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Heritability and sex differences in  
experimental neuropathic pain with special  
emphasis on the role of the major  
histocompatibility complex

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*To my mother and Marcelo*

## **ABSTRACT**

Neuropathic pain caused by injury to the nervous system is a difficult clinical problem and a large proportion of patients experience no or only partial relief from existing treatments. Pain sensitivity and the development of neuropathic pain are complex biological and physiological entities that are known to be affected by genetics and sex. Although many theories have been put forward to explain neuropathic pain, the mechanisms are still largely unknown.

In the present thesis we have used a genetic approach to explore the potential involvement of the major histocompatibility complex (MHC) genes in the development of neuropathic pain-like behavior following a photochemically induced sciatic nerve or spinal cord injury in inbred and/or congenic rat strains with different MHC haplotypes. We were able to show that following peripheral nerve injury, certain allelic variants of MHC have stronger influence on the susceptibility for the development and maintenance of neuropathic pain in both males and females. We could also demonstrate that both MHC and non-MHC genes are implicated in the genetic regulation of susceptibility to neuropathic pain. However, after spinal cord injury, MHC genes do not appear to be involved in the development of central neuropathic pain.

We have also studied sex difference in the development of mechanical hypersensitivity and wide spread pain after infraorbital and sciatic nerve injury in Sprague-Dawley rats. Our results demonstrated that sex differences in the development of neuropathic pain-like behavior are dependent on site of injury as well as site of testing with females being more susceptible to develop widespread mechanical hypersensitivity, particularly after infraorbital nerve injury. This finding may lead to a rat model of the human condition of fibromyalgia and other forms of spread pain that is mostly observed in woman.

Key words: genetics, major histocompatibility complex, neuropathic pain, rat strains sex difference,

## LIST OF PUBLICATIONS

- I. **Dominguez CA**, Lidman O, Hao JX, Diez M, Tuncel J, Olsson T, Wiesenfeld-Hallin Z, Piehl F, Xu XJ. Genetic analysis of neuropathic pain-like behavior following peripheral nerve injury suggests a role of the major histocompatibility complex in development of allodynia. *Pain* 2008,136:313-319.
- II. **Dominguez CA**, Lidman O, Olsson T, Wiesenfeld-Hallin Z, Piehl F, Xu XJ. Contrasting genetic effects of major histocompatibility complex on ischemic peripheral nerve and spinal cord injury in female rats. *Neuroscience Letters* 2008, 443:95-98.
- III. **Dominguez CA**, Li L, Lidman O, Olsson T, Wiesenfeld-Hallin Z, Piehl F, Xu XJ. Both MHC and non-MHC genes regulates development of experimental neuropathic pain in rats. *Neuroscience Letters* 2008, 442:284-286.
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# CONTENTS

1	Introduction .....	1
1.1	Pain: some definitions and terms .....	1
1.2	Pain transmission.....	2
1.3	Neuropathic pain .....	3
1.3.1	Some potential mechanisms .....	3
1.3.2	Animal models of neuropathic pain .....	4
1.3.3	The role of immune system in neuropathic pain .....	4
1.4	The major histocompatibility complex (MHC).....	5
1.4.1	MHC and its functions .....	5
1.4.2	The MHC and neuropathic pain .....	7
1.5	Genetic and sex differences in pain .....	8
2	Aims of this thesis .....	10
3	Materials and methods .....	11
3.1	Animals.....	11
3.2	MHC haplotype definition and MHC congenic rats .....	12
3.3	Photochemically-induced ischemic injury.....	13
3.3.1	Sciatic nerve .....	13
3.3.2	Infraorbital nerve.....	13
3.3.3	Spinal cord injury.....	13
3.4	Behavioral testing.....	14
3.4.1	Hypersensitivity of the hindpaws .....	14
3.4.2	Hypersensitivity of the snout .....	14
3.4.3	Spread mechanical hypersensitivity after nerve injury .....	14
3.4.4	Cutaneous hypersensitivity after spinal cord injury .....	15
3.5	Morphology .....	15
3.6	Genotyping .....	15
3.7	RT-PCR analysis .....	16
3.8	Immunohistochemistry.....	16
3.9	Statistics.....	17
4	Results .....	18
4.1	Strain differences in response to sciatic nerve injury in DA, PVG and MHC congenic PVG-RT1 <sup>av1</sup> rats of both sexes .....	18
4.2	Development of mechanical hypersensitivity in male F2 rats (PVG × PVG-RT1 <sup>av1</sup> ) (Paper I) .....	18
4.3	Morphological analysis of F2 rats (Paper I) .....	19
4.4	Effects of peripheral nerve injury on the expression of MHC class I,II and TNF- $\alpha$ in PVG and congenic PVG-RT1 <sup>av1</sup> rats (unpublished results) .....	19
4.5	Strain differences in response to sciatic nerve injury in BN, PVG and MHC congenics PVG-RT1 <sup>av1</sup> and PVG-RT1 <sup>n</sup> (Paper III) .....	22
4.6	Strain difference in response to spinal cord injury in DA, PVG and MHC-congenic PVG-RT1 <sup>av1</sup> females (Paper II).....	22
4.7	Sex difference in the development of hypersensitivity in SD rats after injury to the infraorbital or sciatic nerves (paper IV) .....	23

4.7.1	Snout hypersensitivity after injury to the infraorbital nerve in male and female rats.....	23
4.7.2	Spread hypersensitivity after infraorbital nerve injury in male and female rats.....	24
4.7.3	Hindpaw hypersensitivity after sciatic nerve injury in male and female rats.....	24
4.7.4	Spread hypersensitivity after sciatic nerve injury in male and female rats.....	24
5	Discussion.....	26
5.1	The involvement of MHC in neuropathic pain-like behavior following peripheral nerve injury.....	26
5.2	The involvement of non-MHC genes in neuropathic pain-like behavior following peripheral nerve injury .....	27
5.3	The potential mechanisms of the role of MHC genes in neuropathic pain.....	27
5.4	Sex difference in the development of localized and spread mechanical hypersensitivity in rats after injury to the infraorbital or sciatic nerves .....	29
6	Conclusions.....	31
7	Acknowledgements .....	32
8	References.....	34

## LIST OF ABBREVIATIONS

Aif	Allograft inflammatory function
ANOVA	Analysis of variance
BN	Brown Norway
CNS	Central nervous system
C	Complement component
DA	Dark Agouti
DRG	Dorsal root ganglion
Hsp	Heat shock protein
IL	Interleukin
IASP	International Association for the Study of Pain
IoN	Infraorbital nerve
i.p.	Intra peritoneal
i.v.	Intravenous
MAD	Median absolute deviation
MHC	Major histocompatibility complex
NMDA	N-Methyl-D-Aspartat glutamate receptor
NSAID	Non steroidal anti-inflammatory drugs
PAG	Periaqueductal gray
PCR	Polymerase chain reaction
PVG	Piebald Virol Glaxo
RT1	Rat MHC
RT-PCR	Reverse transcriptase polymerase chain reaction
RVM	Rostral ventral medulla
SCI	Spinal cord injury
SEM	Standard error of the mean
SD	Sprague-Dawley
Th	T-helper
TNF	Tumor necrosis factor
WDR	Wide dynamic range neuron

# 1 INTRODUCTION

## 1.1 PAIN: SOME DEFINITIONS AND TERMS

The sensation of pain acts as an alarm to protect our body from external and internal harm and thus is necessary for organisms to survive. In academic terms, pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential damage, or described in terms of such damage” according to the International Association for Study of Pain (IASP) (Merskey and Bogduk 1994).

Pain can be classified into different categories using various criteria. Most commonly, pain can be classed as nociceptive, inflammatory, neuropathic and idiopathic based on its causes. Nociceptive pain is caused by activation of nociceptors responding to noxious stimulation. Nociceptive pain occurs after acute trauma and is usually temporary. However, pain can become prolonged and chronic where it serves little purpose as a warning signal. Chronic pain is mostly seen in conditions of inflammation and neuropathy.

Inflammatory pain is associated with inflammatory processes that can arise from, for example, minor infections, tissue damage, burns and various forms of chronic inflammatory diseases, including autoimmune diseases. Inflammatory pain is characterized by local release of chemical mediators from damaged tissues and inflammatory cells activating and sensitizing nociceptors. In the majority of the cases, inflammatory pain can be adequately managed by nonsteroidal anti-inflammatory drugs (NSAIDs) or opiates (Basbaum et al. 2008).

