significant if the obtained p value was less than 0.01.

8.4. Paper IV

Change in expression levels in all strains (AKR, C58, C57, CBA and C3H) were analyzed by two-way ANOVA followed by Newman Keuls test ($p<0.05$ was considered significant). Fisher exact probabilities test was used when only the direction of change within all five strains and not its magnitude was analysed ($p<0.05$ was considered significant). Correlation analysis was made comparing mRNA levels in the injured DRG with pain behavior (strain autotomy rankings from the animals followed 35 dpo).

RESULTS AND DISCUSSION

1. Expression of VGSC mRNAs during the development of the rat TG - Paper I

In previous investigations on DRGs from segmental spinal levels, the ontogeny and maturation of mRNA encoding for primary sensory neuron VGSC α-subunits have been mapped, and related to electrical activity, establishment of target innervation and neuronal sensitivity to trophic factors (Benn et al., 2001). However, several pieces of evidence would argue that these findings cannot readily be translated to trigeminal territories. The embryonic origin is to some extent different between DRG and TG. The anatomical position also differs with a closer association between the primary sensory neurons and target organs in the TG. Furthermore, it is obvious that functional demands related to environmental exploration and suckling might necessitate a developed sensitivity earlier in the orofacial region than in other parts of the body.

1.1. $Na_v1.3$

With regard to the TTX-sensitive VGSC $Na_v1.3$, which is mainly expressed during development and considered to be responsible for the spontaneous activity that can be recorded from primary sensory neurons during embryogenesis (Fitzgerald, 1987), we did not observe any obvious differences in developmental expression patterns between TG and DRG (Felts et al., 1997). Thus, mRNA encoding for the VGSC $Na_v1.3$ was expressed at high levels in the TG at Embryonic day (E)15, and then decreased during development (E19: 65%± 8.9, Postnatal day (P)1: 32%±8.3 and P5/6: 4%±2.5 of total number of neurons).

1.2. $Na_v1.8$

During development, $Na_v1.8$ mRNA was expressed in the TG from E15, and increased during development with a peak at P1. This was followed by a down-regulation which was detected at P5/6 (E19: 38%± 4.8, P1: 58%±3.6 and P5/6: 48 %±2.5 of total number of neurons). In DRG there is a similar onset of expression beginning at E15 but the peak of expression (both in terms of proportion and intensity) beginning at P1 in the TG is several days earlier than the corresponding peak in the DRG. (Benn et al., 2001).

Following P1, $Na_v1.8$ levels in TG continue to decrease until they reach adult values which seem to be similar in both types of ganglia.

1.3. $Na_v1.9$

$Na_v1.9$ was first found in the TG at E19. This was followed by a rapid increased in expression with maximum proportional and intensity values at P1(E19: 35 %±2.5, P1: 52%±3.6 and P5/P6 42%± 2.3 of total number of neurons). Compared to spinal levels, the expression in the TG started later but showed a more rapid development towards adult levels. In the DRG, the expression begins at E19 and reaches its highest expression values at P5-P6 (Benn et al., 2001) when trigeminal $Na_v1.9$ mRNA levels already have begun to decrease.

1.4. VGSC α-subunits in TG maxillary/mandibular vs ophthalmic regions

$Na_v1.8$ and $Na_v1.9$ mRNA displayed differential expression levels within different regions of the TG. Expression levels were higher in the ophthalmic region as compared to the maxillary/mandibular region of the TG at E19. The difference was still present at P5/P6 (Table 1).

$Na_v1.8$	N. Ophtalmicus	N Maxillaris/ Mandibularis	**$Na_v1.9$**	N. Ophtalmicus	N Maxillaris/ Mandibularis
E19	58%±4.2	38%±3.4	E19	47%±2.9	33%±2.7
P5/6	62%±3.6	46%±3.3	P5/6	59%±3.3	41%±4.2

Table 1. Percentage of $Na_v1.8$- or $Na_v1.9$ mRNA-positive neurons in different TG regions during development.

