Nucleus pulposus application onto rat spinal dorsal nerve roots leads to a persistent increase in spinal C-fibre responses, possibly due to upregulation of IL1α, IL1β and TNF

by

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Master Thesis

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Abstract

Sensitization of sensory neurons after noxious conditioning may be involved in many chronic pain states, including radiating low back pain and sciatica following disc herniation. Here, we examine such sensitization induced by two types of noxious conditioning: I) electrical sciatic high frequency stimulation (HFS), and II) nucleus pulposus (NP), harvested from intervertebral discs, applied onto the spinal dorsal nerve roots. In addition, we investigate the gene expression of the proinflammatory cytokines IL1α, IL1β, TNF and the protease MMP1 in NP tissue.

Electrophysiological extracellular potentials were recorded from the spinal dorsal horn in anaesthetized Sprague-Dawley- or Lewis rats. A single test stimulus was applied to the sciatic nerve every 4th minute and the A- and C-fibre responses were separated according to latencies. For the gene expression analysis, total RNA was isolated from NP tissue and mRNA expression quantified by RT-qPCR.

First, the spinal neuronal responses were studied by field potential recordings following HFS conditioning of the sciatic nerve. The HFS conditioning produced a clear long-term potentiation (LTP), which outlasted the experimental time period of 180 minutes. Next, the spinal neuronal responses were studied by single unit recordings following NP application onto the dorsal nerve roots. The NP conditioning produced a persistent increase in the spinal C-fibre responses, also outlasting the experimental time period of 180 minutes. In addition, the present study demonstrated a significant upregulation in the gene expression of IL1α, IL1β and TNF 180 minutes after application of NP onto the spinal dorsal nerve roots. No changes, however, were seen in the expression of MMP1.

In summary, the HFS caused a robust LTP in the spinal cord. Furthermore, application of NP onto the spinal dorsal nerve roots induced an LTP-like phenomenon which was also associated with an increase in gene expression of IL1α, IL1β and TNF in NP tissue 180 minutes after application. The present data suggests that herniated NP in contact with the dorsal nerve roots may cause a persistent spinal hyperexcitability of nociceptive neurons, possibly due to biochemical mediators intrinsic to the NP tissue.

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Abbreviations

5-HT	5-hydroxytryptamine/serotonin
ACC	anterior cingulate cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate
ANOVA	analysis of variance
AP	action potential
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BK	bradykinin
Bp	base pair
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
cAMP	cytosolic adenosine monophosphate
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CGRP	calcitonin-gene-related protein
CNS	central nervous system
COX	cyclooxygenase
CRE	cAMP response element
CREB	cAMP response element-binding protein
Ct	threshold cycle
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
EDTA	ethylenediaminetetraacetic acid
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
GABA	γ-aminobutyric acid

GC	guanosine/cytosine
GDNF	glial cell line-derived neurotrophic factor
Glu	glutamate
HFS	high-frequency stimulation
HSD	honestly significant difference
IFN-γ	interferon-γ
iGluR	ionotropic glutamate receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
IP_3	inositol 1,4,5-trisphosphate
KCC2	K^+/Cl^- co-transporter 2
LC	locus coeruleus
LFS	low-frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NK1	neurokinin 1
NMDA	*N*-methyl-$_D$-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NP	nucleus pulposus
NS	nociceptive specific
OD	optical density
P2XR	purinergic 2X receptor
PAG	periaqueductal grey

PB	parabrachial
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PI3	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
RIN	RNA integrity number
RNA	ribonucleic acid
RNase	ribonuclease
RT-qPCR	reverse transcription quantitative PCR
RVM	rostral ventromedial medulla
SEM	standard error of the mean
SP	substance P
TE	Tris-EDTA
Tm	melting temperature
TNF	tumour necrosis factor
Trk	tropomyosin receptor kinase
TRPV1	transient receptor potential vanilloid receptor-1
VEGF	vascular endothelial growth factor
WDR	wide dynamic range

1 Introduction

1.1 Pain versus nociception

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Loeser and Treede, 2008). According to this definition, pain is a subjective experience that involves not only the perception of sensory signals, but also higher brain functions, cognitive analysis and processing, as well as subsequent associated emotional responses. It is dependent on emotions, context, and experience.

Nociception, on the other hand, is the neural processing of noxious stimuli, i.e. detection and signalling of an actual or potential tissue-damaging event (Loeser and Treede, 2008). Noxious stimuli may be chemical, thermal or mechanical. More specifically, nociception is the objective signalling arising from activation of nociceptors, and the transmission of such input through specialized pathways. A nociceptive neuron is defined as “a central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli” (Loeser and Treede, 2008).

The fact that most phyla in the animal kingdom possess some sort of nocifensive system, underlines the evolutionary importance of such a function. This is evident when one looks at individuals lacking the sense of pain, as seen in the rare condition of congenital insensitivity to pain, or in some patients suffering from leprosy. The affected individuals are unable to feel physical pain, causing devastating effects.

The distinction between pain and nociception is important when it comes to pain research. To experience pain one has to be conscious, and possess the necessary brain functions to perceive and process it. To measure and assess pain in an experimental manner, the subjects have to have the ability to communicate the experience. This makes it impossible to study pain in anaesthetized laboratory animals, but the physiological nociceptive process can still be studied.

One may have pain without the activation of nociceptors, and nociception may occur without pain. This is due to the complex neurobiology of the pain pathways, and the ability of the central nervous systems to modulate sensory signalling and input.

1.2 Adaptive and maladaptive pain

In many cases pain is temporary, and only lasts for as long as the painful stimulus is present. Clearly, acute pain in response to an injury has an adaptive function, as it draws attention to the site of injury and urges immobilization of the damaged body part. Withdrawal reflexes and inflammatory pain in response to an infection are other examples of adaptive pain. It promotes survival through avoidance of harmful objects and substances, thereby optimizing the healing process.

In maladaptive pain, the experienced pain no longer serve as a protective mechanism and give no biological advantage to the organism, it just causes suffering. Chronic pain, as in long lasting inflammatory or neuropathic pain, is an example of maladaptive pain. Sometimes pain persists long after the initial injury has healed, or even without a clear cause. There is no clear definition as to when the pain may be characterized as being “chronic”, but the term is often used when pain persists for more than three months. Chronic pain affects about 20 % of the population and is a major health care problem in Europe, with severe negative effects both for the individual´s quality of life and their work abilities through long-term sick leave. In this manner, chronic pain has negative impact on an individual-, societal- and economic level (Breivik et al., 2006).

Hyperalgesia and allodynia are two behavioural manifestations of pain that may occur in both adaptive and maladaptive pain. Hyperalgesia is an increase in the response of the nociceptive system following an injury or inflammation, leading to an exaggerated pain response to subsequent noxious stimuli. On the other hand, allodynia, i.e. reduced pain threshold, is a painful experience due to non-noxious stimulation. Like hyperalgesia, allodynia may also occur after injury or inflammation.

Long lasting musculoskeletal pain is a common cause of long-term sick leave and is an example of the aforementioned maladaptive pain. Such pain may be low back pain and sciatica, which is often caused by lumbar intervertebral disc disease and disc herniation. Low back pain and sciatica may be debilitating conditions, lasting for weeks, months or even years.

1.3 Nociceptive signalling and the modulatory system

The following section gives an overview of the nociceptive signalling system, which comprise the nociceptors, the ascending system from the spinal dorsal horn, the supraspinal brain regions, and the descending modulatory pathways from the brain to the spinal cord.

1.3.1 Primary afferent nerve fibres

The primary afferent nerve fibres associated with pain are called nociceptors. These are specialized high-threshold sensory receptors in the peripheral somatosensory nervous system, which react specifically to potentially damaging stimuli. The nociceptors are capable of transducing and encoding such noxious stimuli. Primary afferent nerve fibres carrying nociceptive information are divided into two classes, myelinated, Aδ fibres, and the unmyelinated, thinner C-fibres. The Aδ fibres are responsible for the sharp and pricking, acute “first pain” (speed of conduction 5-30 m/s). The C-fibres are responsible for the throbbing and burning, slower “second pain” (speed of conduction 1-2 m/s), which is often associated with chronic pain.

Innocuous stimuli, such as light touch, pressure, vibration and warmth/cool, normally do not exceed the intensity required to activate nociceptors. These stimuli will under normal conditions activate low-threshold sensory fibres, and is conducted through myelinated Aα- and Aβ fibres, with large diameter (speed of conduction 50-100 m/s).

Activation of the pain system often starts in the periphery with activation of nociceptors in the skin, muscles, joints, or connective tissue. The initial event could be tissue damage, injury or an inflammation, with thermal, chemical or mechanical stimuli of potential harmful intensity, thus reaching nociceptor threshold. Nociceptors are excited by irritant chemical stimuli, noxious heat or cold and mechanical impact. The resulting signal transduction is mediated through a range of ion channels and G-protein-couples receptors, GPCRs. A variety of factors are able to activate the nociceptors through their relevant receptors, including glutamate, substance P, bradykinin (BK), adenosine triphosphate (ATP), protons (H^+), heat, capsaicin, calcitonin gene-related protein (CGRP), neurotrophins, prostaglandin (PG) and serotonin (5HT). If activation threshold is reached, the transduction generates an action potential (AP) which is conducted through the afferent nociceptive neurons to the spinal cord dorsal horn. For review on peripheral nociceptive mechanisms, see (D'Mello and Dickenson, 2008; Woolf and Salter, 2000).

1.3.2 The spinal dorsal horn

Nociceptive information from the peripheral afferent nerve endings of the skin, muscles, joints and connective tissue, is conducted through the nociceptive axons via the dorsal nerve roots, and eventually into the dorsal horn of the spinal cord. In the dorsal horn the peripheral afferent fibres terminate and synapse with various second order neurons. Based on differences in cytoarchitecture, the dorsal horn grey matter is organized into laminae I-V. Aδ and C-fibres terminate predominantly in the superficial lamina I and lamina II, also called the substantia gelatinosa, with some innervations of the deeper lamina V (Sugiura et al., 1986). Within the dorsal horn, the primary afferents synapse with different classes of second order neurons in the various laminae. The nociceptive specific cells (NS) are located in lamina I-II and receive input from C-fibres only. The so-called wide-dynamic-range neurons (WDR) are able to respond to both Aβ-, Aδ- and C-fibres, and thus the full range of sensory input, from innocuous touch to nociceptive stimulus. WDR neurons are located throughout the dorsal horn, but are mainly found in the deeper laminae.

Upon activation in their peripheral nerve endings, the terminals of nociceptive afferents in the spinal dorsal horn release neurotransmitters, primarily the excitatory neurotransmitters glutamate and substance P. Multiple receptors are expressed on dorsal horn neurons, including ionotropic- and metabotropic glutamate receptors (iGluR and mGluR, respectively), and the neurokinin1 (NK1)-receptor for substance P. The release of glutamate from the pre-synaptic neuron leads to its binding to the iGlu receptors kainate receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionate (AMPA) receptor. This results in an influx of cations, mostly Na^+, and a subsequent depolarization of the post-synaptic cell membrane, an excitatory post-synaptic potential (EPSP). If the temporal summation of the EPSPs results in a sufficiently strong depolarization, an AP is generated.

Apart from the central terminals of primary afferents, the spinal cord contains other neuronal cell types as well. These are the interneurons, the propriospinal neurons, and the projection neurons, which are connected to both each other and to other neurons in the spinal cord, involved in processing of the incoming information. The A-fibres carrying non-noxious sensory information also terminate in the dorsal horn, but mainly in other laminae. Still, these can make connections to the same interneurons as the nociceptors.

Interneurons constitute a major part of the neurons in the dorsal horn, and are local intrinsic neurons whose axons and dendrites extend no further than to nearby neurons; they are restricted within the segments of the spinal cord. They act as local relays in spinal processing, and are involved in modulation of nociceptive activity (Huang et al., 2005). Interneurons are tonically active, and can be either excitatory (glutamatergic) or inhibitory (γ-aminobutyric acid (GABA)- or glycinergic). Although most interneurons are inhibitory, some of the substantia gelatinosa interneurons may be excitatory (Santos et al., 2007). Propriospinal neurons send their axons across the spinal cord segments, mediating information transfer between these segments and are also involved in spinal reflex responses.

Projection neurons, however, have longer axons which terminate in various brainstem areas, before the nociceptive information eventually is relayed to higher

brain regions. Most projection neurons located in lamina I express the NK1 receptor for substance P (Bester et al., 2000; Ikeda et al., 2003; Marshall et al., 1996; Mouton and Holstege, 1998). However, NK1-expressing cells are also found in the deeper lamina V and X (Li et al., 1998). Previous studies have demonstrated that most lamina I neurons are nociceptive specific (Bester et al., 2000; Ikeda et al., 2003). For review on spinal dorsal horn and projection neurons, see (Todd, 2002).

In addition to the neuronal cells, the spinal cord also contains a substantial amount of the non-neuronal glial cells, which have important supportive, nutritional, immune-related and homeostatic functions. In recent years, it has become evident that astrocytes and microglia also play a role in the modulation of synaptic transmission, and it has also been accepted that they partake in nociceptive transmission in the spinal dorsal horn. Spinal microglia can produce and secrete cytokines, chemokines and neurotrophins, which may alter neuronal excitability. Studies using fluorocitrate, a glial metabolic inhibitor, demonstrated this interaction of neurons and glia by attenuating hyperalgesia in a rat inflammation model (Meller et al., 1994). In addition, specific inhibition of microglia by minocycline treatment, attenuate the development of allodynia and hyperalgesia in a rat model of neuropathic pain (Raghavendra et al., 2003), as well as inflammation-induced hyperalgesia (Hua et al., 2005).