Neuropathic pain is caused by a primary lesion or dysfunction in the nervous system (Merskey and Bogduk 1994). Examples of disorders that might give rise to neuropathic pain include traumatic nerve injury, diabetes, trigeminal neuralgia and postherpetic neuralgia. Central pain is a special form of neuropathic pain resulting from injury or diseases in the central nervous system (CNS), such as spinal cord injury, stroke or multiple sclerosis. People suffering from neuropathic pain often have spontaneous, ongoing pain of burning or stabbing quality. Allodynia (pain to innocuous stimulation) is common in patients with neuropathic pain such that a simple movement or light

touch can be perceived as very painful (Jensen et al. 2001). The mechanisms of neuropathic pain are not well understood and it is considered difficult to manage.

## **1.2 PAIN TRANSMISSION**

The experience of pain starts from the detection of the noxious stimulus by nociceptors. The stimulus can be of various modalities, such as mechanical, thermal or chemical. Some nociceptors are stimulus specific whereas some are polymodal. The activation of nociceptors triggers impulses that travel along axons of sensory nerves through the dorsal root ganglion (DRG) and reach the dorsal horn of the spinal cord. There are three types of sensory fibers that respond preferably to different types of stimuli. The large diameter A $\beta$ -fibers are highly myelinated and respond preferably to mechanical stimulation of low intensity such as light touch or vibration. The A $\delta$ -fibers are thinly myelinated and they respond to mechanical stimulation of higher intensity (including in the noxious range) and thermal stimulation. The C-fibers which, are unmyelinated respond to noxious stimuli of various modalities (D'Mello and Dickenson 2008).

The primary afferents terminate in the spinal cord in a highly organized fashion. A $\delta$ - and C- fibers terminates in laminae I and II although a few reach deeper laminae (Light and Perl 1979), whereas large myelinated fibers terminate in the deeper laminae. Many neurons within laminae I and II are nociceptive specific and comprise projection neurons to different areas of the brain, namely to thalamus, periaqueductal grey (PAG) and spinoparabrachial area (Bester et al. 1997; Todd 2002). Laminae III and IV contains cells that respond to mechanical stimulation mediated by the A $\beta$ -fibers. Wide dynamic range (WDR) neurons are a particular type of dorsal horn neuron, which have large receptive fields and respond to a range of stimuli coming from all three types of sensory fibers. The WDR neurons are located in large numbers in lamina V and project to the brain stem and to regions of the thalamus via the spinothalamic tract.

When the neuronal network in the dorsal horn is activated the processing and integration of incoming signals will lead to spinal level responses, often in the form of reflexes. Furthermore, neurons in the dorsal horn will also transmit the information to the brain through ascending tracks, terminating in different targets in the midbrain and thalamus. The thalamus is the main brain region responsible for the integration of pain input and for the generation of the sensory component of pain. The thalamus also

forwards the pain signals to other structures of the brain, the somatosensory cortex (where pain awareness occurs) and the limbic system (the emotional aspects of pain is determined) (Bester et al. 2000).

Brainstem structures like the PAG and rostral ventral medulla (RVM) also influence pain modulation in the dorsal horn via descending pathways and the release of monoaminergic neurotransmitters such as norepinephrine or 5-hydroxytryptamine that have either facilitatory or inhibitory properties (Dickenson and Bee 2008, Millan 2002).

### **1.3 NEUROPATHIC PAIN**

#### **1.3.1 Some potential mechanisms**

Neuropathic pain is a difficult clinical situation for which effective treatments are lacking and the mechanisms are not well understood. Complex changes in the peripheral and central nervous system occur in response to peripheral nerve injury. Injured nerve fibers and sensory neurons have altered function, threshold, excitability and transmission properties. Neighboring non-injured sensory neurons may also be affected chemically and electrophysiologically (Ali et al. 1999). Profound plasticity in gene expressions can be observed at various levels of the nervous system after an injury (Costigan et al. 2002) resulting in, for example in the DRG, a rather markedly different peptidergic make-up of sensory neurons following nerve injury (Hökfelt et al. 1994). Sodium channels will be accumulated at the site of injury as well as in the DRG, which may be responsible for the generation of spontaneous action potentials (ectopic firings) (Devor 2006). Nerve injury may also lead to an activity-dependent increase in spinal cord excitability known as central sensitization, which may be responsible for the allodynia and hyperalgesia seen in patients with neuropathic pain (Decosterd et al. 2002). Activation of N-methyl-D-aspartate (NMDA) receptors by glutamate binding, initiates an influx of calcium into the postsynaptic neuron leading to a cascade of biochemical changes that ultimately contribute to strengthening of central sensitization (Woolf and Mannion 1999).

### **1.3.2 Animal models of neuropathic pain**

Animal models are of major significance to our understanding of the underlying mechanisms of neuropathic pain. They are also useful in developing new analgesics and treatments. There are several well-established animal models of neuropathic pain with peripheral and central nervous system injuries that mimic various aspects of the human symptoms. Several commonly used rodent models of peripheral nerve injury involve partial mechanical injury to somatic nerves innervating the hindpaw, such as the sciatic nerve or spinal nerve (Bennet and Xie 1988; Seltzer et al. 1990; Kim and Chung 1992). Nevertheless, models using other forms of injury (such as ischemia, toxin or viral infection) or affecting nerves innervating other tissues (such as facial region) has also been developed (Xu and Wiesenfeld-Hallin 2003). There are also several animal models of central neuropathic pain, such as spinal cord injury pain as produced by weight drop injury (Siddall et al. 1995), surgical hemisection (Christensen et al. 1996) and ischemia (Hao et al. 1991).

In my studies, I have used several well-characterized rat models of ischemic injury by a photochemical method. These models include injury to the sciatic (Gazelius et al. 1996; Kupers et al. 1998) and infraorbital nerves (IoN) (Eriksson et al. 2005) and to the spinal cord (Xu et al. 1992). We have studied mainly mechanical hypersensitivity associated with these models reminiscent of the human condition of mechanical allodynia. The advantage of the model is that the level of injury can be adjusted by the intensity and duration of photochemical exposure to the laser, leading to consistent and reproducible injury. In the peripheral nerve, the injury affects both myelinated and unmyelinated fibers (Yu et al. 2000; Eriksson et al. 2005).

### **1.3.3 The role of immune system in neuropathic pain**

It has been recognized that the immune cells and glia play an important role in the development and maintenance of neuropathic pain both in the periphery and in the CNS (Watkins and Maier 2002; Marchand et al. 2005; McMahon et al. 2005). Although, glia cells are not involved in pain processing under normal conditions, they become activated after nerve injury. During Wallerian degeneration, Schwann cells start to synthesize a cocktail of mediators that recruit macrophages, neutrophils and T-cells. These in turn release chemoattractant signals that recruit proinflammatory agents, resulting in sensitized nociceptors in the periphery (Thacker et al. 2007). Neutrophils

further reinforce the recruitment of macrophages by an early release of cytokines and a depletion of the circulating neutrophils has been demonstrated to reduce the development of neuropathic pain (Perkins and Tracey 2000). In the DRG there is an increased synthesis and release of proinflammatory cytokines including interleukins (ILs) 1, 6 and tumor necrosis factor alpha (TNF- $\alpha$ ), which modulates the neuronal activity and evokes ectopic discharge (Scholz and Woolf 2007).

Although for a long time it was believed that the central nervous system (CNS) was isolated from the immune system, it is known today that immune cells can gain access to the CNS (Moalem et al. 1999). Microglia, astrocytes and oligodendrocytes are examples of glia cell present in the CNS and act as immune cells when activated. It has been well established in recent years that following an injury to the peripheral nerve, microglia and astrocytes start to produce inflammatory cytokines, and chemokines in the spinal cord that has been shown to play an important role in altered spinal pain processing after nerve injury (Watkins and Maier 2002; McMahon et al. 2005).

## **1.4 THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)**

### **1.4.1 MHC and its functions**

The major histocompatibility complex (MHC) is a cluster of approximately 200 genes with a size of about 4Mb and is located on chromosome 6 in humans, chromosome 17 in mice and chromosome 20 in rats (Murphy et al. 2001). The majority of the genes within the MHC have immunoregulatory function and have been associated with several autoimmune conditions, such as multiple sclerosis, rheumatoid arthritis and diabetes, in which pain symptoms are often very common (Weissert et al. 1998; Murphy et al. 2001).

In the rat, the MHC (denoted RT1) is divided into the following regions: the centromeric class I region, the class II region, the class III regions and the telomeric class I region (Figure 1). The centromeric class I region encodes for the classical MHC class I genes, also referred to as class Ia genes. These genes are well characterized and their main function is processing and presentation of antigens to cytotoxic T-cells. The Ia genes can vary in numbers depending on the haplotype of the rat (Dressel et al.