Our finding that VGSC develop according to separate chronologies in ophthalmic vs mandibular/maxillary TG regions fits with recent data which show that neurons that innervate distinct areas of the face possess different characteristics. Pax3-expressing cells in the early mouse neurogenic placode, for example, are committed to populate ophthalmic regions only (Baker et al., 1999), indicating that precursor cells from neural crest and placode regions, respectively, could be targeted to specific TG areas.

Our results show that the early prenatal differential expression of genes throughout the TG continues during late prenatal and also postnatal stages, at least with regard to VGSCs. This likely enables the sensory TG neurons to adapt to functional requirements. These requirements should be different in maxillary/mandibular-innervated mouth areas, which are vital for feeding and exploring already at birth, as compared to the ophthalmic nerve-supplied eye and forehead, with eye-opening occuring as late as P12-P15. On these grounds, it seems reasonable to suggest that the early maturation of VGSC in nerve cells of maxillary/mandibular TG areas reflects the need for a well-developed sensory system in the target territory of these neurons.

1.5. β-subunits

In our material, we noted that the β1, the β2 and the β3 subunits displayed different developmental patterns in the TG. Thus, β1 mRNA expression was first evident at E17 and then increased in extent and intensity (E19: 43%± 12.9, P1: 66%±17.9 and P5/6 80%±11.1 of total number of neurons). Expression of β2 mRNA in the TG was absent at stages before E19. At E19, a very weak signal was detected in some cells, but at P1 and P5/P6 a strong upregulation had occurred, and at these time points a widespread ganglionic expression of β2 mRNA was observed. Both β1 and β2 showed rapid perinatal increases in neuronal expression, perhaps reflecting important roles as modulators and/or adhesion factors during an intense period of craniofacial axon terminal establishment. β1 continued to increase in expression, to reach an adult value far exceeding that reported for β1 mRNA in the adult rat DRG (Takahashi et al., 2003). β2, however, underwent a postnatal decrease, and the adult proportion of TG β2 mRNA expressing neurons was low and similar to that observed in adult DRG (Takahashi et al., 2003). The VGSC subunit β3 has been linked to small C-fiber neurons in sensory ganglia. During development, β3 mRNA was strongly expressed in the TG at E15, but the proportion of β3-labelled cells decreased during development (E19: 84%± 5.2 P1: 78%±4.0 and P5/6 62%±4.3 of total number of neurons).

1.6. p11

p11 - annexin II light chain - is a regulatory co-factor which influences the insertion of $Na_v1.8$ protein into the neurolemma. Considering its important role in determining the functional state of $Na_v1.8$, we examined whether the development of p11 expression occurred *pari passu* with that of $Na_v1.8$. However, this was not the case - ISH sections of the TG demonstrated that p11 mRNA was abundantly expressed in virtually all neurons throughout all developmental stages examined.

2. Expression of the sodium channel transcripts $Na_v1.8$ and $Na_v1.9$ in injured dorsal root ganglion neurons of interferon-γ or interferon-γ receptor deficient mice - Paper II