1.3.3 Ascending pathways and supraspinal areas

There are two primary ascending nociceptive pathways from the dorsal horn: the spinoparabrachial and the spinothalamic pathways projecting to the parabrachial (PB) area and the thalamus, respectively. Neurons from lamina I mainly project via the spinoparabrachial tract to the PB nucleus, which again project to brain regions involved in the autonomic and homeostatic aspects of pain, like the ventrolateral medulla and hypothalamus. Lamina I neurons also project to brain regions involved in emotional affect, fear and avoidance, like the periaqueductal grey (PAG), amygdala, insular cortex and the anterior cingulate cortex (ACC).

Lamina V projection neurons largely convey information directly to the thalamus through the spinothalamic tract, and subsequently to the somatosensory cortex, providing the sensory and discriminatory component of the pain experience. For review on projection neurons and the supraspinal areas involved in nociception, see (Gauriau and Bernard, 2002; Mantyh and Hunt, 2004; Treede et al., 1999).

1.3.4 Descending modulatory system

The central nervous system also has a well-developed system of descending modulatory pathways from the brain to the spinal cord. In fact, the incoming nociceptive information from the dorsal horn is continuously being regulated by tonically active inhibitory and excitatory pathways, modulating the activity of nociception and thereby the perception of pain.

There are descending projections from both cortical, subcortical and brainstem regions that influence the nociceptive signalling in the dorsal horn, modulating the spinal nociceptive activity. Important structures in this respect are the midbrain PAG, hypothalamus, the brainstem rostral ventromedial medulla (RVM), and the locus coeruleus (LC), which together control the activity in serotonergic, noradrenergic and enkephalinergic descending projections. The PAG is interconnected with various regions of the CNS, like the prefrontal cortex, ACC, insula, hypothalamus and the amygdala, which have descending pathways converging on PAG. Neurons in the PAG project to the RVM, which is able to exert both pro- and antinociceptive effects through its so-called “on cells” and “off cells”. Output from these cells controls the excitatory or inhibitory information, which in turn modulate the spinal cord activity. Insular cortex and amygdala also contribute to the regulation of nociceptive signalling, through projections to the LC, whose projection neurons release noradrenalin onto the dorsal horn. For review on the descending modulatory system, see (Mantyh and Hunt, 2004; Willis and Westlund, 1997). For a simplified presentation of the ascending and descending nociceptive system, see Figure 1.1.

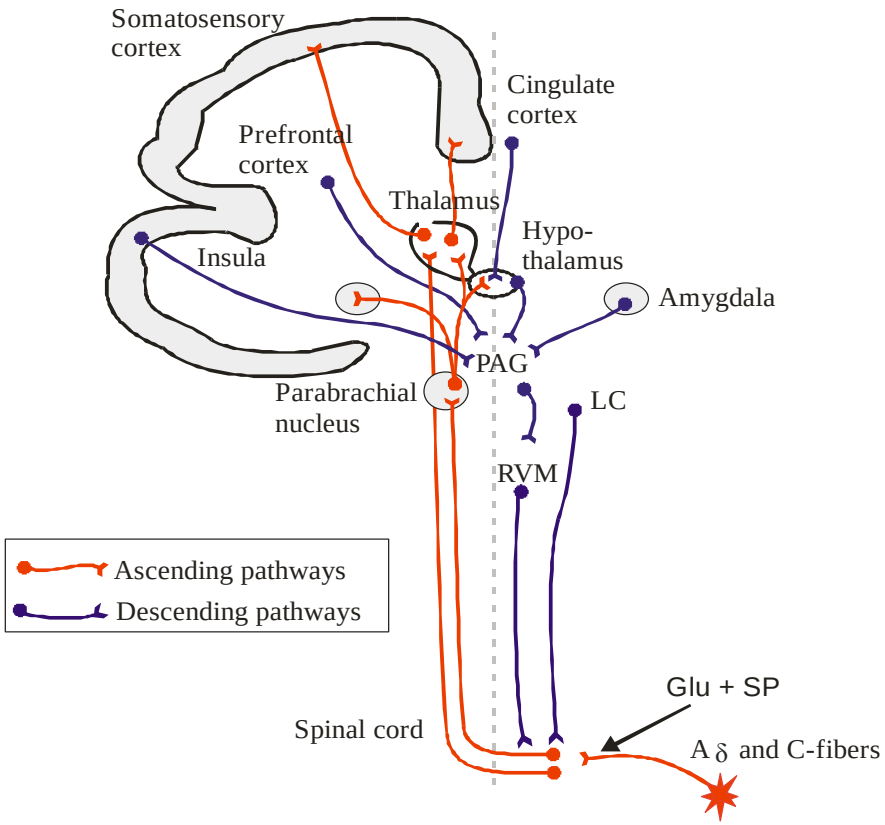


Figure 1.1 The ascending and descending nociceptive signalling system. Noxious input from activated Aδ- and C-fibre afferents are conducted to the spinal dorsal horn, where the excitatory neurotransmitters glutamate (Glu) and substance P (SP) are released. From the dorsal horn, the nociceptive signals are transferred via spinoparabrachial- and spinothalamic projection neurons to various brainstem-, subcortical- and cortical areas. In addition, a complex descending system modulates the spinal activity, by exerting excitatory and inhibitory output from cortical- and brainstem areas. Important structures in this respect are the hypothalamus, periaqueductal grey (PAG), rostral ventromedial medulla (RVM), and the locus coeruleus (LC). Adapted from (Gjerstad, 2007).

1.4 Inflammatory and neuropathic pain

Trauma and tissue damage, as well as pathological conditions of disease and infection, may all lead to inflammation. The pain hypersensitivity associated with inflammation is caused by posttranslational changes both in the peripheral nerve endings and in dorsal horn neurons, but this usually returns to normal as the tissue

repairs and the disease process is reversed. Numerous chemical mediators are released in the affected tissue during the inflammatory process, such as pro-inflammatory cytokines, PG, BK, nerve growth factor (NGF), ATP, 5HT, H^+ and histamine. These endogenous substances are capable of inducing pain either directly, by having excitatory effects on the nociceptive afferents, or indirectly, by sensitizing the nociceptors, giving rise to spontaneous pain, hyperalgesia and allodynia. These mediators may work in synergy to produce their effects, brought about by both post-translational and transcriptional changes. For review on inflammatory pain and its mediators, see (Woolf and Costigan, 1999).

The transient receptor potential vanilloid receptor-1 (TRPV1), for instance, usually requires heat (>42 °C) to be activated. It is a transduction ion channel found both on primary afferent nociceptors and in NK1-expressing dorsal horn neurons (Doly et al., 2004). Prostaglandin E_2 (PGE_2) (Moriyama et al., 2005) and BK (Chuang et al., 2001) released during inflammation, sensitizes TRPV1 so that it becomes activated at a lower temperature. This is an example of peripheral sensitization. In tissue inflammation, previous studies have shown that the neurotrophin NGF is also an important mediator for sensitization of primary afferent nociceptors (Koltzenburg et al., 1999). NGF, acting on the tropomyosin receptor kinase A (TrkA) receptor, activates a signalling pathway involving phosphoinositide 3-kinase (PI3) kinase and the tyrosine kinase Src, which subsequently increases the expression and peripheral transport of TRPV1 to the membrane, augmenting heat hyperalgesia (Zhang et al., 2005).

Peripheral injection of the substance complete Freund's adjuvant (CFA) is a well established experimental method for inducing peripheral inflammation. Previous studies of murine CFA-induced inflammation have demonstrated a critical role for the cytokines tumour necrosis factor (TNF, formerly known as TNFα) and interleukin 1β (IL1β) in upregulating the expression of NGF (Safieh-Garabedian et al., 1995; Woolf et al., 1997). CFA-induced inflammatory pain may also be associated with up-regulation of the neuropeptides substance P and calcitonin gene-related protein (CGRP) in the primary afferent nerve fibres of lamina I, contributing to central inflammatory responses (Honore et al., 2000).

Trauma, tissue damage and in particular nerve injury may also lead to neuropathic pain. Such neuropathic pain is defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Loeser and Treede, 2008). Thus, neuropathic pain is caused by pathological changes or abnormal function in the peripheral or central nervous system itself. For instance, previous studies have demonstrated that peripheral nerve injury induces sprouting of A-fibre central terminals into lamina II, which normally receives nociceptive information (Woolf et al., 1992). Such sprouting could be induced by the neurotrophin NGF. Moreover, previous studies have also demonstrated that sciatic nerve injury may induce spinal hyperexcitability and spontaneous discharges in dorsal root ganglion (DRG) cells (Xie et al., 1995). Peripheral nerve injury may also lead to reduced inhibitory control of spinal neurons, i.e. disinhibition (Seltzer et al., 1991). Neuropathic pain may manifest in different ways, for instance as spontaneous pain or hyperalgesia, that is hypersensitivity to further stimuli. Also, neuropathic pain is often associated with allodynia, in which a non-nociceptive input gives rise to pain. Allodynia is found in diabetic neuropathy, multiple sclerosis, cancer (compression of nerves), leprosy, and in phantom pain after amputations. Neuropathic pain is often difficult to treat with conventional analgesics like non-steroidal anti-inflammatory drugs (NSAIDs) and opioids because of its often complex pathophysiology. Apart from the underlying nerve injury, non-neuronal microglia in the spinal cord have been suggested to be important mediators in neuropathic pain. In peripheral neuropathic pain sensory afferents may display ectopic firing, or spontaneous firing of action potentials. For review on neuropathic pain, see (Woolf and Mannion, 1999). As discussed below, long-lasting low-back pain and sciatica is often caused by lumbar disc herniation, and may have both inflammatory and neuropathic characteristics.

1.5 Spinal disc herniation

The intervertebral discs in between the vertebrae are filled with a gelatinous substance called nucleus pulposus (NP), which functions as a shock-absorber. It has a cushioning effect on the vertebrae, dissipates the mechanical load, and allows for twisting and bending of the upper torso. Although degeneration of the lumbar discs

is a natural consequence of aging, in some individuals these changes include small cracks in the outer fibrous ring of the intervertebral disc, the annulus fibrosus. General wear and tear or trauma due to heavy load lifting may also injure the discs in the lumbar region. In disc herniation, the annulus fibrosus ruptures and NP protrudes into the spinal canal. This leads to mechanical compression of the surrounding spinal nerve roots resulting in low back pain or sciatica. As such, it is a form of radiculopathy, a condition specifically affecting the nerve roots. In recent years it has been accepted that NP also has an inflammatory effect on the neuronal tissue, which contributes to the pain experience by sensitizing primary afferents and spinal neurons.

1.5.1 Nucleus pulposus

NP consists of a matrix of mainly type II collagen and the proteoglycan aggrecan, which is negatively charged and highly hydrophilic, and thus draws water into the disc. Under normal circumstances, the intervertebral disc is free of both blood vessels and neurons, except the outer annulus fibrosus and the vertebral endplates (Grönblad et al., 1991; Hirsch et al., 1963). In patients with disc degeneration and low back pain, however, microfractures of the bone and trauma to the annulus fibrosus allows growth of blood vessels and primary afferent fibres into the NP. Actually, several studies have found ingrowths of nociceptive neurons into pain-generating damaged intervertebral discs (Freemont et al., 1997; Freemont et al., 2002; Peng et al., 2005). This is supported by studies showing that the cytokines TNF and IL1β may cause an increase in the release of NGF (Safieh-Garabedian et al., 1995), which subsequently leads to sprouting of peripheral nerve fibres. In NP cells isolated from patients with intervertebral disc degeneration, IL1β and TNF may stimulate the gene expression of vascular endothelial growth factor (VEGF), NGF, and brain-derived neurotrophic factor (BDNF). This suggests that IL1β generated during intervertebral disc degeneration results in angiogenesis and innervations (Lee et al., 2011).

Matrix metalloproteinases (MMPs), also found in the discs, are enzymes that break down extracellular matrix. They contribute, among other factors, to the disc

degeneration, leading to loss of structural integrity, decreased hydration and reduced ability to withstand load (Le Maitre et al., 2005). The interstitial collagenase MMP1 is the main collagenase to degrade collagen fibres in the NP. Previous data show that disc cells in culture stimulated with cytokines may display enhanced production of matrix metalloproteinases, which play an important role in spontaneous regression of the disc materials (Doita et al., 2001; Kang et al., 1997; Le Maitre et al., 2005).

Previous animal studies have demonstrated that puncture of a lumbar intervertebral disc and subsequent leakage of NP, causes spontaneous pain behaviour (Olmarker, 2008), and mechanical hyperalgesia has been reported after application of NP onto nerve roots (Kawakami et al., 2000). Several studies have also shown direct effects of NP on the neuronal tissue in experimental models for disc herniation. Hence, it has been suggested that NP have proinflammatory and sensitizing effects on neuronal tissue, promoting pain due to cells in the discs producing and secreting cytokines (Ahn et al., 2002; Aoki et al., 2002; Burke et al., 2002; Igarashi et al., 2000), MMPs, PGE_2 (Kang et al., 1997), nitric oxide (NO) (Kang et al., 1997), and phospholipase A_2 (Kawakami et al., 1996).