2001). However, the telomeric class I genes, also referred to as non-classical class I genes, or Ib genes, encode for genes whose functions are still largely unknown. In general the MHC class I genes are present on most nucleated cells and apart from interacting with CD8<sup>+</sup> T-cells also play a crucial role in synaptic plasticity following peripheral nerve lesion (Oliveira et al. 2004).

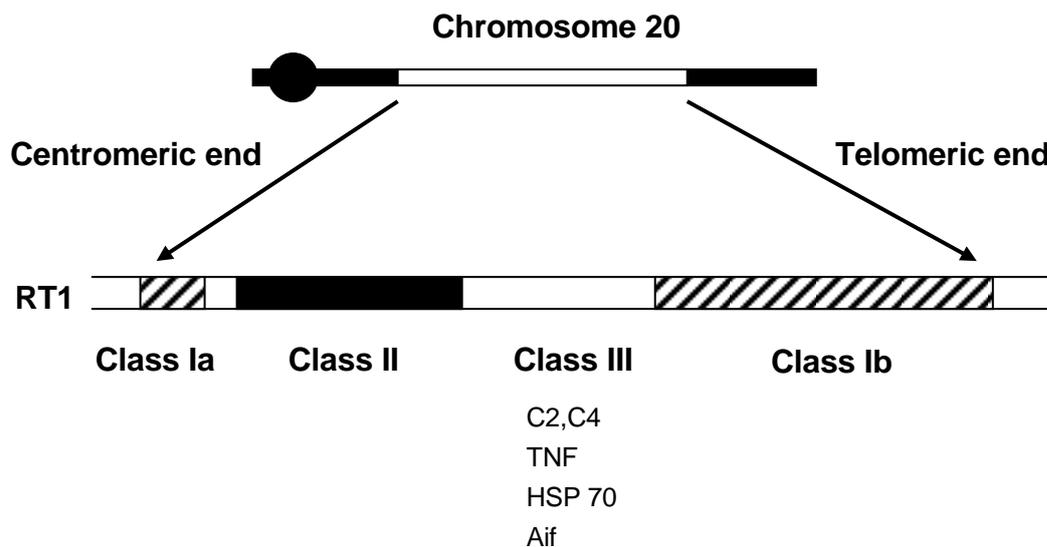


Figure 1. Scheme of the rat MHC (RT1) on chromosome 20.

The MHC is divided into the following regions: the centromeric class I region (Ia), the class II region, the class III region and the telomeric class I region (Ib). Some of the genes expressed within Class III are complement components (C2 and C4), tumor necrosis factor (TNF), heat shock proteins (HSP 70) and allograft inflammatory function (Aif). The regions are approximately drawn to scale. (Modified picture from (Gunther and Walter 2001).

The MHC class II molecules are only present in antigen presenting cells and their main functions involve interacting with CD4<sup>+</sup> T-cells. Upon activation, the CD4<sup>+</sup> T-cells differentiate to type 1 helper cells (Th1) and type 2 helper cells (Th2). The Th1 cells will produce proinflammatory cytokines like interferon- $\gamma$ , TNF- $\alpha$  and IL-2. This, in turn will stimulate phagocytosis by activating macrophages, neutrophils and natural killer cells. On the other hand Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, IL-10 and IL-13, that when activated act as cell regulators of the immune response by inhibiting the production of proinflammatory cytokines (London et al.

1998). MHC class II does not only serve as antigen presenter to T-lymphocytes but is also used as a marker for microglia activation.

Genes within the MHC class III encode for molecules with other roles in the immune system. There are cytokines, such as TNF- $\alpha$ , lymphotoxin (TNF- $\beta$ ) and complement components like C2, C4, and factor B, as well as the heat shock protein (Hsp)70 family (Dressel et al. 2001).

There are, however, many more genes within the MHC cluster that do not yet have any characterized functions. Because of the variety of diseases associated with the MHC, the region has been extensively studied and many inbred, congenic and recombinant strains of rat models have been developed (Gill et al. 1989; Weissert et al. 1998; Joe et al. 1999).

#### **1.4.2 The MHC and neuropathic pain**

As mentioned previously, the immune system has been shown to play an important factor in neuropathic pain, which suggests the possibility that MHC genes may be involved to some extent in neuropathic pain mechanisms. The MHC has a role as antigen presenting cells in the immune system and acts as mediator between activated glia cells and the immune system (Sweitzer et al. 2002; Griffin et al. 2007).

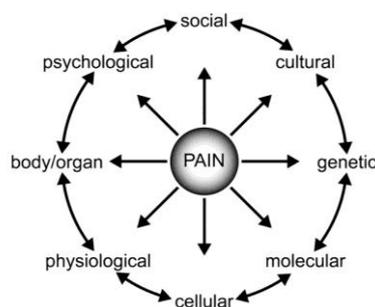
In the normal nervous system no or only low levels of MHC class I and class II molecules have been detected in the DRG and spinal cord. Following a peripheral nerve lesion, there is an increased expression of both MHC class I and class II on neurons and glia cells (Maehlen et al. 1988; Streit et al. 1989; Moalem et al. 1999). MHC class II may contribute to the development of neuropathic pain through further release of mediators that enhance central sensitisation. Mice lacking the MHC class II have been shown to exhibit decreased allodynia after peripheral nerve transection compared to wild type mice (Sweitzer et al. 2002). TNF- $\alpha$  is also regulated following peripheral nerve injury and has been suggested to be an important mediator in neuropathic pain (Wagner and Myers 1996; DeLeo et al. 1997). TNF antagonists counteract the increased levels of TNF- $\alpha$  after nerve injury and reduce the development of mechanical allodynia (Sweitzer et al. 2001; Schafers et al. 2003).

In a recent study with three different neuropathic pain models it was observed that 54 genes were commonly regulated across the models and that the main functions of the largest regulated group were associated with immune function (Griffin et al. 2007). Among the most regulated ones were allograft inflammatory function (Aif) and C4, which are two molecules found within the MHC cluster. There is also behavioral evidence that the complement cascade contributes to the development of mechanical hypersensitivity and is involved in spinal sensitization of nociception (Twining et al. 2005).

## **1.5 GENETIC AND SEX DIFFERENCES IN PAIN**

Pain is a complex biological and physiological entity that is affected by a large number of factors. One of the important aspects of pain is genetic factors in pain sensitivity and in the development of chronic pain. It is well established that there is wide variability in threshold sensitivity and tolerance level to noxious stimulation among human populations which cannot be fully explained by environmental and cultural causes (Mogil 1999). Genetic studies have identified that in humans, acute pain sensitivity and tolerance as well as many chronic pain conditions have genetic factors (Mogil and Devor, 2004). There is also ample evidence that this is also the case for experimental animals, including in neuropathic pain models (Mogil and Devor, 2004, Xu and Wiesenfeld-Hallin, 2004). Different strains of rodents that have been subjected to the same degree of injury readily show differential development of neuropathic pain-like behavior even when environmental factors have been controlled (Wiesenfeld-Hallin et al. 1993; Mogil et al. 1999; Xu et al. 2001). Autotomy behavior, a sign of neuropathic pain, has been demonstrated to be inherited as a single-gene autosomal recessive trait (Devor and Raber 1990). In fact, two genomic regions containing one gene or genes influencing susceptibility to neuropathic pain-like behavior have been identified in rodents with the help of genetic approaches. The region found in mouse is denoted *Pain 1* and is located on chromosome 15 (Devor et al. 2005). The second one is located on rat chromosome 2 and is denoted *Pain 2* (Nissenbaum et al. 2007). Thus, by dissecting genes correlated to pain, not only does this produce better understanding of pain mechanisms, it may also lead to the discovery of new pain processing molecules.

Another factor influencing pain sensitivity that was studied in this thesis is related to sex differences in pain. Abundant experimental and clinical studies have demonstrated that there are sex differences in pain sensitivity in that females usually have lower pain thresholds and pain tolerance to different types of noxious stimuli (Aloisi et al. 1994; Berkley 1997; Fillingim et al. 1999; Barrett et al. 2002; Greenspan et al. 2007). Many clinical chronic pain conditions, such as trigeminal neuralgia and fibromyalgia are also more prevalent in women than in men (Greenspan et al. 2007). Women also report more anatomically diffuse pain states, which are of longer duration than men (Hurley and Adams 2008). The response to analgesia has also been demonstrated to be sex dependent. For example, morphine appears to have a stronger analgesic response in males compared to females (Mogil et al 200; Berkley et al. 2006). Studies of sex differences in pain in humans can however be complicated since it is influenced by the stereotypical social gender roles (Levine and De Simone 1991) as well as psychological factors (eg, anxiety and depression) in addition to biological factors (eg, gonadal hormones, genetics and endogenous pain inhibition) (Wiesenfeld-Hallin 2005) (Figure 2).