Following peripheral nerve damage, DRG neurons reduce the levels of mRNA for some sodium channel genes, while the mRNA for others are increased. This changes the setup of sodium channels that is available for insertion in DRG neuronal cell membranes following injury. Functionally, this is reflected in changes in the physiological properties of the affected cells (Cummins et al., 2000). The DRG expression of the tetrodoxin-resistant sodium channels $Na_v1.8$ and $Na_v1.9$, are down-regulated following either axotomy or chronic constriction of the sciatic nerve (Dib-Hajj et al., 1996;1998; Sleeper et al., 2000). Following nerve injury there is a subsequent loss of target innervation and neurotrophic support to the neuron which can effect the expression of VGSC. NGF and GDNF have been shown to differentially regulate $Na_v1.8$ and $Na_v1.9$ in DRG (Fjell et al., 1999b). In studies of mice with a targeted disruption of the IFN-γ receptor (IFN-γR) gene, it was noted that these animals displayed a reduced pain-related behavior in response to peripheral nerve injury (Robertson et al., 1997). Cytokines such as IL-1, IL-6, TNF-α and IFN-γ have been implicated as contributing factors in chronic pain states after tissue injury (DeLeo et al., 2001). In normal adult peripheral nerves, IFN-γ is absent. However, after nerve injury, IFN-γ mRNA, which most likely is T-cell-derived, is expressed at the lesion site between 5 days and 2 weeks post operation (Taskinen et al., 2000). Direct effects of IFN-γ on the axons of primary sensory nerves should be possible, since these cells possess IFN-γR (Robertson et al., 1997; Vikman et al., 1998). A potential role for IFN-γ in sensory neuron sodium channel regulation is indicated by the fact that exposure of PC12 cells to this substance causes $Na_v1.7$ gene induction, through molecular signaling pathways that IFN-γ share with NGF (Toledo-Aral, et al., 1995). However, our results show that at least $Na_v1.8$ and $Na_v1.9$ mRNA seem to be regulated independently of IFN-γ after nerve injury. The down-regulation of these channels in our mouse material followed the pattern previously established in the rat (Cummins et al., 2000), and was similar in IFN-γ−/− or IFN-γR−/− animals when compared to wild type control mice. This may indicate that the change in pain behavior observed in IFN-γR−/− mice with peripheral nerve damage is purely the result of changes in central pain processing, and is unrelated to ectopic electrical activity in the DRG. On the other hand, electrophysiological characteristics may indeed differ in injured DRG between wild-type and IFN-γR−/− mice, but this may be due to changes in genes other than $Na_v1.8$ and $Na_v1.9$. Compensatory changes that are seen in other knockout models also have to be considered. However,

Vikman et al., 2003 showed that IFN-γ is involved in disinhibition of synaptic activity and primary afferent input in the dorsal horn, which consequently results in central sensitization, indicating CNS effects.

3. Behavioral changes and trigeminal ganglion sodium channel regulation in an orofacial neuropathic pain model – Paper III

3.1. Behavioral characterizations: mechanical hypersensitivity

In rats irradiated for 3, 4.5 or 6 min. there was a significant bilateral decrease in withdrawal threshold lasting 28-45 days. Sham operation as well as 1.5 min irradiation did not significantly alter the withdrawal threshold. The bell-shaped relationship between irradiation time and hypersensitivity has previously been noted in rats and mice (Yu et al., 2000; Hao et al., 2000). We have previously argued, based on electron microscopic analysis, that the development of neuropathic pain-like behavior in this model may require injury to the unmyelinated fibers, which are relatively resistant to ischemic injury (Yu et al. 2000). Thus, irradiation of 1.5 min may not be sufficient to damage unmyelinated fibers. The lack of mechanical hypersensitivity in 10 min irradiated rats may be explained by a lack of myelinated afferent input (Yu et al., 2000). The mechanical hypersensitivity was bilateral, although the ipsilateral effect was somewhat stronger than that on the contralateral side. This also agrees with results from some rodent models of hindlimb nerve, IoN injury or inflammation (Benoliel et al., 2002; Hao et al., 2000). Several mechanisms have been proposed to account for the contralateral pain including possible anatomic connections between the two sides of the spinal cord and brainstem, a supraspinal loop involving bilateral descending facilitation and possible bilateral glial cell activation (Koltzenburg et al., 1999; Porreca et al., 2002; Milligan et al., 2003). It is interesting to note that at least in the rat, the bilateral effect seems to be more consistent after IoN nerve injury than after hindlimb nerve injury. This may be related to the well-documented projections to contralateral medullary dorsal horn by trigeminal primary afferents (Jacquin et al., 1990; Panneton et al., 1991).

3.2. Behavioral characterizations: heat hypersensitivity

In contrast to the marked mechanical hypersensitivity, the response latency of the IoN-innervated facial region to heat stimulation was not consistently affected. The relatively lack of heat hypersensitivity may be due to differences in level of injury to myelinated vs. unmyelinated afferents in responses to ischemia (Yu et al., 2000; Hao et al., 2000).