1.5.2 Proinflammatory cytokines

Cytokines belong to a large family of small protein molecules that can be produced and secreted by numerous cell types throughout the body, especially in response to infection, inflammation, injury or trauma. They are involved in cell signalling and intercellular communication, and can have both homeostatic, proinflammatory and anti-inflammatory activity, depending on the biological processes in which they are involved. The different cytokines can have overlapping roles, and often work in synergy. Important proinflammatory cytokines are interleukin1 (IL1) (comprising both IL1α and IL1β), IL6, TNF, interferon γ (IFNγ) and the chemokine IL8. Chemokines refer to a class of cytokines that are able to induce chemotaxis in nearby responsive cells, and recruit immune cells such as leukocytes to the site of an infection.

Inflammation is a noxious event capable of stimulating cytokine expression, whereupon cytokines may be released locally in the inflamed tissue. The cytokines act on nearby cells which express their various receptors and activate them, having an excitatory effect on synaptic transmission. Activated cytokines may also lead to the release of even more cytokines, leading to a perpetuation of the inflammatory response. However, inflammation also increases the levels of circulating cytokines. Peripheral inflammatory responses may therefore increase the cytokine levels in the CNS. Moreover, several previous studies have demonstrated the presence of proinflammatory cytokines, such as IL1β and TNF, in herniated lumbar discs (Brisby et al., 2002; Takahashi et al., 1996; Yoshida et al., 2005).

Notably, in the spinal cord both neurons and glia express receptors for cytokines (Sawada et al., 1993), and both circulating and locally released proinflammatory cytokines are thought to increase the neuronal excitability. Cytokines interact in a complex network, and may work in synergy to stimulate release of other mediators and further drive the sensitization of dorsal horn neurons. For review on cytokines, see (Dinarello, 2000).

1.6 Neuroplasticity

The nervous system is not a static apparatus of rigidly fixed wires and contacts; rather it is a dynamic arrangement, which is able to change its structure, function and organization in response to the changing environment and in response to experiences. This plasticity is fundamental in early development and during critical period in childhood, and is now known to be the basis of learning and memory. Neuroplasticity also manifests in relation to nociception, in which increased or decreased activity leads to functional changes of synaptic connections resulting in for example changed efficiency of synaptic strength of the nociceptive pathways. Increased input to a primary afferent neuron may lead to an often increased magnitude of response of the secondary neuron. Such changes may be caused by alterations in quantity and type of neurotransmitter release, trafficking of receptors or ion channels, the phosphorylation of receptors and ion channels, and even changes in the number of synaptic connections between neurons.

This plasticity varies in timescale from seconds up to years, and it is the underlying mechanism of sensitization. Peripheral sensitization implies a reduced threshold and an increased responsiveness of peripheral nociceptive neurons, due to various chemicals localized at the site of tissue damage. In contrast, central sensitization is characterized by increased excitability of neurons in the CNS, i.e. the spinal cord or the brain.

1.6.1 Central sensitization

Central sensitization means an increased responsiveness of nociceptive neurons in the central nervous system, and is considered to be of critical importance for the development of chronic pain. Central sensitization includes spontaneous firing, reduction in activation threshold, and enlargement of the receptive fields, all of which come into play in response to a prolonged intense noxious stimulus, inflammation or nerve injury. This may manifest as hyperalgesia and allodynia, which appears in various pain states.

Central sensitization is initiated by intense or prolonged stimulation of nociceptive neurons, leading to release of not only glutamate, but also substance P and BDNF from the central afferent terminals (Lever et al., 2001). These neurotransmitters and neuropeptides will then activate their respective receptors, and subsequently activates various intracellular kinases and transduction cascades, resulting in a substantial rise in intracellular Ca^{2+} concentration. Short-term effects (<2.5 hours), rely on rapid changes and posttranslational modifications of existing proteins, such as activation of intracellular kinases that phosphorylate iGluRs (AMPAR, NMDAR), increasing their conduction efficacy. This may also be associated with trafficking and insertion of additional AMPA receptors into the membrane, and activation of previously silent synapses. More long-term effects (>2.5 hours) require changes in gene expression and *de novo* synthesis of proteins. Central sensitization may also include anatomical reorganization or dysfunction in the endogenous pain control system, due to for instance central sprouting, loss of inhibitory interneurons or reduced synthesis or action of inhibitory neurotransmitters. For review on central

sensitization, see (Ji et al., 2003; Latremoliere and Woolf, 2009; Woolf and Costigan, 1999; Woolf and Salter, 2000).

1.6.2 Cellular mechanisms of spinal long-term potentiation

Long-term potentiation (LTP) is a phenomenon strongly associated with central sensitization. It was first described by Bliss and Lømo in 1973, when they discovered that short, but high-frequent electrical stimulation of neurons in the hippocampus, led to a persistent increase in synaptic transmission (Bliss and Lømo, 1973). Its counterpart long-term depression (LTD), in which low-frequency stimulation (LFS) may decrease the synaptic transmission, is of equal importance in the dynamics of synaptic plasticity. In later years LTP has also been demonstrated in other parts of the central nervous system, such as in the spinal cord.

The induction of LTP is dependent on activation of both the AMPA and the NMDA receptor (Pedersen and Gjerstad, 2008; Svendsen et al., 1998). LTP is initiated by intense excitation of nociceptors, leading to co-release of glutamate and substance P from the afferent nociceptors (Afrah et al., 2002), which then stimulate the postsynaptic AMPA-, NK1-, and mGlu receptors. Activation of these receptors leads to a long-lasting depolarization of the membrane due to influx of cations. At normal resting potential, the voltage-gated NMDA receptor is blocked by a Mg^{2+} ion. The NMDA receptors require both glutamate binding and a sufficient depolarization of the membrane to become activated. The prolonged depolarization thus expels the Mg^{2+} ion, allowing a substantial influx of Ca^{2+} into the postsynaptic cell. Additionally Ca^{2+} influx is mediated through voltage-gated T-type Ca^{2+} channels (Ikeda et al., 2003), as well as activation of the group I mGlu receptor and NK1 receptor, together with the intracellular inositol triphosphate (IP_3) receptor. Collectively, this results in a substantial increase of intracellular Ca^{2+} concentration. A rise in the intracellular Ca^{2+} level seems to be a key event for induction of LTP. Ca^{2+} is an important second messenger that activates signal transduction cascades in the postsynaptic cell, including subsequent activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Pedersen et al., 2005), protein kinase C (PKC) and protein kinase A (PKA) (Yang et al., 2004), and members of the mitogen-activated protein kinase (MAPK) family,

including extracellular signal-regulated kinase (ERK) (Xin et al., 2006). ERK has the ability to be translocated to the nucleus, where it phosphorylates the transcription factor cAMP response element binding protein (CREB). CREB binds to the cAMP response element (CRE) on DNA, and increases transcription of its downstream gene. Genes containing CRE sites in their promoter regions includes the immediate early genes encoding c-fos, cyclooxygenase 2 (COX2) and Zif268, as well as the late response genes encoding NK1, TrkB, BDNF and prodynorphin. Spinal LTP has also been associated with an increased gene expression of Il1β, glial cell-line derived neurotrophic factor (GDNF) and inducible nitric oxide synthase (iNOS) (Pedersen et al., 2010).

A well established *in vivo* method for inducing spinal LTP is based on electrical high frequency stimulation (HFS) conditioning, or tetanus, of the sciatic nerve. Several studies have demonstrated that HFS-induced LTP can also be induced at C-fibre synapses in the spinal dorsal horn, and thus contribute to hyperalgesia (Liu and Sandkühler, 1997; Liu and Sandkühler, 1995; Randic et al., 1993; Svendsen et al., 1997). In addition to electrical HFS, natural noxious stimuli such as crushing of tissue (Rygh et al., 1999), chemically induced inflammation, nerve injury, and heating or pinching of the skin, have also been shown to induce LTP in the dorsal horn (Sandkühler and Liu, 1998). However, using electrical HFS conditioning as stimulus has been criticized for not being biologically relevant. More relevant models are therefore needed to study the mechanisms underlying long-lasting pain conditions.

1.6.3 Glial cells and central sensitization

Microglia-neuronal signalling may be critical in the development of hypersensitivity, and is now considered to be an important component in driving central sensitization. A key molecule in microglia-neuron interactions, especially in neuropathic pain, has been shown to be ATP acting through the ionotropic P2X4 receptor, a nonselective purinergic cation-channel, whose expression is strongly upregulated in response to peripheral nerve injury (Tsuda et al., 2003). ATP binds to P2X4 receptor causing influx of Ca^{2+}, which subsequently activates the p38 MAPK,

leading to synthesis and release of BDNF from the activated microglia (Trang et al., 2009). BDNF, upon binding to its receptor TrkB, causes disinhibition of nociceptive transmission in lamina I neurons by downregulation of the K^+/Cl^- co-transporter, KCC2 (Coull et al., 2005). This leads to an excess of intracellular Cl^-, which subsequently renders the inhibitory actions of GABA- and glycine channels much less effective. This change in inhibitory response produces a phenotypic switch, where the neurons start to transmit innocuous mechanical input, display spontaneous activity, and increase their firing of noxious stimulus (Coull et al., 2003; Keller et al., 2007).

Previous data show that the glial metabolic inhibitor fluorocitrate may block induction of spinal LTP (Ma and Zhao, 2002). Hence, spinal LTP also seems to be dependent on activation of nearby glial cells. The non-neuronal glial cells are also considered to take part in LTP by releasing chemical mediators such as cytokines, whose downstream effects act back on both neurons and glia. The cytokine IL1β for instance, stimulates the production of COX, which synthesizes PG in dorsal horn neurons. Increased level of PG can augment neuronal excitability by sensitizing neurons to BK, resulting in subsequent neuropeptide release (Vasko et al., 1994), by activating neurons directly (Baba et al., 2001), or by reducing inhibitory activity (Ahmadi et al., 2002). For review on the role of glia cells in nociception, see (Hansson, 2006).

For an overview of possible signalling mechanisms involved in spinal hyperexcitability, see Figure 1.2.