Holdcroft, A.I. and Berkley, K. J.  
 In: Wall and Melzack's Textbook of Pain, 2006

Figure 2. Many factors contribute to the gender differences in pain.

In animal models of neuropathic pain, the role of sex has been unclear. Some studies have shown differences in baseline withdrawal frequency following mechanical stimulation, but no difference in the development of mechanical hypersensitivity following injury (Bourquin et al. 2006). Others have found that sex-differences in the development of neuropathic pain in rodents are strain-dependent (DeLeo and Rutkowski 2000; Mogil et al. 2000).

## **2 AIMS OF THIS THESIS**

The overall aim of this thesis was to further elucidate the role of genetic and sex factors on the development of neuropathic pain with special emphasis on the MHC.

In particular,

- **1. To study the role of MHC and sex in the development of neuropathic pain-like behavior in response to photochemically induced sciatic nerve injury in rats.**
- **2. To study and compare the role of MHC in the development of neuropathic pain-like behavior in peripheral vs. central nervous system injury.**
- **3. To study and compare sex differences in the development of mechanical hypersensitivity and widespread pain after a photochemically-induced infraorbital vs. sciatic nerve injury.**

### 3 MATERIALS AND METHODS

#### 3.1 ANIMALS

The protocol of all experiments conducted within the scope of this thesis have been approved by the local ethical committee (Stockholms Södra Försöksdjursetiska Nämnd) and the experiments were performed in agreement with the Ethical Guidelines of the International Association for the Study of Pain.

All animals were kept under specific pathogen-free and climate-controlled conditions with 12 h light/dark cycles. They were housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water was available ad libitum. The experiments were conducted on male or female rats of various inbred and outbred strains weighing between 150-300g at the beginning of each experiment as specified in Table 1.

Strains	Description (MHC haplotype)	Paper	Source
Piebald Virol Glaxo (PVG)	RT1 <sup>c</sup>	I-III	Scanbur BK AB (Sollentuna, Sweden)
Dark-Agouti (DA)	RT1 <sup>av1</sup>	I-II	Scanbur BK AB (Sollentuna, Sweden)
MHC-congenic PVG-RT1av1	RT1 <sup>av1</sup>	I-III	Harlan UK Ltd. (Blackthorn, UK)
F2 (PVG × PVG-RT1av1)	Homozygot/Heterozygot of RT1 <sup>c</sup> or RT1 <sup>av1</sup>	I	Center for Molecular Medicine (Solna, Sweden)
Brown-Norway (BN)	RT1 <sup>n</sup>	III	Harlan UK Ltd. (Blackthorn, UK)
MHC-congenic PVG-RT1n	RT1 <sup>n</sup>	III	Provided by Prof G. Butcher (Cambridge, UK)
Sprague-Dawley (SD)	outbred rats	IV	Mollegård, Denmark

Table 1. Rat strains included in each paper.

### 3.2 MHC HAPLOTYPE DEFINITION AND MHC CONGENIC RATS

The different variation of genes at a specific position is termed allele and the different combination of MHC alleles found on a single chromosome is known as MHC haplotype. Throughout Papers I-III we have studied different inbred strains of rats carrying strain-specific haplotypes of the MHC (Table 1).

Congenic rats are of importance to study since they carry a specific genomic region from a donor rat (Figure 3). In our congenic rats (PVG-RT1<sup>av1</sup> and PVG-RT1<sup>n</sup>) this specific genomic region is the MHC. The MHC congenic rats are created by backcrossing the MHC haplotype from a donor strain (eg, DA or BN rats) into a recipient strain (eg, PVG rats) for over 10 generations. By including MHC-congenic rats in Papers I-III we have been able to study the inserted genomic region and its behavioral effects.

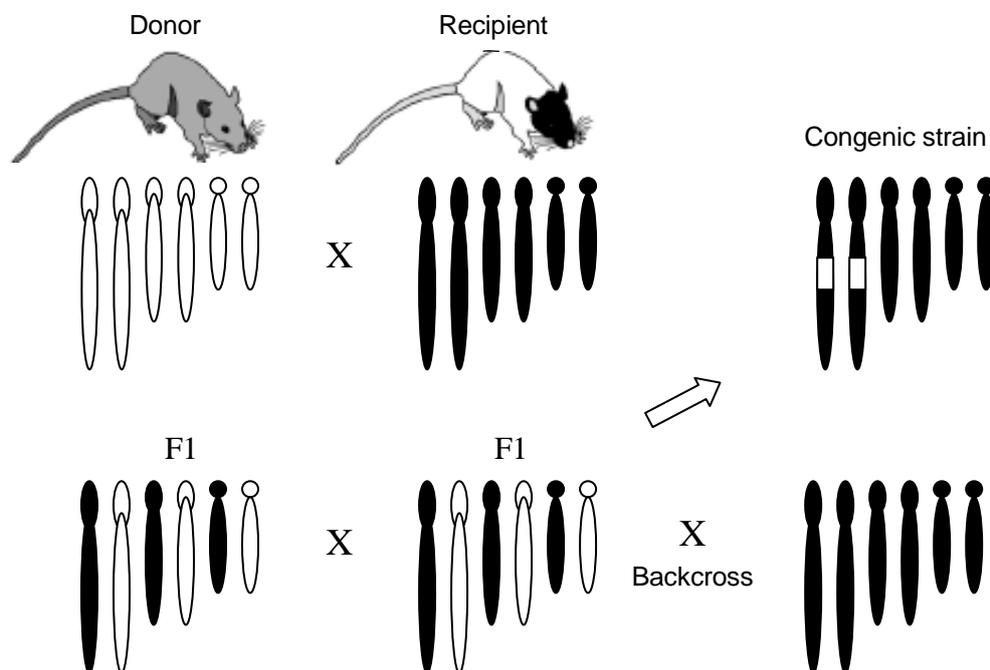


Figure 3. Illustration of the creation of a congenic strain. The congenic strains carry the donor's MHC haplotype but in the recipient's genome.

### **3.3 PHOTOCHEMICALLY-INDUCED ISCHEMIC INJURY**

#### **3.3.1 Sciatic nerve**

The common sciatic nerve was exposed at midhigh level under chloral hydrate anesthesia (Sigma, 300 mg.kg i.p.) and gently dissected from the surrounding tissue over a distance of about 1 cm proximal to the trifurcation. The part of the nerve just proximal to the trifurcation was irradiated for 90 s with a tunable argon ion laser (Innova model 70, Coherent Laser Products Division, Palo Alto, CA, USA) operating at 514 nM and with an output of 0.16 W. The rat was positioned so that the laser beam was perpendicular and transversal to the exposed nerve. Immediately before irradiation, the photosensitizing dye, Erythrosin B (Aldrich, 32.5 mg/kg) was injected intravenously (i.v.) via the tail vein. The interaction between the dye and the laser beam causes vascular occlusion and focal ischemia (Gazelius et al.1996; Kupers et al. 1998). After irradiation the wound was closed in layers and the rats were returned to their home cages.

#### **3.3.2 Infraorbital nerve**

Under chloral hydrate anesthesia the left IoN was exposed via a longitudinal incision at the maxillary region. The different branches of the IoN were held together and lifted by a glass hook while a piece of aluminium foil was placed under the nerve. The nerve was irradiated for 6 min as described above. Immediately before irradiation Erythrosin B was injected i.v. via the tail vein and the injection was repeated after 5 min. After irradiation the wound was closed in layers and the rats were returned to their home cage for recovery.

#### **3.3.3 Spinal cord injury**

Under chloral hydrate anesthesia, a midline incision was made on the skin overlying T12-L1 vertebral segments. The rats were placed under the laser beam and irradiated as described above at vertebral segment T13 (spinal cord segments L4-5), for 10 min. A jugular vein was cannulated and just prior to irradiation Erythrosin B was injected i.v at the same dose as for sciatic nerve irradiation. Erythrosin B injection was repeated after 5 min of irradiation. After irradiation the wound was closed and the rats were returned to their home cage.

## **3.4 BEHAVIORAL TESTING**

For all behavioral tests the animals were always habituated for several days before the start of the experiments in the testing situation. Baseline measurements were usually taken for 3 days before the operation procedure and the development of neuropathic pain-like behaviors was then followed for various time periods after nerve injury. In some experiments, the behavioral testers were blind to the experimental groups.

### **3.4.1 Hypersensitivity of the hindpaws**

Rats were placed in testing chambers with a metal mesh floor 10 min before the experiments. A set of calibrated nylon monofilament (von Frey hairs, Stoelting, IL) was applied to the glabrous skin of the paws with increasing force until the animal withdrew the limb. Each monofilament was applied 5 times and the force at which the rat withdrew the paw from at least 3 out of 5 consecutive applications was taken as pain threshold.