3.3. Behavioral characterization: facial grooming/scratching

we observed hair loss and/or facial injury in the IoN territory, suggesting increased asymmetric excessive facial grooming and/or scratching. This is unlikely to be a direct result of sensory loss since this behavior coexists with mechanical hypersensitivity, but rather may be indicative of reactions of rats to some forms of spontaneous sensations in this region. Noxious facial stimulation or chemical irritation of the trigeminal nucleus caudalis also generates persistent and intense face-grooming which is distinctively different from that produced by some non-painful facial sensory disturbances (such as vibrssae removal, anesthetic IoN blockade or dripping of mineral oil**)** (Vos et al., 1998; Benoliel et al., 2002). Hence, excessive grooming/scratching may reflect the presence of ongoing pain referred to the partially denervated IoN distribution area. Alternatively, facial scratching may also be a response to other types of disturbing sensory inputs such as itch or dysesthesia.

3.4. Morphological changes

Extensive occlusion of the blood vessels were seen within the irradiated segment of the IoN, confirming previous observations made in the rat sciatic nerve (Kupers et al., 1998; Yu et al., 2000), and verifying the validity of the photochemical method to produce ischemic injury within peripheral nerves. The extent of injury appears to be related to the duration of laser irradiation, which is consistent with the previous results in rats or mice (Yu et al., 2000; Hao et al., 2000).

3.5. Expression of sodium channel mRNAs in the injured ggl V

Examination of sodium channel transcript expression in the TG 14 days after IoN injury when neuropathic pain signs were most intense revealed complex changes, including a de novo $Na_v1.3$ mRNA expression in TG cells. The up-regulation of $Na_v1.3$, which is responsible for a TTX-sensitive current, may be associated with the injury-induced increase in such currents that appear in affected DRG neurons (Waxman et al., 1994). The impact of this upregulation in neuropathic pain is debated. Boucher et al., 2000 showed that intrathecal infusion of GDNF prevented the increase of DRG $Na_v1.3$, and at the same time it prevented hypersensitivity and blocked ectopic impulse discharge in nerve-injured rats. However, GDNF reverses a number of neurochemical changes that

take place in injured DRG nerve cells (Wang et al., 2003), so an explicit relationship between an up-regulation of $Na_v1.3$ and ectopic discharge/neuropathic pain is still not provided. In a study by Nassar et al., 2006, $Na_v1.3$ null mutant mice displayed nerve injury induces allodynia and ectopic discharges with no difference compared to wildtype mice.

The mRNA expression of $Na_v1.8$- and $Na_v1.9$ decreased in the affected TG. This is in accordance with results from spinal nerve lesions (Cummins et al., 2000; Lai et al., 2004; Wood et al., 2004). The consequences of these downregulations seem less apparent. An upregulation of the channel protein associated with a functional relocalization has been reported in uninjured neighboring neurons following injury at spinal level (Gold et al., 2003). Following nerve damage, uninjured neurons will be exposed to increased levels of neurotrophic factors from the denervated organs, causing modulation of expression and channel function. It has been argued that $Na_v1.8$ is necessary for the development of neuropathic pain (Lai et al., 2004). This is supported by data showing that treatment with $Na_v1.8$ antisense oligonucleotides reverses behavior signs of pain in animal models (Lai et al., 2002). A similar treatment with $Na_v1.9$ antisense oligonucleotides does not seem to affect hyperalgesia or hypersensitivity, perhaps indicating that $Na_v1.9$ is less involved in the development of neurogenic pain after sciatic nerve trauma (Lai et al., 2004).

β3 mRNA was upregulated both with regard to proportions of positively labeled neurons and mRNA levels in individual cells. This is also in accordance with injury at the spinal level (Shah et al., 2000; Takahashi et al., 2003). The β subunits are essential modulators of sodium channel function (Isom, 2001). As a consequence an increased level of β3 might contribute to the generation of ectopic impulses in axotomized DRG cells (Takahashi et al., 2003).