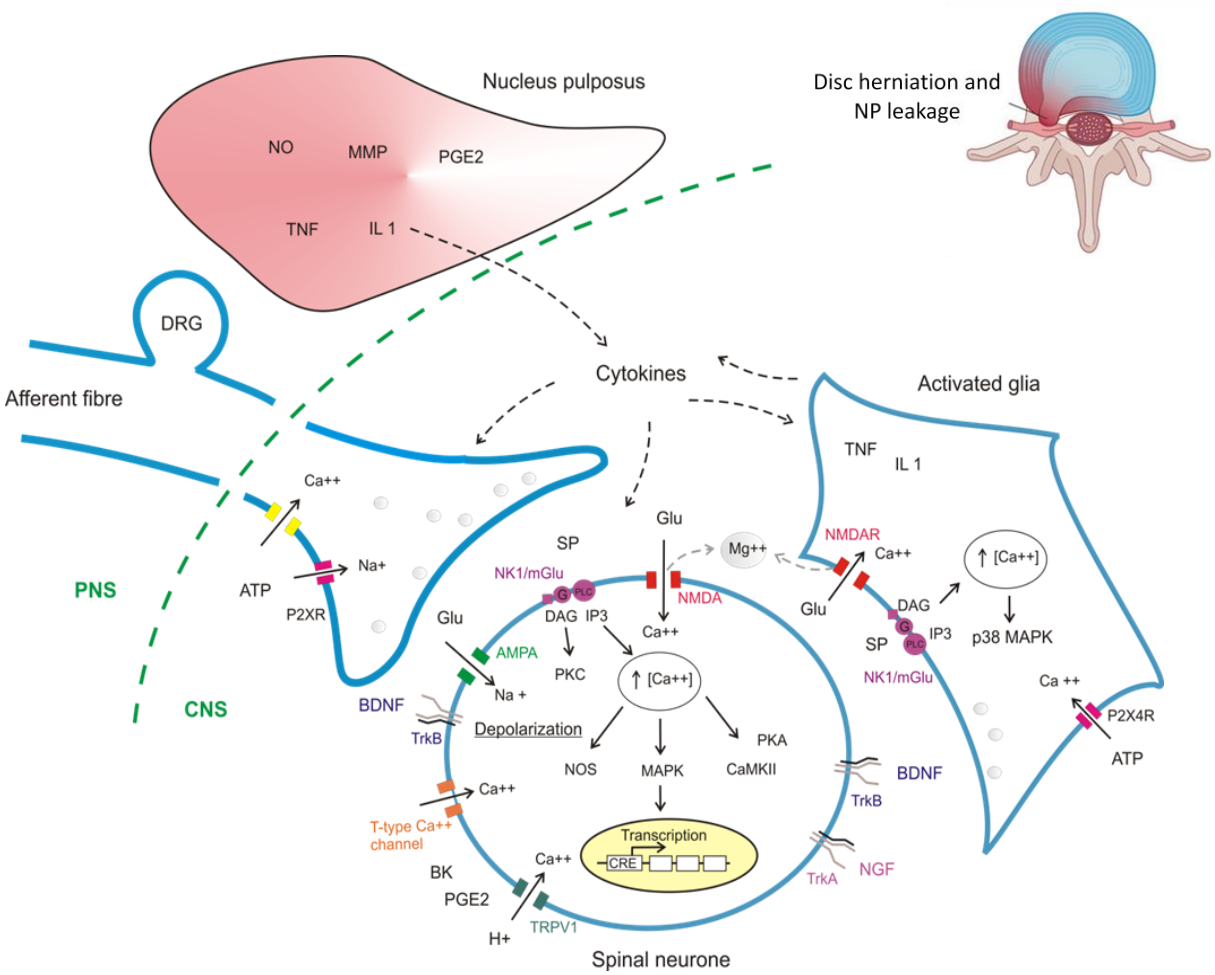


Figure 1.2. Mechanisms involved in spinal hyperexcitability. Following a strong input to the spinal cord, the primary afferents release Glu, SP and BNDF. This activates postsynaptic AMPAR, mGluR, NK1 receptors, TrkB and intracellular IP_3 receptors, leading to a sustained postsynaptic depolarization. In sequence, this removes the Mg^{2+} block of the NMDAR, and activates voltage-gated cation channels such as T-type Ca^{2+} channel. The following postsynaptic increase in cytosolic Ca^{2+}-concentration results in activation of NOS, and of intracellular kinases such as PKA, PKC, CaMKII and ERK. Activated ERK may translocate into the nucleus and activate transcription of genes encoding proteins important for synaptic transmission. In addition, nucleus pulposus (NP) may release various proinflammatory substances such as IL1β, TNF and MMP1, which again may induce an enhanced spinal signalling by affecting afferent fibres, spinal neurons and glial cell excitability. Upon activation by Glu, SP, ATP and cytokines, glial cells may synthesize and release BDNF and additional cytokines, which again contribute to enhance synaptic transmission. For instance, cytokines may stimulate the production of PG, NGF and BDNF. In addition, H^+, PGE_2 and BK released from inflammatory processes in the surrounding area may sensitize the cation-conducting TRPV1 receptor. Together, these actions may enhance the synaptic transmission in dorsal horn neurons, contributing to spinal hyperexcitability. AMPA: α-amino-3-hydroxy-5-methyl-isoxazoleproprionic acid, BDNF: brain-derived neurotrophic factor, CaMKII: calcium/calmodulin—dependent kinase II, DRG: dorsal root ganglion, ERK: extracellular signal-regulated kinase, IL: interleukin, IP3: inositol triphosphate, MMP: matrix metalloproteinase, NGF: nerve growth factor, NMDA: *N*-methyl-D-aspartate, NOS: nitric oxide synthase, PGE_2: prostaglandin E_2, PKA/C: protein kinase A/C, SP: substance P, TNF: tumour necrosis factor, TRPV1: transient receptor potential vanilloid receptor1. Adapted from (Gjerstad, 2007; Iordanova et al., 2010).

2 Aims

The main purpose of this study was to investigate the mechanisms underlying the development of sensitization relevant for low back pain and sciatica after lumbar disc herniation. Therefore, the conditioning effect of electrical HFS, and NP applied onto neuronal tissue, was examined. More specifically the study aimed to:

I) Demonstrate that electrical HFS conditioning of the sciatic nerve may induce spinal LTP as previously shown in a well established animal model.

II) Explore whether conditioning with NP applied onto spinal dorsal nerve roots could induce a spinal LTP-like phenomenon, thereby developing a more clinically relevant animal model for studying sensitization that is likely to occur following intervertebral disc herniation.

III) Examine the gene expression in the NP tissue of the proinflammatory cytokines IL1α, IL1β, TNF and MMP1 after application of NP onto the spinal dorsal nerve roots.

3 Materials and Methods

The examination of the spinal nociceptive neuronal activity was based on two animal models: I) an established LTP-model following electrical HFS-conditioning, and II) a novel spinal disc herniation model following NP application onto the spinal dorsal nerve roots. Extracellular field potential recordings were used to study the effect of the HFS conditioning in the LTP model, whereas single cell recordings were used to study the effect of NP application onto the spinal dorsal nerve roots.

In the gene expression analysis of NP tissue, the gene transcripts of the target genes interleukin 1α (IL1α), IL1β, TNF and MMP1 was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

All animal experiments were approved by the Norwegian Animal Research Authority (NARA) and were performed in conformity with the laws and regulations controlling experiments and procedures on live animals in Norway. These are in accordance with the European convention for the protection of vertebrate animals used in experimental and other scientific purposes.

3.1 Animals

Adult female outbred Sprague-Dawley rats (210-270 g) were used in the experiments with electrical sciatic conditioning, whereas adult female inbred Lewis rats (170-215 g) were used in the experiments with nucleus pulposus application onto the spinal dorsal nerve roots. Upon arrival the rats (Taconic Farms Inc., Harlan Laboratories Inc.) were housed in the animal facility at the National Institute of Occupational Health. The rats had free access to food and water and were acclimatized for at least one week before the experiments were performed. The air temperature was kept at 20-22 °C, the relative humidity at 45-55 %, and air ventilation rate was 15 x the room volume per hour. All experiments were

performed during the light period of a 14-hour day / 10-hour night cycle. The rats were euthanized immediately after the end of the experiments.

3.2 Surgery

The animals were initially anaesthetized by isoflurane (Baxter International Inc., USA) gas anaesthesia, followed by intraperitoneal administration of 250 mg/ml urethane (~2.1 g/kg body weight) (Sigma-Aldrich Co., USA; Alfa Aesar, Germany). Absence of hind paw withdrawal, eye reflexes, and ear wriggling to pinch was considered to indicate adequate anaesthesia. The rat's core temperature was kept at a constant level of 36-37 °C by means of a feedback heating pad (Harvard homeothermic blanket control unit, Harvard Apparatus Ltd. Kent, UK). Simplex eye salve (80 % Vaseline, 20 % paraffin) was applied to the eyes to prevent them from drying. Two ear bars attached to a rigid frame were used to hold the head in a steady position. A microscope and fibre optic light were used for better precision during surgery.

At the mid-thigh level, an 8-10 mm section of the left sciatic nerve was dissected free and then isolated from the surrounding tissue by a plastic film (Parafilm). A bipolar silver hook electrode (1.5 mm distance between the hooks) was placed proximal to the main branches of the sciatic nerve for electrical stimulation.

A laminectomy was performed at vertebrae Th13-L1, corresponding to the spinal cord segments L3-S1, where the sciatic nerve roots enter the spinal cord. To ensure stability during the experiments, the vertebral column was rigidly fixed by clamps rostral and caudal to the exposed spinal cord segments. The meninges, i.e. dura mater and arachnoidea, were carefully punctured by a cannula and removed by two tweezers.

In the experiments involving NP, a caudectomy was performed on genetically identically donor rats immediately after they were sacrificed, and NP was harvested from 3-8 caudal intervertebral discs.

3.3 In vivo electrophysiological recordings

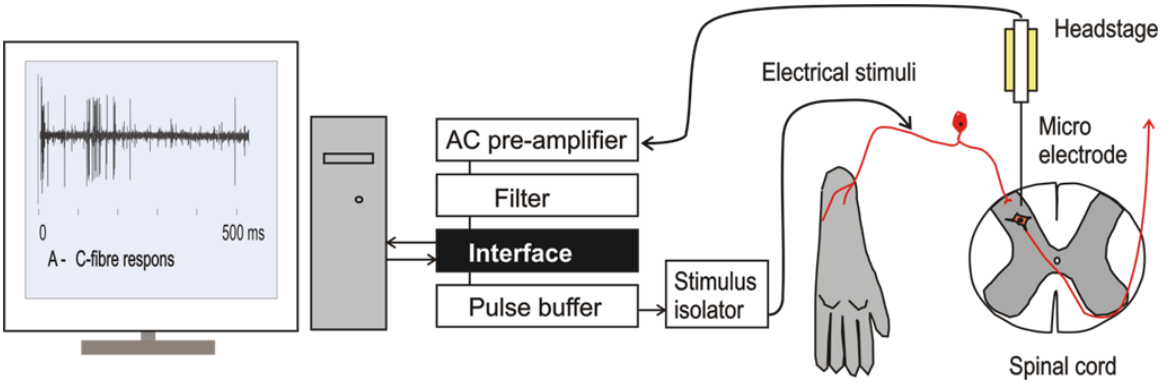
A parylene-coated tungsten microelectrode (impedance 1.0-4.0 MΩ) (Frederick Haer & Co., Bowdoinham, USA) was lowered vertically into the left dorsal horn of the spinal cord by an electrically controlled micromanipulator (Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar, Germany), whereas a reference electrode was placed subcutaneously. The spinal cord segments L3-S1 were identified by the neuronal responses to left hind paw finger tapping and pinching. The neuronal activity was monitored both graphically on the computer screen and acoustically through a loudspeaker, to assist the search for relevant neuronal activity.

First, the recorded signals were captured with a headstage and amplified (x 5000) with an AC preamplifier. Next, the signals were band-pass filtered (NeuroLog by Digitimer Ltd, Hertfordshire, UK), digitalized with the interface CED Micro1401, and stored by the software CED Spike 2.2 (Cambridge Electronic Design, Cambridge, UK). The sampling frequency was 20 000 Hz.

The software Spike 2.2 and interface CED Micro1401 was also used to control the electrical stimuli frequency given to the sciatic nerve by the hook electrode.

A pulse buffer connected to a stimulus isolator unit (NeuroLog System, Digitimer Ltd, Hertfordshire, UK) controlled the stimuli intensities. C-fibre threshold was defined at the start of each experiment as the lowest stimulus intensity that evoked the first visible C-fibre response. Every 4^{th} minute a single test stimulus (2 ms rectangular pulse, 1.5 x C-fibre response threshold) was delivered to the left sciatic nerve, and the A- and C-fibre responses were defined according to latencies. Six stable C-fibre responses served as baseline for the subsequent experiments. The response signals were recorded for 180 minutes after conditioning. Rats receiving no conditioning served as control. The test stimulus intensity given in the experiments varied from 1.0 mA up to 3.0 mA. For the experimental set-up, and the protocol for the electrophysiological experiments, see Figure 3.1. a, and Figure 3.1. b, respectively.

a



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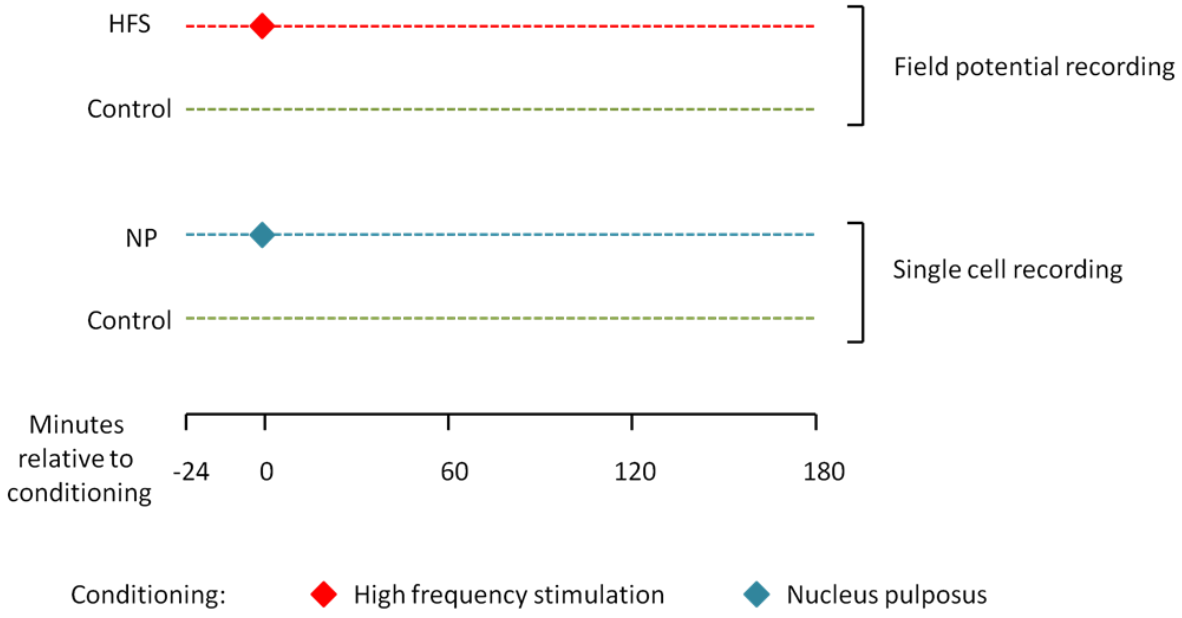

Figure 3.1. Experimental set-up and protocol for electrophysiological experiments. a) Set-up for electrophysiological recordings in the left spinal dorsal horn. A bipolar electrode was placed on the left sciatic nerve for electrical stimulation. The neuronal responses were recorded by a microelectrode in the spinal dorsal horn, before the signals were amplified, filtered and digitalized. **b)** Protocols of electrophysiological experiments. The two experimental protocols are indicated by different colours; field potential recordings in red and single cell recordings in blue. Controls, i.e. no conditioning, are shown in green. Diamonds indicate I) conditioning with HFS and II) conditioning with NP application.

3.3.1 Conditioning with HFS and field potential recordings

Extracellular field potentials, i.e. the negative extracellular potential caused by cation-influx in the dendritic nerve-endings, were recorded at depths of 100-500 µm from the surface of the spinal cord in anaesthetized Sprague-Dawley rats. The microelectrodes had an impedance of 1.0-2.0 MΩ and the signals were band filtered with a band-width of 1-100 Hz corresponding to a wavelength of 10-1000 ms. The A- and C-fibre volleys were defined according to latencies, and the C-fibre responses defined as the amplitude of the volleys (see Figure 3.2).

Spinal LTP was induced using HFS conditioning applied to the sciatic nerve (1 ms rectangular pulses, 4.5 mA, five trains of 1 s duration, 100 Hz, 10 s intervals between the trains).