### **3.4.2 Hypersensitivity of the snout**

Von Frey filaments were applied in ascending order to the bilateral IoN territory on the hairy skin of the vibrissal pad. The stimulation with each filament consisted of five consecutive stimuli at 1s intervals on the injured and then on the contralateral side. The response threshold was taken as the force at which the rat demonstrated either a withdrawal reaction or escape/attack in 3 out of the 5 stimulations.

### **3.4.3 Spread mechanical hypersensitivity after nerve injury**

The skin areas of the neck, upper and lower back and flanks were tested. 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.

#### 3.4.4 Cutaneous hypersensitivity after spinal cord injury

Vocalization thresholds to mechanical stimuli were tested after spinal cord injury. The animals were gently restrained in a standing position and the von Frey hairs were pushed onto the skin of the upper or lower back area, in dermatomes rostral to the irradiated spinal segments, with increasing force until the filament became bent. The intensity of stimulation that induced consistent vocalization (>75% response rate) was considered as pain threshold.

### 3.5 MORPHOLOGY

In rats that had undergone sciatic nerve injury a 2-3 mm piece of the lesioned area of the nerve, as well as from a corresponding part of the intact contralateral nerve, was removed and immersion-fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH7.2) with 3 mM CaCl<sub>2</sub> and 0.1 M sucrose. The tissue was rinsed in 0.15 M sodium cacodylate buffer containing 3 mM CaCl<sub>2</sub> and subsequently osmicated in 2% OsO<sub>4</sub> diluted in 0.07 sodium cacodylate buffer containing 1.5 mM CaCl<sub>2</sub> for 2 hours at 0°C. Thereafter, it was dehydrated in several alcohol and acetone steps, and finally embedded in LX-112 plastic (Ladd Research Industries Inc., Williston, VT). One-micron sections were collected at four different levels throughout the injury area and the sections were thereafter stained with Toluidine blue.

### 3.6 GENOTYPING

Genomic DNA was extracted from tissue sampled from F2 (PVG × PVG-*RT1<sup>av1</sup>*) rats using a standard protocol (Laird et al. 1991). PCR primers for polymorphic simple sequence length polymorphisms (SSLPs), flanking and spanning the *RT1* locus, were selected from two Internet databases (Rat Genome Database (<http://rgd.mcw.edu>) and the Ensembl Genome Browser ([http://www.ensembl.org/Rattus\\_norvegicus](http://www.ensembl.org/Rattus_norvegicus)). The following SSLPs were used: D20Wox18, D20Rat47, D20Arb2, D20Wox17, D20Wox4, D20Got4, D20Rat42, D20Rat66, and D20Rat45. Primers were purchased from GENSET (Paris, France). One primer in each pair was labeled with [ $\gamma$ -<sup>33</sup>P]ATP (PerkinElmer, Boston, MA). PCR amplification of genomic DNA was performed as described previously (Jacob et al. 1995), and the amplified fragments were separated on 6% polyacrylamide gels. Genotypes were recorded manually from autoradiographic films independently by two investigators. DNA from PVG and PVG-*RT1<sup>av1</sup>* were

included for every marker to verify expected polymorphisms between the two alleles. F2 rats having recombinations within the *RTI* were not further included in the experiment. In addition, the number of heterozygous rats was reduced to be approximately one third of the total number of animals. Following the peripheral nerve injury experiments the rats were re-genotyped with three of the microsatellites in order to verify the genotypes. In this analysis one out of 66 rats displayed an uncertain genotype for one of the markers and this rat was excluded from further analysis.

### **3.7 RT-PCR ANALYSIS**

The ipsilateral and contralateral nerve, DRGs L4 and L5 and spinal cord segments L4 and L5 were dissected from 4 controls and 6 injured PVG and the congenic PVG-*RTI<sup>av1</sup>* at 7 days post injury by deeply anaesthetizing the rats with chloral hydrate and perfusion through the ascending aorta with PBS containing Heparin (Leo Pharma A/S, Ballerup, Denmark) The tissue was used for quantitative analysis of the following markers:  $\beta$ -microglobuline, Cd74, and TNF- $\alpha$  on the RNA levels. The tissue was homogenised with FastPrep Lysing Matrix D (QBiogene, CA) and RNA was obtained by the use of RNeasy Mini Kit (Qiagen, Hilden, Germany). To promote degradation of genomic DNA the samples were DNase treated with RNase-Free DNase Set, Qiagen. The obtained RNA was eluted in 35 $\mu$ l RNase free water and reverse transcription was performed by further processing the samples with 10  $\mu$ l of total RNA, random hexamer primers (Invitrogen, Carlsbad, CA), nucleotides (GE Helthcare) and Superscript Reverse Transcriptase (Invitrogen). The mRNA expression was calculated as the ratio of the target and the corresponding housekeeping genes, Gapdh and Hprt.

### **3.8 IMMUNOHISTOCHEMISTRY**

At 7 days following photochemical injury to the sciatic nerve, spinal cord tissue was dissected from PVG and congenic PVG-*RTI<sup>av1</sup>* rats for immunohistochemical analysis of MHC class I and MHC class II. Fourteen  $\mu$ m transverse sections were cut on a cryostat and mounted 4 animals per slide. The sections were washed in PBS and incubated for 30 min in blocking-solution (3% normal donkey serum, 5% bovine serum albumin (BSA), 0.3% Triton X-100, 0.1% Natriumazid and PBS) at room temperature and followed by incubation overnight with the primary antibody mouse anti rat OX-18 or OX-6 (1:200, AbD serotec, Oxford, UK), at 4<sup>o</sup> C. Next, the sections were washed

with PBS followed by a 45 min incubation in Cy3-conjugated donkey anti-mouse (1:500 Jackson immunoResearch , West Grove, TA, USA), washed in PBS and finally coverslipped with a drop of glycerol (9:1).

### **3.9 STATISTICS**

The data are presented as mean $\pm$ SEM or median $\pm$ median absolute deviation (MAD) and analyzed accordingly. Statistical analysis was conducted using the Statview computer program (SAS institute, Cary, NC). The data were analysed with ANOVA and ANOVA with repeated measures followed by Fisher PLSD test or by Dunnett's test. Individual comparison was made with paired t-test.

## 4 RESULTS

### 4.1 STRAIN DIFFERENCES IN RESPONSE TO SCIATIC NERVE INJURY IN DA, PVG AND MHC CONGENIC PVG-RT1<sup>AV1</sup> RATS OF BOTH SEXES

In the first series of experiments, mechanical sensitivity was assessed with von Frey hairs before and for 6 weeks after unilateral nerve injury in adult inbred male DA, PVG and MHC-congenic PVG-RT1<sup>av1</sup> rats. No differences were observed for baseline mechanical withdrawal threshold among the strains (Figure 1, Paper I). After nerve injury, all strains developed bilateral mechanical hypersensitivity, although the severity and duration was greater on the ipsilateral side compared to the contralateral side. There was significantly less ipsilateral mechanical hypersensitivity in the DA and congenic PVG-RT1<sup>av1</sup> strains in comparison to the more sensitive PVG rats (Figure 1, Paper I).

As in the males, among the female rats of these three strains there were no significant differences in baseline response threshold (Figure 1, Paper II). All three strains developed mechanical hypersensitivity, although the magnitude and duration differed. As observed in the male rats, there was a significant difference in the females as well, where again DA and congenic PVG-RT1<sup>av1</sup> showed less hypersensitivity than the more sensitive PVG rats (Figure 1, Paper II). Furthermore, no significant difference was found between male and female rats in the development of hypersensitivity after sciatic nerve injury in these three strains.

### 4.2 DEVELOPMENT OF MECHANICAL HYPERSENSITIVITY IN MALE F2 RATS (PVG × PVG-RT1AV1) (PAPER I)

Differences in phenotypes between a congenic strain and the recipient parental strain may depend on contaminating genes from the donor strain of the congenic fragment or the accumulation of genetic differences outside of the congenic fragment through breeding. Therefore, an intercross experiment between PVG and PVG-RT1<sup>av1</sup> was set up in order to test for the possibility that phenotypic differences depended on potentially contaminating genes residing in the background genome of the PVG-RT1<sup>av1</sup> rat, or in other words, to verify that the difference in neuropathic pain-like behavior

observed between PVG and PVG-*RTI<sup>av1</sup>* was actually linked to the MHC locus. The F2 rats were subjected to sciatic nerve injury and the behavioral tests were the same as in the previous experiments. The study revealed that rats that were either homozygous or heterozygous for the *RTI<sup>av1</sup>* exhibited reduced development of mechanical hypersensitivity in comparison to the *RTI<sup>c</sup>* homozygous rats, thus establishing a linkage to the MHC complex for this phenotype (Figure 2, Paper I).