Photochemically-induced ischemia produces a graded injury of the rat IoN, which is dependent on the duration of laser irradiation (Hao et al., 2000). The dose-dependency and the characteristics of the injury in the IoN as seen in the present study are reminiscent of those observed after a similar photochemical lesion to the rat sciatic nerve (Gazelius et al., 1996; Kupers et al., 1998; Yu et al., 2000). The photochemically-induced IoN injury produced behavioral changes indicative of evoked and possible ongoing pain-like responses in the facial region innervated by the IoN and a spread of mechanical hypersensitivity to the body. In the TG, which sends axons to the IoN, robust changes in

the mRNA expression of some nociceptive neuron-associated sodium channel subunits were seen after the ischemic IoN injury. It seems reasonable to assume that these changes are related to the development of pain-related behavior in the affected animals.

4. Pain behavior induced by nerve injury correlates with the expression of specific sodium channels in inbred mouse strains - paper IV

The pain rank order of the 5 mouse strains were determined based on the autotomy behavior assessed weekly until 35 dpo. Autotomy behavior was highest in C3H mice, followed by CBA, C58, B6 and AKR. Our autotomy data from 7 dpo correlates well with the ranking obtained 35 dpo, with $r=0.9$; $p<0.05$, which further strengthens the validity of our strain rank order underlying the correlation analysis of pain behavior versus gene expression.

Seven dpo sciatic and saphenous nerve transaction, induced a statistically significant downregulation of Contactin mRNA levels (n=2-9) in all five strains ($p<0,05$ AKR, C58, C57 and $p<0,001$ C3H; two-way ANOVA followed by Newman Keuls test). This finding is consistent with ISH observations of a reduction both in the number of Contactin mRNA-positive cells and in signal intensity of individual neurons. This observation implies a link between Contactin functions and the development of ectopic discharge and pain signaling observed in damaged sensory neurons. Contactin is involved in trafficking of VGSC to the axolemma (Rush *et al.*, 2005), and the repetitive firing capability of injured neurons depends on the disposition of VGSCs (Matzner & Devor, 1994). It is therefore reasonable to assume that disturbances in the functioning of Contactin might influence the electrical properties of affected neurons.

$Na_v1.3$ transcript is expressed in DRGs during development and re-appears in adult DRG cells after nerve injury (Waxman *et al.*, 1994). This fact, and the biophysical properties of $Na_v1.3$, makes it a plausible candidate for being responsible for the ectopic action potential generation and associated hyperexcitability that occurs in injured afferent neurons and that is implicated in neuropathic pain (Boucher *et al.*, 2000). However, we found a significant upregulation of $Na_v1.3$ only in one strain (using RT-PCR), and the signal was small, below detection levels with ISH. The rank order of $Na_v1.3$ expression levels post axotomy did not correlate with that of pain behavior. Taken together, our data do not support a significant role for $Na_v1.3$ in pain behavior in the neuroma model in mice. Similar conclusions were made by (Nassar et al., 2006), who demonstrated allodynia-like behavior as well as ectopic firing in damaged nerves of genetically manipulated mice that lacked $Na_v1.3$. This finding was rather surprising to us, due to the

vast amount of literature suggesting an involvement of $Na_v1.3$ in pain aroused after nerve injury. Also, we have previously showed an up-regulation of $Na_v1.3$ in the TG following an injury to the infraorbital branch of the trigeminal nerve in allodynic rats (Eriksson et al., 2005). However, that study along with most previous work on the DRG was performed on rats and it is possible that the divergent findings reflect species differences with $Na_v1.3$ contributing to the injury induced pain behavior in rats but not in mice.

$Na_v1.6$ was downregulated in the DRG in a majority of the strains 7 days after nerve injury. Levels correlated with pain behavior across strains, with the highest residual level of expression being found in the most pain-prone strains, suggests that this VGSC may make a particular contribution to ectopic firing in axotomized neurons. $Na_v1.6$ is the main VGSC isoform present at the nodes of Ranvier in myelinated axons, but it is also distributed along the axons of pain-transducing unmyelinated C-fibers (Black et al., 2002).