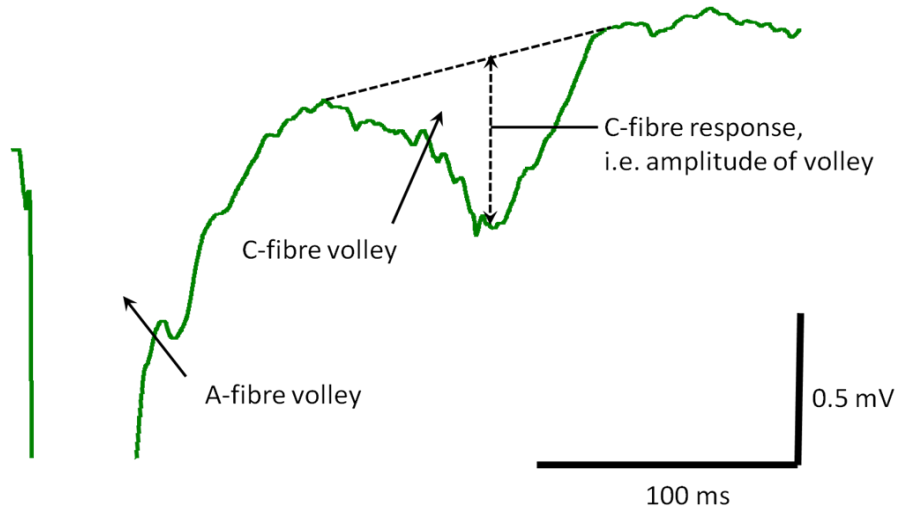

Figure 3.2. Extracellular field potential recording. The figure shows the A- and C-fibre volleys generated by the cation influx causing excitatory postsynaptic potentials (EPSPs) in a field of neurons following electrical stimulation. The C-fibre response is defined by the amplitude of the C-fibre volley.

3.3.2 Conditioning with NP and single cell recordings

Electrophysiological extracellular single cell potentials, i.e. the electrical activity of single neurons, were recorded from the dorsal horn at depths of 100-500 μm from the surface of the spinal cord in anaesthetized Lewis rats. The microelectrodes used had an impedance of 2.0-4.0 MΩ. The signals were filtered with a band-width of 500-1250 Hz corresponding to the wave length of 0.8-2 ms.

The A- and C-fibre responses were defined according to latencies, where spikes 50-300 ms after stimulus were defined as C-fibre response. As a measurement of the spinal nociceptive response, the C-fibre response on each test stimulus was quantified. Single cell recordings were ensured by the amplitude and shape of the recorded signals (see Figure 3.3., inset).

NP was harvested from 3-4 caudal intervertebral discs of a genetically identical donor rat, and the isograft was then applied directly onto the spinal dorsal nerve roots, after a baseline of 6 stabile C-fibre responses. The NP was applied 0.5-2 mm caudally to the recording electrode, covering the left dorsal nerve roots.

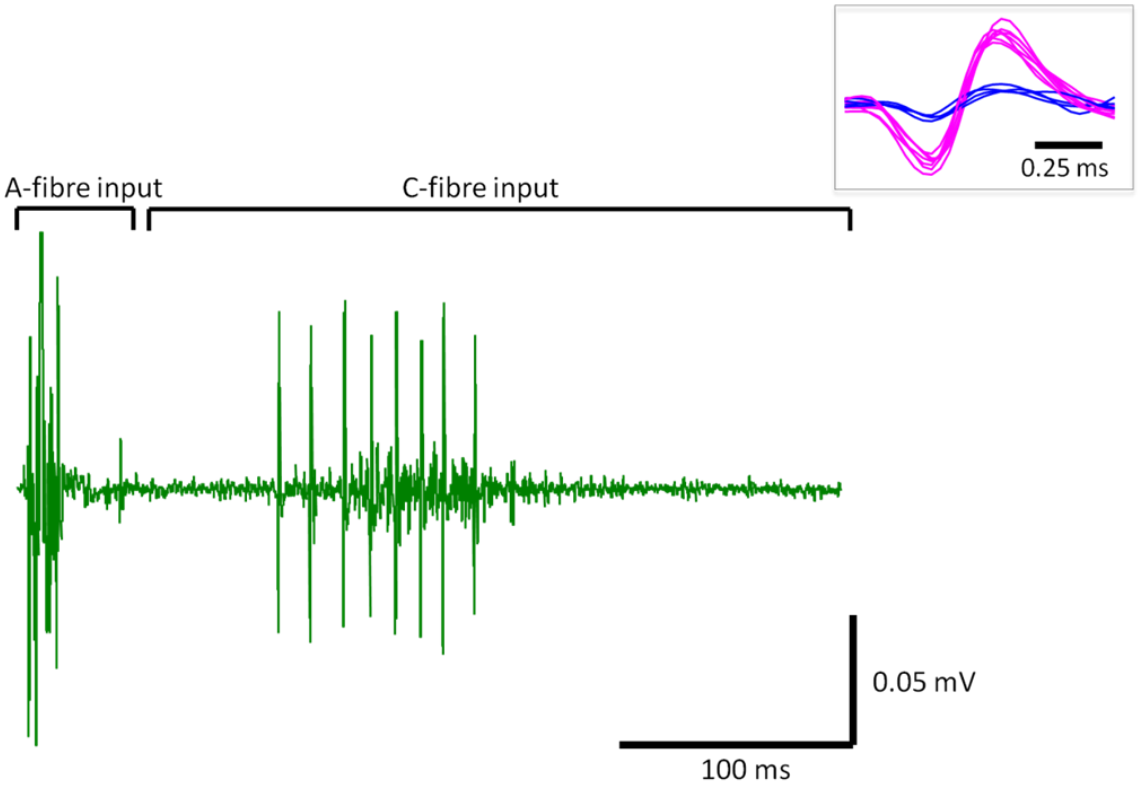

Figure 3.3. Extracellular single cell recording. Neuronal activity evoked by a single test pulse applied to the left sciatic nerve. Spikes 50-300 milliseconds (ms) after stimulus were defined as the C-fibre response. Inset figure: Comparison of shape and amplitude of the action potentials

3.4 Gene expression analysis

Following the harvesting of NP tissue from caudal vertebrae, total RNA was isolated from the tissue, before being converted into complementary DNA (cDNA). To determine the amount of target gene RNA in the NP tissue, RT-qPCR analysis was performed.

3.4.1 Tissue harvesting

NP tissue was harvested from 3-8 caudal intervertebral discs following four series of experiments defined as; I) native, II) control, III) isolated and IV) exposed. In the native experiments NP tissues were harvested and frozen immediately after surgery,

whereas in the control experiments NP tissues were dissected out from the caudal intervertebral discs 180 minutes after sham operation. For the isolated and the exposed group, NP tissue from the caudal intervertebral discs was removed from a donor rat and then bisected. One piece of NP was put in a tube with a droplet of saline 180 minutes prior to freezing. The other piece was applied onto the spinal dorsal nerve roots for 180 minutes prior to freezing (for experimental protocol, see Figure 3.4.). The experiments were performed in a randomized order.

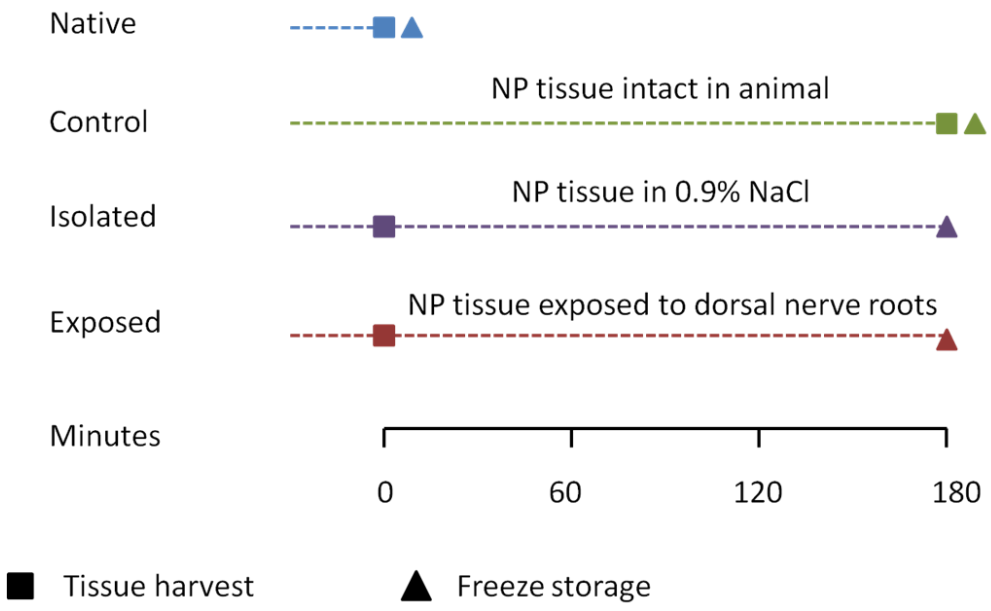

Figure 3.4. Protocol for tissue harvesting of NP. The four different series are indicated by different colours. Tissue harvest and freeze storage are indicated by squares and triangles, respectively. NP tissue was frozen and stored immediately after caudectomy for the native tissue, and at time 180 minutes for the control tissue. For the NP tissue that was isolated in 0.9 % NaCl or exposed to the spinal dorsal nerve roots, the tissues were harvested from donor rats at time 0, then collected and frozen at time 180 minutes. NP: nucleus pulposus.

3.4.2 RNA isolation from NP tissue

Total RNA was isolated from frozen (-80 °C) tissue samples of NP. Isol-RNA Lysis Agent (5 PRIME) was added to the frozen tissues before the tissues were homogenized by a mixer mill (Retsch MM301, Haan, Germany). The tissue samples were then centrifuged and any non-solubilised cell material was removed. Chloroform was added to separate the sample in three phases; one organic phase with lipids and proteins, one intermediate phase with DNA, and one upper aqueous phase containing RNA. This upper phase was carefully extracted, before isopropanol was added to it for RNA precipitation. The resulting pellet was washed with 75 % ethanol, dried and re-dissolved in ribonuclease (RNase)-free water. The amount of RNA in each sample was quantified by optical densitometry (Eppendorf AG, Hamburg, Germany). TE-buffer was added to the samples in a 1:70 µl dilution. An optical density (OD)-value of 1 at 260 nm corresponded to 40 ng/µl RNA. Finally, the samples were diluted in DEPC-water to a final concentration of 0.5 µg/µl (for further details, see Appendix I).

3.4.3 Evaluation of RNA quality

The quality of the isolated RNA was analyzed by on-chip gel electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbronn, Germany). RNA from 2-4 different samples were mixed together, denatured at 70 °C and applied into different wells on a microchip pre-treated with gel matrix and a fluorescent dye. All reagents were obtained from the Agilent RNA 6000 NanoKit (Agilent Technologies, Waldbronn, Germany).

In the analysis each sample was injected into a separation channel where the ribosomal subunits were electrophoretically separated and detected with laser induced fluorescence detection. Since the fluorescent dye emitted fluorescence upon binding to RNA, the fluorescence intensity was used to detect the ribosomal subunits.

The Bioanalyzer instrument generated an electropherogram (see Figure 3.5), which was used by the software to define the RNA Integrity Number (RIN) and to

determine the RNA quality. RIN is an algorithm based on the ratio of 18S to 28S ribosomal subunits, and is used to evaluate the degree of RNA degradation. It is based on a numbering system from 1 to 10, with the value 1 being the most degraded RNA, and 10 being completely intact RNA (for further details, see Appendix II).

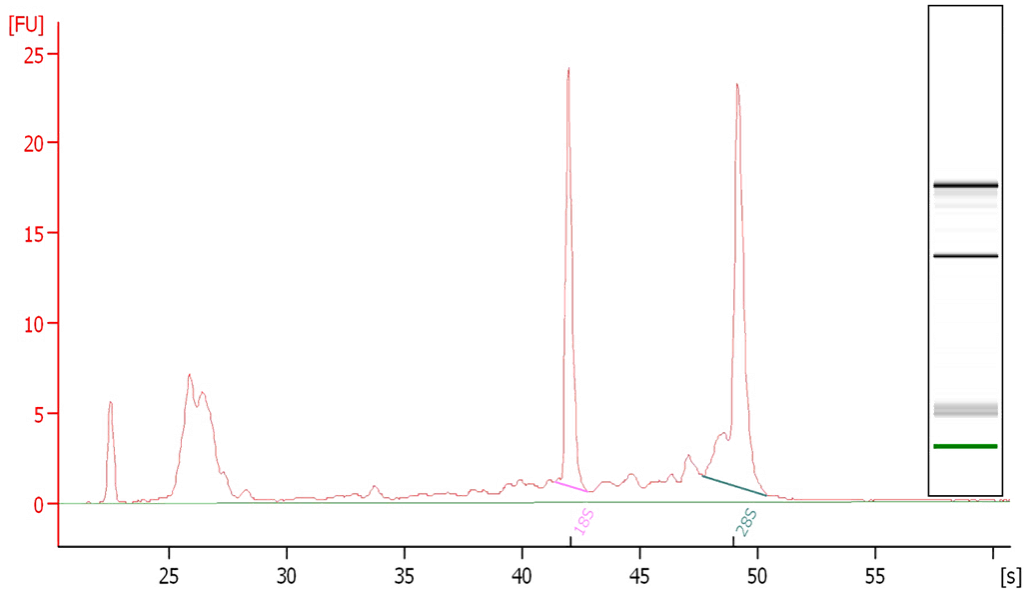

Figure 3.5. Example of an electropherogram. Electropherogram of an RNA sample obtained from nucleus pulposus tissue, showing two peaks representing the 18S and 28S rRNA subunits. The RIN-value of this particular sample was 8.5. Bar represents ladder.