### **4.3 MORPHOLOGICAL ANALYSIS OF F2 RATS (PAPER I)**

A group of rats from the F2 cohort were randomly selected for assessments of morphological changes in the epicenter of the injury in the sciatic nerve 7 days following irradiation. Although all rats examined showed morphological alterations consistent with an ischemic injury, no structural differences could be observed in terms of occluded blood vessels, amount of myelin or axonal degeneration between RT1av1/av1 and RT1c/c (Figure 3, Paper I).

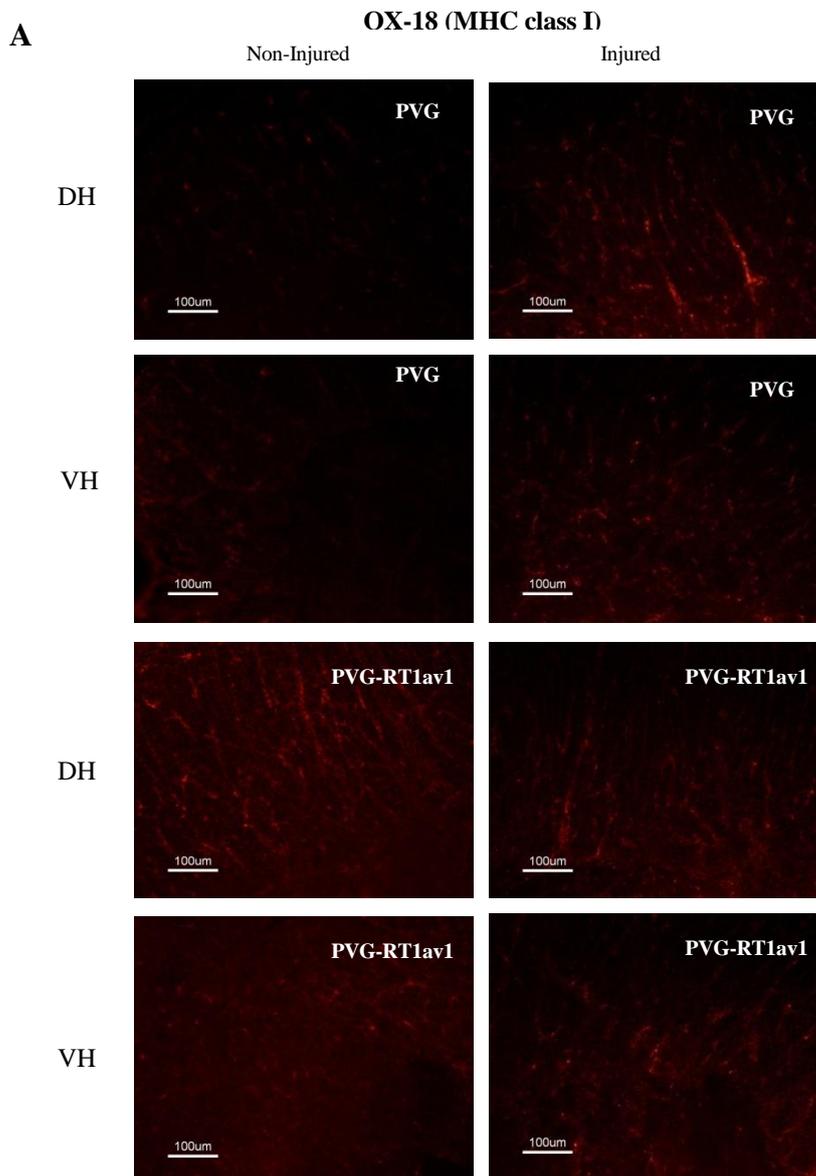
### **4.4 EFFECTS OF PERIPHERAL NERVE INJURY ON THE EXPRESSION OF MHC CLASS I,II AND TNF- $\alpha$ IN PVG AND CONGENIC PVG-RT1<sup>AV1</sup> RATS (UNPUBLISHED RESULTS)**

In an attempt to explore the mechanisms of MHC involvement of nerve injury-induced neuropathic pain-like behaviors, we collected tissue from peripheral nerve, DRG and spinal cord at 7 days following sciatic nerve injury from PVG and the congenic PVG-*RTI<sup>av1</sup>* rats, for RT-PCR and immunohistochemical analysis of MHC class I, MHC class II and for TNF- $\alpha$ .

The MHC class I levels were determined with beta-2-microglobulin ( $\beta$ -2m) which is co-expressed when MHC class I complex is formed. No significant differences were detected before and after injury in either PVG or congenic PVG-*RTI<sup>av1</sup>* in the nerve, DRG and spinal cord before and after injury. The MHC class II levels were detected with Cd74, which is an integral membrane protein, co-expressed with MHC class II. In the nerve there was a significant upregulation of Cd74 after injury compared to the uninjured side in both PVG and congenic PVG-*RTI<sup>av1</sup>*. In the DRG there was only a significant upregulation in the injured congenic PVG-*RTI<sup>av1</sup>* compared to non-injured, but not in the PVG. In the spinal cord there were no differences between non-injured

and injured animals in the two strains. TNF- $\alpha$  levels showed a tendency to be upregulated after injury in PVG rats in the nerve and was significantly upregulated in the DRG and spinal cord. No changes were detected before and after injury in any of the three structures in the congenic PVG-RT1<sup>av1</sup>.

Because of the limited expression of MHC class I and class II in the spinal cord in non-injured and injured rats, as observed in the RT-PCR analysis, we conducted further immunohistochemical analysis on the spinal cord 7 days following sciatic nerve injury. We observed MHC class I and class II immunolabeling in the spinal cord (segments L4-5) both in the dorsal and ventral horns, but again at very low levels and no clear upregulation was detected in the injured rats compared to non-injured in both strains (Figure 4).



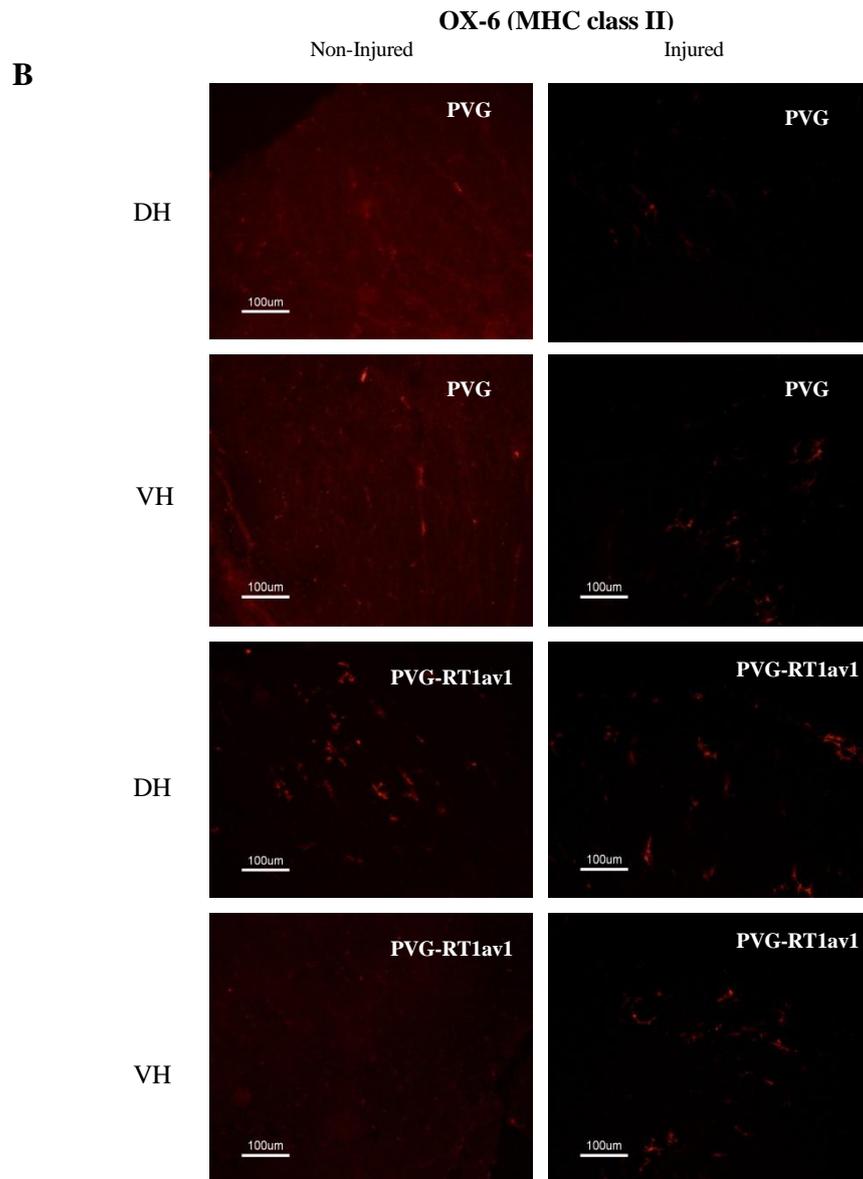


Figure 4. Immunohistochemical analysis of MHC class I (OX-18) (A) and MHC class II (OX-6) (B) in injured and non-injured PVG-RT1<sup>C</sup> and PVG-RT1<sup>av1</sup> rats. The immunoreactivity is observed in ipsilateral dorsal horns (DH) and ventral horns (VH) in spinal cord segments L4-5. Calibration is 100 µm.