The expression of $Na_v1.7$ was significantly down-regulated only in C58 although a tendency towards a decreased expression pattern was seen in all of the strains.
There was no obvious change in the size distribution of the mRNA positive neurons following axotomy for any of the genes assayed. The low basal mRNA levels of $Na_v1.5$ were significantly changed after injury in one strain, AKR. On the ISH processed sections only a small number of $Na_v1.5$ positive cells could be visualized in the DRG on the lesioned side.

In the spinal cord, the most intriguing finding was the upregulation of $Na_v1.7$. ISH showed the clearest indication of increased $Na_v1.7$ expression to be localized to motorneurons in the ventral horn. However, it is not unlikely that some of the increase also occurs in the dorsal horn. Be that as it may, post axotomy levels of $Na_v1.7$ in the spinal cord were strongly correlated to the levels of pain behavior.

SPECIFIC CONCLUSIONS

- Specific TG VGSC transcripts, some of which are specifically associated with pain-transmitting neurons, mature in waves that are particular for each channel. The results suggest that some craniofacial primary sensory neuron VGSC mature earlier than their counterparts at segmental levels during development. They also clearly indicate that different facial regions may differ in the ability to transmit sensory signals during early life.

- The down-regulation of $Na_v1.8$ and $Na_v1.9$ mRNA that occurs in injured DRG cells is not influenced by IFN-γ. Thus, the reduced pain-related behavior seen in nerve-injured IFN-γ receptor knockout mice is not due to changes in the regulation of $Na_v1.8$ and $Na_v1.9$.

- Photochemically induced IoN injury produced behavioral changes indicative of evoked and possible ongoing pain-like responses in the facial region innervated by the IoN and a spread of mechanical hypersensitivity to the body. In the TG, which sends axons to the IoN, robust changes in the mRNA expression of some nociceptive neuron-associated sodium channel subunits were seen after the ischemic IoN injury. It seems reasonable to assume that these changes are related to the development of pain-related behavior in the affected animals.

- Expression of specific VGSC isoforms after injury in the DRG and the dorsal horn of the spinal cord correlate across mouse strains with pain behavior (autotomy) in the neuroma model of neuropathic pain. Nerve injury induced significantly altered levels of $Na_v1.3$, $Na_v1.5$ and $Na_v1.7$ in one strain only,. However, Contactin and $Na_v1.6$ were down-regulated in a majority of the strains to expression levels that tightly correlated to autotomy behavior. In the spinal cord, $Na_v1.7$ expression was strongly up-regulated following nerve injury to levels well correlated with pain-like behavior.

FINAL COMMENTS

When the existence of (more or less) pain-neuron specific VGSC was recognized some ten years ago, it was initially thought that this would rapidly lead to the identification of new targets for pain therapy. However, although much effort was put into the field, clearcut identification of the roles of various VGSC in different acute, inflammatory and neuropathic pain states turned out to be complicated. Thus, findings have been conflicting and as of yet no consensus seems to exist, which demonstrates the need for further studies. The results from the present thesis have in general confirmed previous observations with regard to the injury-related gene regulation in DRG and TG of some channels which have been put forward as candidates responsible for neuropathic pain behavior. Such VGSC include $Na_v1.3$, $Na_v1.8$ and $Na_v1.9$. At TG levels, the results presented here show that the VGSC are regulated much in the same way as at DRG levels after nerve injury, perhaps suggesting, assuming that VGSC are pivotal proteins in this context, a common mechanism for spinal and craniofacial neuropathic pain. However, through correlations with behavior, the findings here indicate that $Na_v1.3$ is not primarily responsible for the pain phenotype seen in rodents after nerve damage at spinal levels. They do however suggest that $Na_v1.6$ could be involved. It is anticipated that these results can be discussed in view of a body of new findings within the near future. Thus, research in the field of pain-associated VGSC and related channelopathies is at present intense, which might lead to major breakthroughs in terms of pharmacological VGSC-blocking agents to alleviate pain.

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