3.4.4 cDNA synthesis

The isolated RNA was converted to cDNA by the aid of a first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) for RT-qPCR. A mixture of 1.5 µg of RNA, deoxynucleotides and random sequence primers was incubated at 65 °C for 15 minutes. AMV reverse transcriptase was added and the cDNA synthesis performed at the following schedule: 42 °C for 60 minutes, 99 °C for 5 minutes and 4 °C for 5 minutes (Perkin-Elmer Cetus DNA Thermal Cycler 480). The cDNA product was diluted in TE-buffer to a final concentration of 10 ng/µl and stored at -80 °C (for further details, see Appendix III).

3.4.5 Quantitative polymerase chain reaction (q-PCR)

In NP tissue, the expression of four different target genes; IL1α, IL1β, TNF and MMP1, was examined. The amount of template used in the qPCR reaction was cDNA corresponding to 40 ng reverse-transcribed total RNA for the target genes, and 4 ng reverse-transcribed total RNA for the reference gene β-actin. The analyses were performed on ABI 7900 (Applied Biosystems, Foster City, California, USA) with Perfecta SYBR Green FastMix (Quanta Bioscience, Gaithersburg, MD, USA) at the following schedule: 90 °C for 2 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds (for further details, see Appendix IV).

All primers, apart from the MMP1 primers, were designed using the Primer Express 2.0 Software (Applied Biosystems, Foster City, California, USA), and checked for specificity by performing a BLAST search. Primers for the MMP1 were designed using the PrimerBLAST website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). To avoid amplification of possible genomic DNA contamination, PCR primers were designed to span introns. For further details regarding the primers, see Table 3.1.

Primer	Sequence (written 5`→ 3`)	bp	% GC	Tm °C	Product size (bp)
β-actin forward	CTA AGG CCA ACC GTG AAA AGA	21	47.6	58.0	87
β-actin reverse	ACA ACA CAG CCT GGA TGG CTA	21	52.4	59.2	
IL1α forward	AGG GCA CAG AGG GAG TCA ACT	21	57.1	58.8	70
IL1α reverse	GTC AGG AAC TTT GGC CAT CTT G	22	50.0	59.6	
IL1β forward	CGT GGA GCT TCC AGG ATG AG	20	60.0	59.4	90
IL1β reverse	CGT CAT CAT CCC ACG AGT CA	20	55.0	59.1	
TNF forward	GCC ACC ACG CTC TTC TGT CTA	21	57.1	59.1	82
TNF reverse	TGA GAG GGA GCC CAT TTG G	19	57.9	59.6	
MMP1 forward	ATC GCA TCC AGG CTT TAT ATG G	22	45.5	59.0	73
MMP1 reverse	CAT GGA TGT GGT GTT GTT GCA	21	47.6	59.8	

Table 3.1. Primers used for RT-qPCR. bp: basepair, GC: guanosine/cytosine, Tm: melting temperature.

A final melting curve of fluorescence versus temperature was generated for each sample to screen for possible co-amplified products. An amplification plot, indicating the intensity of the fluorescence emitted by the SYBR Green-bound PCR product as a function of number of cycles in the reaction, was generated for each sample. The threshold cycle (Ct) value, i.e. the number of cycles required for the fluorescence signal to reach a computer-defined threshold (dependent on the background fluorescence), was estimated for each sample with the software SDS 2.2 (Applied Biosystems, Foster City, California, USA). The Ct value for each sample corresponds to a specific amount of RNA converted to cDNA, and the lower the Ct value, the greater the amount of PCR product in the sample. A dilution series was made to make a standard curve for each PCR run. The amount of target RNA converted to cDNA in each sample was then calculated using the Ct value and the standard curve. The gene expression of the target genes encoding IL1α, IL1β, TNF and MMP1 was normalized to the expression of the reference gene β-actin (see Figure 3.6).

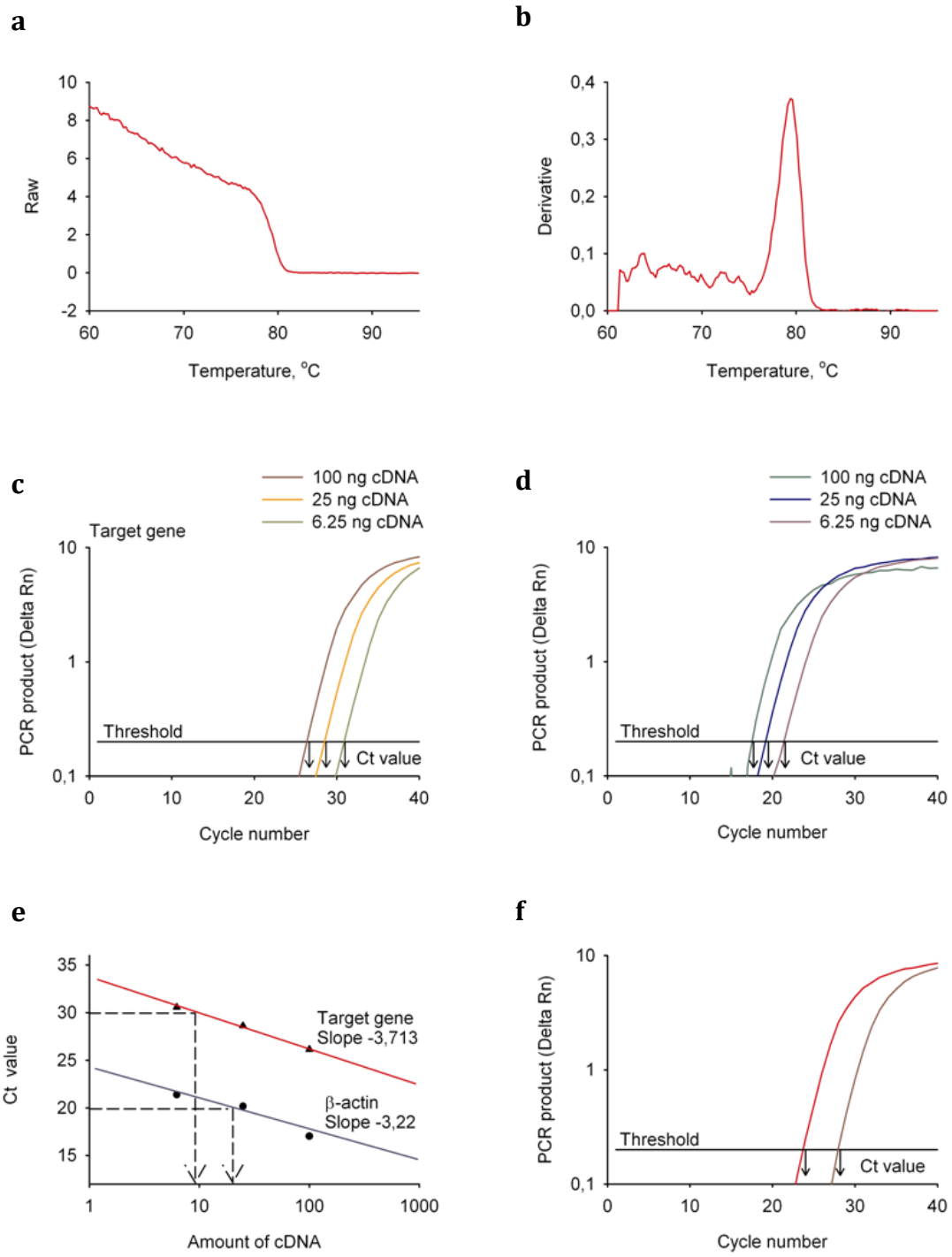

Figure 3.6. RT-qPCR. a) and **b):** Examples of melting curves for one sample; raw data and derivative, respectively. The one-peak shape indicates absence of co-amplification products. **c)** and **d)**: Examples of the amplification plots of the dilution series of the target gene and the reference gene β-actin. The Ct (threshold cycle) value represents the number of amplification cycles required for fluorescence signals to reach a computer-defined threshold. Delta Rn values equals the intensity of the fluorescence emitted by the SYBR Green-bound PCR product. **e)** Example of quantification of gene expression by the standard curve for the target gene Il1β and the reference gene β-actin. The Ct value for each sample corresponds to a specific amount of RNA converted to cDNA indicated by the arrows. Data from analysis of IL1β and β-actin. **f)** Examples of two amplification plots from two different samples of IL1β. The lower Ct value of the left plot indicates a higher amount of target gene compared to the right plot.

3.5 Data analysis and statistics

The spinal nociceptive activity, i.e. the C-fibre response was defined as the amplitude of the C-fibre volley or the number of C-fibre spikes. To determine the baseline of each electrophysiological recording, the mean of six recordings prior to HFS or NP conditioning was used, and the C-fibre responses over time were then presented as percent of baseline.

To examine the effect of HFS and NP tissue on spinal nociceptive response versus the effect of the control, the average C-fibre responses 160-180 minutes after conditioning, i.e. the mean of the last six recordings, were compared with the same responses in the corresponding controls. Group means were compared using Unpaired two-sample Student´s *t*-tests. Data are given as means ± standard error of the mean (SEM). A p-value ≤ 0.05 was set as the level of statistical significance.

For the gene expression studies, fold change values for each sample were defined by the expression of the target gene normalized to the expression of the reference gene β-actin. All values were then normalized to the native group. Statistical analyses were performed on log-transformed data to compensate for non-normal distributions. Group means were compared using one-way ANOVA and Tukey HSD post-hoc comparisons. Statistical analyses were performed by the PASW Statistics 18 (SPSS Inc., Hong Kong). Data are given as means ± SEM. A p-value less than 0.05 was set as the level of statistical significance.

4 Results

In this study extracellular electrophysiological recordings were performed to investigate the C-fibre responses in the rat spinal dorsal horn following conditioning with HFS, and conditioning with NP tissue. In addition, the change in gene expression of proinflammatory cytokines and MMP1 in NP tissue exposed to the spinal dorsal nerve roots was examined using RT-qPCR.

4.1 In vivo electrophysiological recordings

4.1.1 Expression of spinal LTP

In accordance with previous studies using the same experimental set-up, field potential recordings showed that HFS conditioning applied onto the sciatic nerve induced a clear LTP in the spinal dorsal horn (n=7), and the increased C-fibre responses outlasted the experimental time period of 180 minutes. At the end of the experiments, at 160-180 minutes after HFS conditioning, the C-fibre response was 135 % of baseline, whereas the C-fibre response in the corresponding controls (n=6) was 84 % of baseline. The C-fibre responses 160-180 minutes after HFS conditioning were significantly higher than in the unconditioned controls (p=0.01, Unpaired two-tailed Student´s t test) (see Figure 4.1. a and b).

4.1.2 The effect of NP on neuronal activity

Single cell recordings demonstrated that NP applied onto the spinal dorsal nerve roots induced a persistent increase in the C-fibre responses, which also outlasted the experimental time period of 180 minutes. At the end of the experiments, at 160-180 minutes after NP conditioning (n=8), the C-fibre response was 156 % of baseline, whereas the C-fibre response in the corresponding controls (n=5) was 90 % of baseline. The C-fibre responses 160-180 minutes after NP application were

significantly higher than in the control group (p = 0.03, Unpaired two-tailed Student´s t test) (see Figure 4.1. c and d).

We here show two examples of the C-fibre responses before and after HFS conditioning (see Figure 4.2. a), and NP conditioning (see Figure 4.2. b).

In the latter example, the increased C-fibre response following conditioning with NP applied onto the dorsal nerve roots were followed for five hours, demonstrating a long-term inflammatory effect of NP on neuronal spinal excitability (see Figure 4.2. b).