#### **4.5 STRAIN DIFFERENCES IN RESPONSE TO SCIATIC NERVE INJURY IN BN, PVG AND MHC CONGENICS PVG-RT1<sup>AV1</sup> AND PVG-RT1<sup>N</sup> (PAPER III)**

In order to further evaluate the contribution of MHC genes to the development of neuropathic pain-like behaviors after sciatic nerve ischemic injury, we have in an additional experiment studied another MHC-congenic rat strain, PVG-RT1<sup>n</sup> that carries MHC haplotype n on a PVG background, and compared it to its donor strain BN rat as well as to PVG and the congenic PVG-RT1<sup>av1</sup> that have been previously studied.

In contrast to previous results, BN displayed significantly elevated baseline mechanical withdrawal thresholds compared to the other strains (Figure 1, Paper III). Photochemically-induced sciatic nerve injury produced development of mechanical hypersensitivity of the ipsilateral hindpaw in all strains studied. Analysis of the behavioral data with ANOVA with repeated measures followed by Fischer PLSD test revealed significant overall strain differences with PVG and congenic PVG-RT1<sup>n</sup> exhibiting more severe hypersensitivity compared to congenic PVG-RT1<sup>av1</sup> and inbred BN rats (Figure 2, Paper III). There was also significant time versus strain interactions, suggesting that the temporal profile of allodynia development differed among the strains (Figure 2, Paper III).

#### **4.6 STRAIN DIFFERENCE IN RESPONSE TO SPINAL CORD INJURY IN DA, PVG AND MHC-CONGENIC PVG-RT1<sup>AV1</sup> FEMALES (PAPER II)**

Pain following spinal cord injury is a form of central neuropathic pain that is a major clinical problem. While our previous results showed that MHC gene-regulated processes influence the development of neuropathic pain-like behaviors following peripheral nerve injury in rats, it is unclear whether similar mechanisms will also play a role in neuropathic pain occurring after injury to the spinal cord. Thus, we have used a spinal cord injury (SCI) model (Hao et al. 1991) to study the role of the MHC complex in central neuropathic pain using DA rats, PVG rats and its MHC-congenic strain PVG-RT1<sup>av1</sup>. The experiments were conducted in female rats, as the model at that time was only available in this sex.

Similar to baseline mechanical hypersensitivity of the hindpaws, the mechanical response thresholds of the trunk and back area of these three studied strains were not significantly different. All strains developed marked mechanical allodynia over the trunk and back 24 h after photochemically-induced ischemic spinal cord injury, which persisted during the entire period of observation (Figure 3, Paper II). Significant overall strain differences in the magnitude of mechanical hypersensitivity were observed. However, in contrast to sciatic nerve injury, the DA rats exhibited more severe mechanical hypersensitivity after spinal cord injury than PVG rats and PVG-RT1<sup>avl</sup> rats, (Figure 3, Paper II).

#### **4.7 SEX DIFFERENCE IN THE DEVELOPMENT OF HYPERSENSITIVITY IN SD RATS AFTER INJURY TO THE INFRAORBITAL OR SCIATIC NERVES (PAPER IV)**

In Paper II we found no sex difference in localized hindpaw hypersensitivity after sciatic nerve injury in the three MHC strains. We expanded those findings in study IV where we used outbred SD rats to study sex differences in the development of neuropathic pain-like behaviors, namely mechanical hypersensitivity to innocuous stimulation (allodynia), after injury to the infraorbital or sciatic nerves. In addition to localized hypersensitivity seen in the dermatomes of the injured nerves, we have also studied sex differences in areas outside the innervation territories of these nerves (spread hypersensitivity).

##### **4.7.1 Snout hypersensitivity after injury to the infraorbital nerve in male and female rats**

The female rats had significantly lower response threshold on the snout than the males before nerve injury (Figure 1, Paper IV). Unilateral injury to the infraorbital nerve produced a marked bilateral hypersensitivity in both male and female rats in the snout region innervated by the nerve. The initial magnitude of mechanical hypersensitivity was similar in male and female rats, but the hypersensitivity was significantly more prolonged in the females than in the males. There was also a significant interaction between sex and time (Figure 1, Paper IV).

#### **4.7.2 Spread hypersensitivity after infraorbital nerve injury in male and female rats**

Normal female rats have significantly lower vocalization threshold to mechanical stimulation on the neck and upper back region than the male rats before injury (Figure 2, Paper IV). Injury to the infraorbital nerve produced a marked mechanical hypersensitivity in both male and female rats in this region (Figures. 2, 3, Paper IV). However the mechanical hypersensitivity was more severe and much more prolonged in the female than in the male rats. Significant overall differences between male and female rats were observed as well as significant sex x time interaction. The spread hypersensitivity was mainly observed in the neck and upper back region, occasionally extending into the lower back and flank region.

#### **4.7.3 Hindpaw hypersensitivity after sciatic nerve injury in male and female rats**

Again, normal female rats had significantly lower paw withdrawal threshold to mechanical stimulation than males before nerve injury. Sciatic nerve injury produced a bilateral hypersensitivity in both males and females on the hind paws. ANOVA with repeated measures indicated a significant overall difference between male and female rats for the 12-week observation period, with no significant sex x time interaction (Figure 4, Paper IV).

#### **4.7.4 Spread hypersensitivity after sciatic nerve injury in male and female rats**

The female rats had significantly lower vocalization thresholds to mechanical stimulation on the lower back and flank region than the males before sciatic nerve injury (Figure 5, Paper IV). Sciatic nerve injury produced a spread mechanical hypersensitivity in both male and female rats in this region (Figures 3, 5, Paper IV). The mechanical hypersensitivity was more severe and prolonged in the female than in the male rats. ANOVA with repeated measurements indicated a significant overall difference between male and females rats for the 12 week observation period and a significant sex x time interaction was observed. The spread hypersensitivity was mainly

observed in the lower back and flank region, occasionally extending into the upper back (Figure 3, Paper IV).

## 5 DISCUSSION

### 5.1 THE INVOLVEMENT OF MHC IN NEUROPATHIC PAIN-LIKE BEHAVIOR FOLLOWING PERIPHERAL NERVE INJURY

In the first three studies presented in this thesis, we have used a genetic approach to explore the potential involvement of MHC genes in the development of neuropathic pain-like behaviors using a rat model of ischemic injury to the sciatic nerve. In these studies, we are able to show that there are significant strain differences among inbred DA, PVG and MHC-congenic PVG-*RTI<sup>av1</sup>* rats. These differences can be observed both in male and in female rats and were very robust and could be observed repeatedly. The strains tested, BN, DA and PVG, have previously been extensively characterized in models of autoimmune neuroinflammation. The similar neuropathic pain-like behavior in PVG-*RTI<sup>av1</sup>* and DA as compared to ordinary PVG, suggests that the observed strain-dependent effect to a large extent is explained by genetic differences in the MHC locus. This notion was subsequently validated by testing an F2 cohort obtained by intercrossing the wildtype PVG with PVG-*RTI<sup>av1</sup>*. This was conducted due to the concern that differences in phenotypes between a congenic strain and the recipient parental strain may depend on contaminating genes from the donor strain or the accumulation of genetic differences outside of the congenic fragment through breeding. The results that the F2 rats that were either homozygous or heterozygous for *RTI<sup>av1</sup>* were relatively protected from developing neuropathic pain-like behavior compared to animals homozygous for the *RTI<sup>c</sup>* allele indicates a dominant protective role of *RTI<sup>av1</sup>*, or alternatively that the *RTI<sup>c</sup>* allele acts in a recessive fashion for this phenotype. Inter-individual phenotypic variation within the groups of F2 animals was somewhat larger in comparison to the parental strains. This is not surprising since a low degree of genetic material from the donor DA strain persisting in the PVG-*RTI<sup>av1</sup>* background genome subsequent to backcrossing will result in a unique genetic setup of every F2 individual.