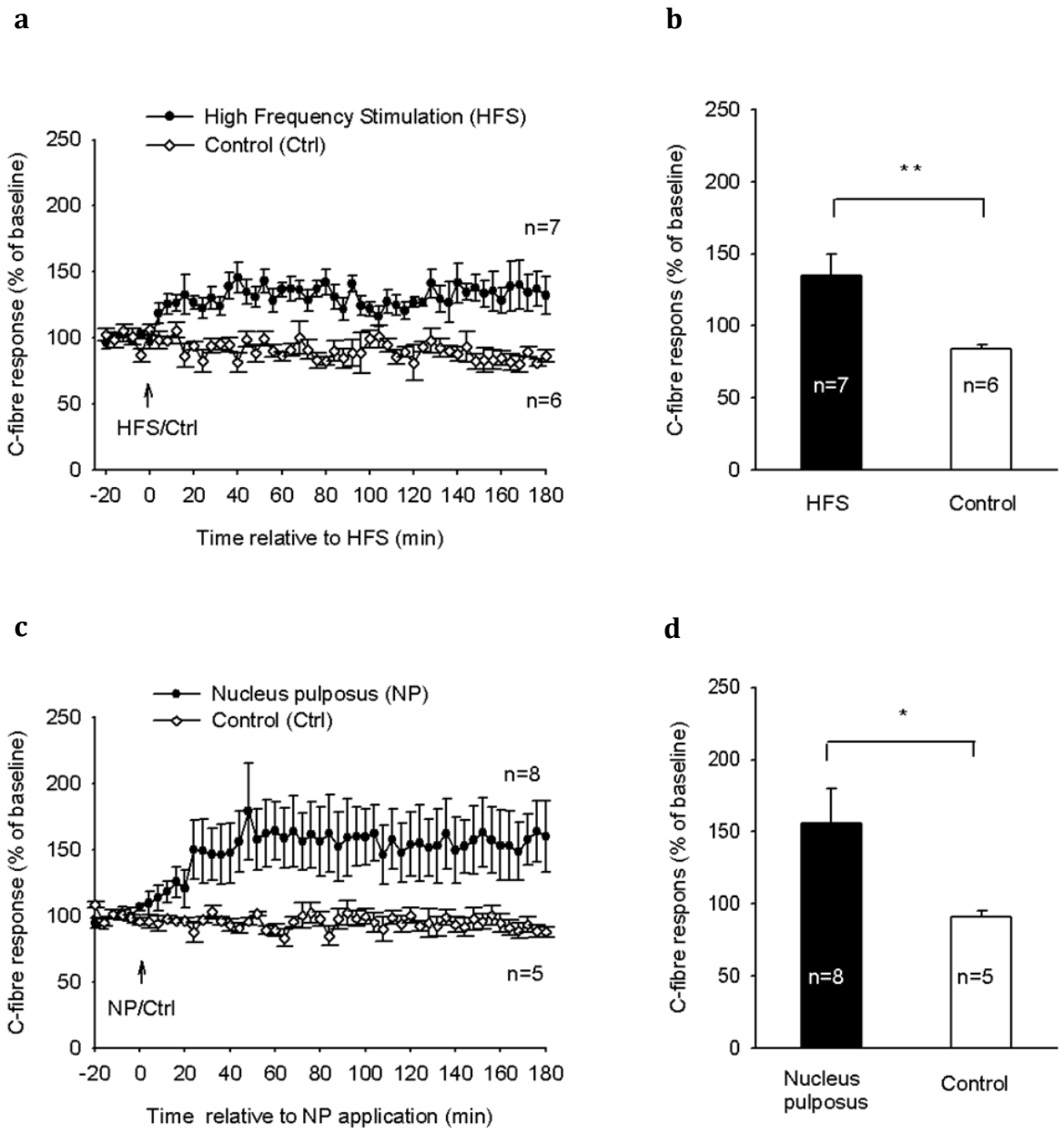

Figure 4.1. C-fibre response in the spinal dorsal horn following HFS conditioning and NP conditioning. a) The time course of the field potential C-fibre response in percent of baseline after sciatic HFS conditioning, and of the corresponding control. **b)** The relative increase in the field potential C-fibre response 160-180 minutes after HFS conditioning, i.e. the mean value of the last six recordings, in the HFS group and the control group. **c)** The time course of the single cell C-fibre response in percent of baseline after application of NP, and of the corresponding control. **d)** The relative increase in the single cell C-fibre response 160-180 minutes after NP conditioning, i.e. the mean value of the last six recordings, in the NP group and the control group. *p ≤ 0.05, **p ≤ 0.01, two-tailed Unpaired Student´s *t*-test. Data are given as mean ± SEM.

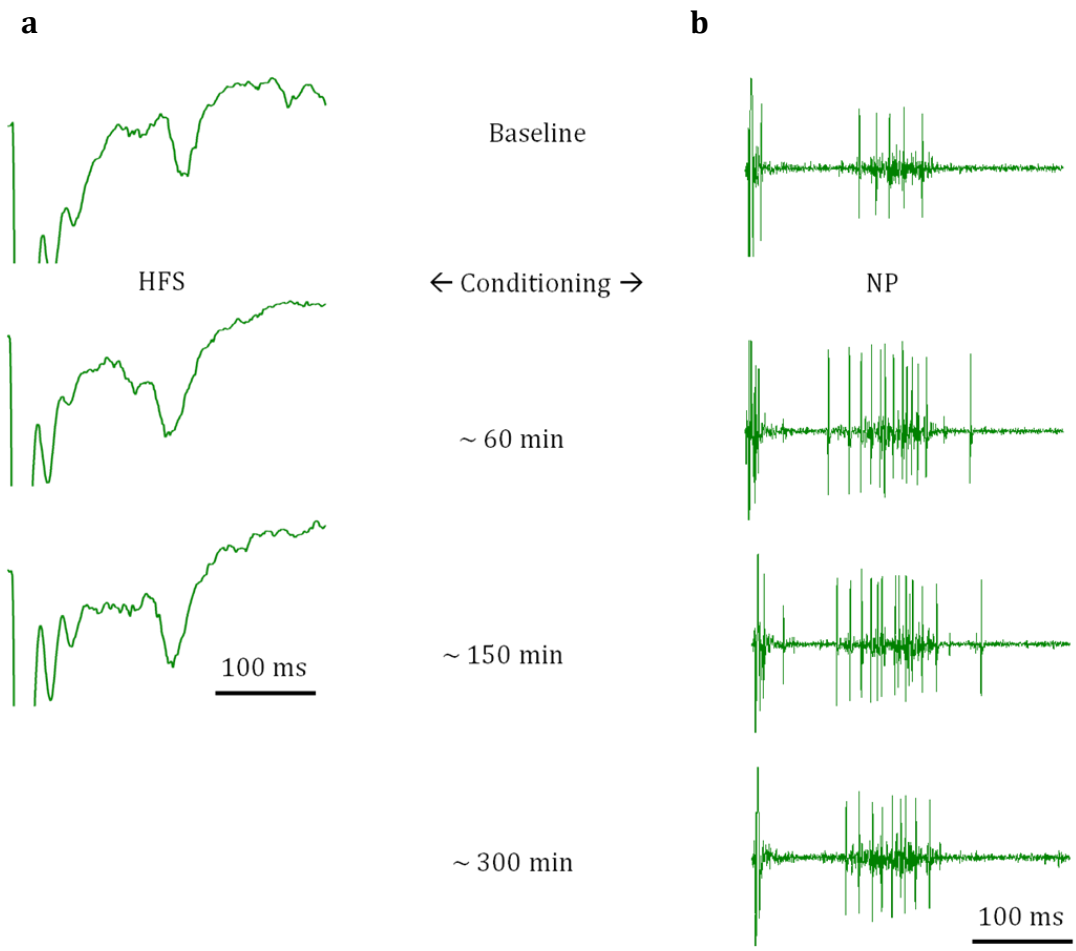

Figure 4.2. C-fibre responses in the spinal dorsal horn following conditioning. a) Example of a field potential recording registered before- , as well as 60- and 150 minutes (min) after sciatic HFS conditioning. The amplitude of the C-fibre volley increases over time. **b)** Example of single cell recordings registered before-, as well as 60-, 150- and 300 minutes after application of NP onto spinal dorsal nerve roots. The number of spikes increased from 5 in baseline to 9-13 spikes 150-300 minutes after conditioning. HFS: high frequency stimulation, NP: nucleus pulposus.

4.2 Gene expression in NP tissue

The gene expression of the target genes IL1α, IL1β, TNF and MMP1 was examined in the NP tissue after I) NP was harvested directly from the caudal intervertebral discs; native (n=5), II) NP was harvested from the discs of sham-operated animals after 180 minutes; control (n=10), III) NP was relocated and placed in 0.9 % NaCl for 180 minutes; isolated (n=10), and IV) NP was relocated and exposed to the spinal dorsal nerve roots for 180 minutes; exposed (n=10). The fold change values for each sample were defined by the expression of the target gene normalized to the expression of the reference gene β-actin, and the level of the corresponding native tissue.

A significant increase in the gene expression of Il1α, IL1β and TNF was demonstrated in NP tissue 180 minutes after NP was exposed to the spinal dorsal nerve roots, compared to the native-, control- and isolated tissues. The relative level of gene expression in NP tissue exposed to the spinal dorsal nerve roots was 9.4 ± 3.3 for IL1α; 5.2 ± 1.8 for IL1β; 14.4 ± 6.6 for TNF (IL1α p=0.000, IL1β p=0.028, TNF p=0.000, one-way ANOVA and Tukey HSD post-hoc comparisons).

No changes in the level of gene expression of MMP1 were observed.

These analyses demonstrate a significant association between the level of IL1α, IL1β and TNF gene expression and the exposure of NP tissue to the spinal dorsal nerve roots. This indicates a proinflammatory role of NP when it is in contact with the neuronal tissue, i.e. of the spinal nerve roots and spinal cord (see Figure 4.3. a-d).

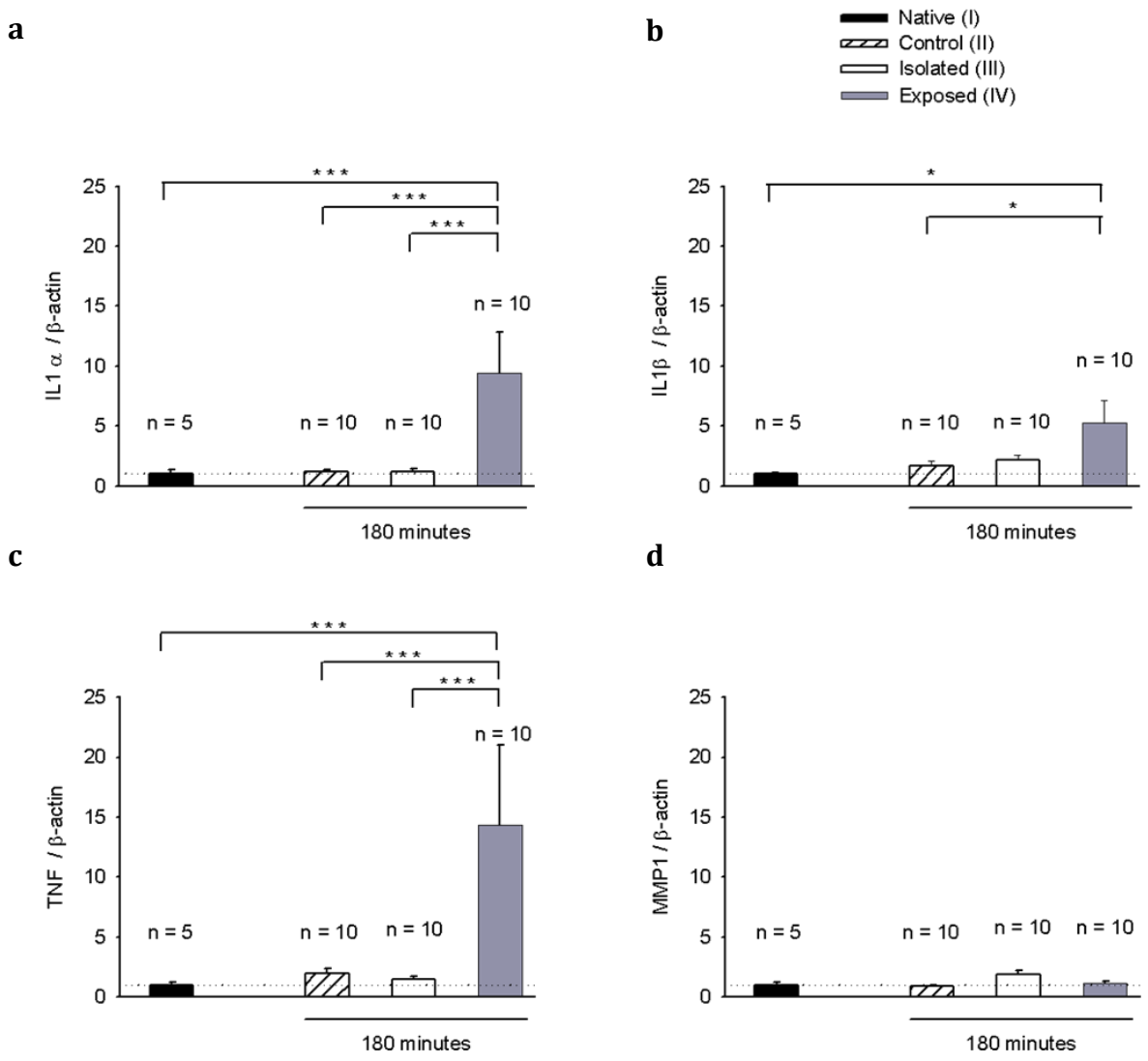

Figure 4.3. Changes in gene expression in NP tissue. Gene expression of target genes relative to the reference gene β-actin in NP tissue I) harvested from native, II) harvested from sham-operated 180 minutes control rats, III) isolated in 0.9 % NaCl for 180 minutes or IV) exposed the spinal dorsal nerve roots for 180 minutes. **a)** Interleukin-1α (IL1α), **b)** Interleukin-1β (IL1β) **c)** Tumour necrosis factor (TNF), and **d)** Matrix metalloproteinase-1 (MMP1). All values are normalized to the native group. *p ≤ 0.05, ***p ≤ 0.001, One-way ANOVA and Tukey HSD post-hoc comparison. Data are given as mean ± SEM.

5 Discussion of methods

5.1 Animals and surgery

The rat is a frequently used model organism in basic pain research. In the present study, female rats were chosen in preference to male owing to the lower risk for the researchers and handlers of developing allergies. Two different strains of rats were used, outbred Sprague-Dawley- and inbred Lewis rats. Both strains have a docile disposition and are widely used in research, including many studies of nociception (Gjerstad et al., 2001; Pender, 1986; Sandkühler and Liu, 1998; Xie et al., 2001). One of the most commonly used strains is the Sprague-Dawley rat. However, since Lewis rats have a more pronounced inflammatory response than other rats, they are often used to study inflammation, in transplantation research and in studies addressing arthritis and allergic encephalitis.