Importantly, the effect of MHC on the development of hypersensitivity is not explained by variations in the level of primary tissue damage, since we could not detect any structural differences in the nerve lesion area among animals having different genotypes. This is in accordance with previous results, where we showed that variation

in mechanical hypersensitivity cannot be explained by degree of nerve damage (Xu et al. 2001).

## **5.2 THE INVOLVEMENT OF NON-MHC GENES IN NEUROPATHIC PAIN-LIKE BEHAVIOR FOLLOWING PERIPHERAL NERVE INJURY**

In study III, we expanded our work from paper I by examining an additional MHC congenic strain, PVG-*RTI<sup>n</sup>*, and its parental strain, the BN rats. Thus the panel of strains used allow for predictions about the genetic contribution of both MHC and non-MHC-genes. Interestingly, the four tested strains segregated into two different groups. The PVG and the congenic PVG-*RTI<sup>n</sup>* strains displayed significantly more allodynia compared to BN and the congenic PVG-*RTI<sup>av1</sup>*. The fact that BN differ from the MHC congenic PVG-*RTI<sup>n</sup>* rat carrying the same MHC haplotype suggests that some of these differences are regulated by non-MHC genes. It is also of interest to note that baseline differences in the tested strains seem to be regulated mainly by non-MHC genes, since the BN strain differed from all the other strains.

## **5.3 THE POTENTIAL MECHANISMS OF THE ROLE OF MHC GENES IN NEUROPATHIC PAIN**

Our results in general support previous suggestion that inflammatory mechanisms may be involved in the development of neuropathic pain (Watkins and Maier 2002; Marchand et al. 2005) and indicate that variability in MHC genes may be an important factor. The observation that PVG-*RTI<sup>av1</sup>* rats are less susceptible to the development of neuropathic pain-like behaviors than PVG strains carrying *RTI<sup>n</sup>* or *RTI<sup>c</sup>* haplotypes may be attributed to the action of products of a single gene or genes in any of the three haplotypes. In the case of PVG-*RTI<sup>av1</sup>*, the effect appears to be dominant and it may be speculated that the PVG-*RTI<sup>av1</sup>* haplotype contains a genetic influence that protects against neuropathic pain development, since both PVG-*RTI<sup>n</sup>* and PVG display similar phenotypes. However, it is not possible to rule out the possibility of actions of gene products that are common between PVG and PVG-*RTI<sup>n</sup>* or bi-directional effects from multiple loci within the MHC.

In addition to the classical antigen presenting molecules, the MHC locus also contains genes such as TNF, Aif1 and a large group of class Ib genes, all of which may be considered candidates for the described MHC effect. TNF- $\alpha$  has, for example, been,

suggested to be a key mediator in neuropathic pain (Watkins et al. 1994; Sorkin et al. 1997; Schafers et al. 2003).

We have attempted to address the potential mechanisms of MHC involvement in neuropathic pain by examining nerve injury-induced regulation of MHC class I, MHC class II and for TNF- $\alpha$  markers in the nerve, DRG and spinal cord using RT-PCR and, in some cases, immunohistochemistry. The results showed that there are no significant differences before and after injury in either PVG or congenic PVG-*RTI<sup>av1</sup>* in the nerve, DRG and spinal cord before and after injury for the marker of MHC class I. In contrast, the MHC class II marker is significantly upregulated in the nerve after injury in both of the strains studied and in the DRG there is also a significant upregulation in injured congenic PVG-*RTI<sup>av1</sup>* rats compared to non-injured rats. The TNF- $\alpha$  levels were significantly upregulated in the DRG and spinal cord in the PVG rats. Together, these preliminary results showed that MHC class II and TNF- $\alpha$ , but not MHC class I, are important in the MHC modulation of neuropathic pain, although the precise roles of these genes need to be further studied.

Several previous studies have shown that the development of pain-like behaviors after spinal cord injury pain is strain dependent (Wiesenfeld-Hallin et al. 1993; Gorman et al. 2001; Mills et al. 2001). In rats subjected to spinal cord injury, we also observed significant inter-strain differences with DA rats exhibiting more allodynia than PVG and PVG-*RTI<sup>av1</sup>* rats. The difference in pain behavior could not be explained by variation in the extent of injury in the examined strains. The pattern of strain differences in pain behavior following spinal cord injury was different from that following peripheral nerve injury and suggests that non-MHC factors may play more important roles in generating spinal cord injury pain. It is interestingly to note that DA rats are particularly prone to develop spinal cord injury pain. Previous studies have shown that DA rats have higher glial activation as well as greater cell loss compared to PVG and PVG-*RTI<sup>av1</sup>* after ventral root avulsion (Lundberg et al. 2001; Lidman et al. 2003) and as mentioned in the Introduction, glial cells are known to play an important role in the enhancement of neuropathic pain. DA rats have also been demonstrated to have more elevated MHC class II immunolabeling compared to PVG in the spinal cord after a weight-drop contusion injury (Birdsall Abrams et al. 2007).

#### **5.4 SEX DIFFERENCE IN THE DEVELOPMENT OF LOCALIZED AND SPREAD MECHANICAL HYPERSENSITIVITY IN RATS AFTER INJURY TO THE INFRAORBITAL OR SCIATIC NERVES**

In studies II and IV we showed that there was no significant sex difference in the development of localized mechanical allodynia after sciatic nerve injury in several rat strains. However, in study IV, we demonstrated a marked sex difference in the response to infraorbital nerve injury in SD rats. Females developed more profound and long-lasting facial hypersensitivity than males. Furthermore, wide-spread mechanical hypersensitivity in body areas outside the innervation territory of the injured nerve was also observed and was more profound after infraorbital than sciatic nerve injury and also showed significant sex difference (female > male). These results suggest that sex differences in the development of neuropathic pain-like behaviors in rats is dependent on site of injury as well as site of testing with female rats being more susceptible to the development of spread mechanical hypersensitivity, particularly after facial nerve injury.

Mechanical hypersensitivity and/or signs of ongoing pain in the innervation territory of peripheral nerves, including the infraorbital and sciatic nerves, have been widely reported with different rodent models with incomplete nerve injury (Vos et al 1994; Idanpaan-Heikkila and Guilbaud 1999; Benoliel et al. 2002; Eriksson et al. 2005). Extraterritorial hypersensitivity has also been reported in some animal models (Tal and Bennett 1994; Vos et al. 1994). In study IV we observed bilateral mechanical hypersensitivity on the snout and of the hindpaws innervated by the injured and contralateral uninjured infraorbital and sciatic nerves, respectively. In addition, we also observed widespread hypersensitivity in a large body area after nerve injury outside the innervation territory of the nerve, which has not been previously reported with other rodent models of neuropathic pain.

The widespread hypersensitivity showed a marked sex difference in that female rats had much more persistent hypersensitivity after injury to both the IoN and sciatic nerve. However, the spread hypersensitivity was more severe after the IoN injury than the sciatic nerve injury. Exactly why the IoN injury gave more profound localized as well as spread hypersensitivity is not clear. It may be due to the more dense innervation of craniofacial tissues than hindlimbs, as well as differences in the central terminal

patterns of IoN and sciatic nerve nociceptors. The afferent properties could also be different between IoN and sciatic nerves after injury (Tal and Devor 1992).

Chronic widespread pain is one of the principal components in fibromyalgia, where low back pain as well as shoulder pain are the most common symptoms. Furthermore, the prevalence of fibromyalgia is much higher in women compared to men (Macfarlane 1999). Thus it remains to be seen whether the present observation of widespread hypersensitivity in rats after localized nerve injury and its sex difference may lead to the development of rat models of the human condition of fibromyalgia or other forms of spread pain that is seen mostly in women.

## 6 CONCLUSIONS

1. Both MHC and non-MHC genes influence the development and maintenance of neuropathic pain following peripheral nerve injury in rats.
2. In particular, certain allelic variants of MHC have strong influence on the susceptibility for the development and maintenance of neuropathic pain.
3. MHC genes do not appear to have a role in the development of acute spinal cord injury pain in rats.
4. MHC genes have no role in sex difference in neuropathic pain after nerve injury.
5. Sex difference in the development of neuropathic pain-like behaviors in rats is dependent on site of injury as well as site of testing with female rats being more susceptible to the development of spread mechanical hypersensitivity, particularly after facial nerve injury
6. Our findings may lead to the development of rat models of the human condition of fibromyalgia and other forms of spread pain that is seen mostly in women.

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