The anaesthetic used in the present study was urethane, -ethyl carbamate, $C_3H_7NO_2$. This drug has been widely used as an anaesthetic in animal experiments, including several studies of the nociceptive system measuring extracellular responses induced by nociceptive stimulation (Gjerstad et al., 2001; Liu and Sandkühler, 1995; Pedersen et al., 2010; Svendsen et al., 1997). The advantages of using urethane is that a single injection gives a steady and long-lasting level of surgical anaesthesia, with minimal effects on the autonomic, cardiovascular, and respiratory systems while maintaining spinal reflexes. This makes it a suitable drug for long-lasting experiments such as the ones performed in this study.

The administered dose of urethane, i.e. ~2.1 g/kg bodyweight, is in accordance with previous studies measuring extracellular electrophysiological recordings (Eriksen et al., 2012; Gjerstad et al., 2005; Jacobsen et al., 2010; Pedersen et al., 2010; Pedersen et al., 2005). It has been assumed that urethane anaesthesia has minimal interference on electrophysiological responses, and that similar physiologic responses would have been seen in awake animals. However, previous studies have shown that urethane may affect neurotransmitter-gated ion channels. For example,

at higher doses (defined as ≥1.5 g/kg) urethane may potentiate the functions of GABA- and glycine channels, and inhibit NMDA- and AMPA receptors (Hara and Harris, 2002). In our animals anaesthetized with urethane, the neurophysiologic measurements may be affected by the drug's effect on neurotransmitters relevant for nociceptive activity in the pain pathways.

It is therefore important to bear in mind that our data obtained from urethane-anaesthetized rats may be different from what the results would have been if measured in awake animals.

5.2 In vivo electrophysiological recordings

The spinal neuronal activity may be measured by recordings of multiple cells, i.e. extracellular field potential recordings, or by recordings of one cell by extracellular single unit recordings. In the present study, extracellular field potential- and single cell recordings from the rat dorsal horn were performed to examine the effect of HFS and NP application, respectively. In order to capture both the short-term (<2.5 hours) and long-term (>2.5 hours) effects of the conditionings, the experimental time period was set to 180 minutes.

Much of the research on the LTP phenomenon has been done in *in vitro* preparations of spinal cord slices by use of patch-clamp recordings (Ikeda et al., 2003; Randic et al., 1993). This provides better control of the intra- and extracellular environment, as well as the ability to study the activity by intracellular recordings. In contrast, in the present study we have applied *in vivo* recordings, with the afferent fibres and descending pathways still intact. This allows a more integral examination of the neuronal events under more physiological conditions.

Both field potential- and single cell recordings have been used to study spinal LTP (Liu and Sandkühler, 1995; Pedersen et al., 2005). Regarding the HFS experiments, we started out with field potential recordings, which reflect the summation of evoked postsynaptic currents into several cells in the area close to the recording electrode. This approach has recently been used in many former studies, and it therefore provides a good basis for comparison with previous findings. The field

potential experiments were also less time-consuming than single cell experiments. However, regarding the NP experiments, we chose to study the neuronal activity by single cell recordings, in which the number of action potentials from one cell is measured. This allowed recording of possible spontaneous neuronal activity that cannot be studied by field potential recordings.

5.2.1 Conditioning with HFS

Induction of spinal LTP by HFS with 100 Hz has been shown in numerous earlier studies, and it is a well-established model for studying synaptic plasticity (Liu and Sandkühler, 1997; Liu and Sandkühler, 1995; Randic et al., 1993; Svendsen et al., 1997). Also, an *in vitro* study of mice spinal cord slices, showed that HFS selectively induced phosphorylation of ERK in spinal dorsal horn laminae (Hartmann et al., 2004). In studies of C-fibre field potential recordings following sciatic HFS conditioning, it has been demonstrated that the excitability of the afferent C-fibres at the site of stimulation is not changed. This indicates that enhancement of synaptic transmission in the spinal cord is responsible for the increased amplitude of the field potentials after HFS (Liu and Sandkühler, 1997). However, the method of inducing increased synaptic efficacy with such an intense electrical stimulation has been criticized. Clearly, HFS is not a natural form of stimulus. Indeed, previous studies have demonstrated that not all C-fibres can follow electrical stimulation as high as 100 Hz (Fang et al., 2005; Liu and Sandkühler, 1997). However, lower frequencies have also been shown to be able to induce LTP. In spinal cord-dorsal root slice preparations, conditioning with 2 Hz for 2-3 minutes at C-fibre strength has been shown to induce LTP at C-fibre synapses with lamina I neurons (Ikeda et al., 2006).

5.2.2 Conditioning with NP

Induction of the LTP-like phenomenon by NP has previously been not very well described. Hence, in the present study we decided to study the activity at the spinal level before and after conditioning with NP. The rationale behind this approach was

to study the effect of a more biologically relevant stimulus for inducing an LTP-like phenomenon, increasing the neuronal activity in the nociceptive pathways.

In this new animal model, we used NP tissue harvested from inbred donor rats. Since immunological response to donor tissue could be elicited due to minor genetic differences, inbred Lewis rats were used. These rats have been inbred for 20 generations, and are as isogenic as identical twins. They are extensively used in studies where genetically identical animals are required, for example in transplantation research. Many other studies have used autologous NP when performing similar experiments, but that was not possible in the present study. In any case, since we used inbred Lewis rats, the observed increase in cell activity most likely were correlated to the conditioning, and not to an immunological response to the donor tissue.

Concerning the location of NP application, we aimed to standardize the procedure of the laminectomy and application of NP tissue. Nonetheless, in working with a biological system there will always be slight differences, such as in the exact location of NP in relation to the exposed dorsal nerve roots.

5.3 Gene expression analysis

In this study, RT-qPCR was applied to investigate the gene expression of IL1α, IL1β, TNF and MMP1 in NP tissue. RT-qPCR has proven to be a highly sensitive method for detection and quantification of even minute amounts of mRNA targets.

The choice of target genes was based on previous findings suggesting an important role of the proteins for these genes in the pathogenesis of disc herniation (Brisby et al., 2002; Matsui et al., 1998; Rand et al., 1997; Takahashi et al., 1996). Hence, a classical candidate gene approach was used in the present study. A gene expression analysis of IL1α, IL1β, TNF and MMP1 in 4 groups of NP tissue was performed. Freshly harvested NP from the caudal intervertebral discs was defined native, and served as the principal control tissue. To correct for any possible effects of the surgical procedure, we included NP tissue from sham-operated animals as a second control. In addition, NP tissue isolated in a tube with 0.9 % NaCl served as a third

control. The rationale behind this approach was to correct for any spontaneous upregulation of the target genes that might occur during the time that NP was away from its natural environment.

Thus, the experimental design included native plus two supplementary 180 minute controls. These were then compared to the gene expression of the target genes in NP tissue that had been in contact with the dorsal nerve roots for 180 minutes. The gene expression analysis was performed on RNA isolated from the NP tissues of 3-8 caudal vertebrae.

For successful expression analysis, the quality of the RNA template is of critical importance. RNA is very delicate, and once it has been removed from its cellular environment it is subject both to degradation by the ubiquitous RNases, and contamination of DNA. Therefore, a number of factors must be considered throughout the purification steps to ensure reliable and reproducible results (Bustin and Nolan, 2004). The RNA quality was assessed by on-chip gel electrophoresis. The analysis displayed separate and intact 18S and 28S ribosomal subunits, indicative of satisfactory RNA quality. Since RNA cannot serve as template for PCR, the RNA template was reverse transcribed into cDNA, which was subsequently amplified exponentially in the qPCR.

To avoid contamination from amplification of possible genomic DNA in the sample, the primers were designed to span introns. Optimal primer length is about 18-24 bases, with a range of 40-60 % GC (guanosine/cytosine) content. High GC content in the primer pair, especially at the 3` end, was avoided as this could lead to false priming. The primer pair melting temperature (Tm) should be between 58-60 °C, and should not differ more than 1-2 °C. All primers used in this study were designed within these parameters. In addition, the possibility of hairpin formation, i.e. primers being self-complementary, was minimized during the design.

The primer concentration should be kept at an optimal level. If the primer concentration is too high, this could promote mispriming and accumulation of non-specific products. Too low primer concentration represents a minor problem at real-time analysis, as the target copy number is calculated at a time point (exponential phase) long before the primer supply is exhausted (Bustin, 2000). Although the

specificity of the primers and parameters for the reaction were optimized, unspecific priming might still occur. For this reason, the final PCR product was checked for formation of unspecific products. To visualize possible co-amplification products, a melting curve with fluorescence as a function of temperature was generated at the end of all PCR reactions. Such bi-products proved not to be present in this study.

The expression of the target genes was normalized to the expression of the internal standard; the β-actin. The gene for β-actin encodes a ubiquitous cytoskeleton protein whose expression is expected to be independent of any conditioning. Ideally, a reference gene should be stably expressed, while its abundance should show strong correlation with the total amount of mRNA present in the samples (Bustin et al., 2009). In the present study this held true for β-actin, which proved to be suitable as reference gene as it showed an invariant expression throughout these experimental conditions. For each target gene, the reference gene was run in parallel to correct for potential sample variations, due to for example tissue weight variation or differences in the efficiency of cDNA synthesis.

One should keep in mind that even though changed amounts of mRNA is detected, this provides no information about whether or not this mRNA would be subsequently translated into a functional protein. However, it does reflect the relative increase of the mRNA.

6 Discussion of results

The objective of the present study was to study the underlying mechanisms of sensitization of spinal cord neurons. In these mechanisms, neuronal hyperexcitability related to proinflammatory cytokines may be important.

6.1 In vivo electrophysiological recordings

6.1.1 Expression of spinal LTP

In accordance with previous studies, we demonstrated that the neuronal activity may be increased in the spinal dorsal horn by HFS conditioning of the sciatic nerve. At the end of the experiments, the C-fibre responses were increased by 35 % following HFS conditioning. This increase was less than reported by other studies using the same experimental set-up (Eriksen et al., 2012; Jacobsen et al., 2010). Still, the spinal hyperexcitability was significantly elevated after HFS. The HFS-induced LTP outlasted the experimental time period of 180 minutes. Similar studies have demonstrated that HFS-induced LTP may last for at least 4-6 hours (Pedersen et al., 2010; Sandkühler and Liu, 1998). Thus, our data confirmed that HFS of the sciatic nerve may induce a persistent increase in spinal nociceptive excitability.

The increase in excitability of nociceptive neurons underlying central sensitization is thought to be mediated partly by LTP. Although tetanic stimulation may be a reliable method for inducing a robust and stabile LTP, electrical HFS is not a natural stimulus. Hence the physiological relevance of the mechanisms underlying HFS-induced LTP can be disputed.

6.1.2 The effect of NP on neuronal activity

Interestingly, NP applied onto the spinal dorsal nerve roots clearly increased the neuronal activity in the spinal dorsal horn, too. We here describe this as an LTP-like phenomenon. At the end of the experiments, the C-fibre responses had increased by 56 % in the rats conditioned with NP. A clear increase in C-fibre responses was observed already within 30 minutes after NP application. This demonstrates that NP, as a natural noxious stimulus, may increase the spinal neuronal excitability. The results obtained by this NP conditioning may be more relevant for the clinical situations than the results obtained in experiments with HFS conditioning. The increase in C-fibre response elicited by NP application, suggests that mediators and biochemical factors intrinsic to NP are able to affect spinal neuronal excitability. To our knowledge, this is the first time NP application onto dorsal nerve roots is combined with 180 minutes of C-fibre single cell recordings in the dorsal horn, and subsequent gene expression analysis.

Previous data have shown a significant reduction in nerve conduction velocity following local application of NP on the cauda equina nerve roots (Aoki et al., 2002; Olmarker et al., 1993). Moreover, in an experimental model of disc herniation, it has been reported that NP induce apoptosis in DRG (Murata et al., 2006). In addition, epidural application of NP may induce a rapid increase in endoneural vascular permeability in spinal nerve roots (Byröd et al., 2000), as well as axonal injury, Schwann cell damage and vesicular swelling (Olmarker et al., 1996). These effects are thought to be caused by structures or substances on the surface of the NP cells (Kayama et al., 1998). Together, these studies suggest that NP has neurotoxic and nerve damaging properties, causing pathological changes to neurons.

The enhancement of neuronal excitability to NP stimuli found in this study is supported by several other studies. One study reported an increase in neuronal activity in thalamus following application of NP onto DRG (Brisby and Hammar, 2007). Other studies have demonstrated increased excitability and mechanical hypersensitivity in the DRG (Takebayashi et al., 2001) or enhanced response of WDR neurons to noxious stimuli (Anzai et al., 2002), after NP application onto the nerve roots. This suggests that NP may cause excitatory changes in neuronal activity.

Moreover, application of NP to DRG have been shown to enhance wind-up (Cuellar et al., 2005), and increase the responses of nociceptive dorsal horn neurons to noxious heat and mechanical stimuli (Cuellar et al., 2004). In accordance with our data, this suggests that NP may induce increased spinal nociceptive signalling.

Interestingly, as shown in a disc herniation model with behavioural tests, the longer the NP is in contact with DRG, the greater is the possibility of rats developing persistent mechanical and thermal hyperalgesia (de Souza Grava et al., 2012). In other words, the NP exerts its effects in a time-dependent manner. In fact, for one of the cells we recorded from, the C-fibre response remained elevated for 5 hours, indicating that NP may have long-term effects on the synaptic transmission.

This enhancement of neuronal excitability may be caused by an inflammatory response elicited by the presence of NP on the nerve roots and the spinal cord. A number of proinflammatory cytokines released from NP may be involved in the pathogenesis of herniated intervertebral disc disease. For example, previous studies have demonstrated that herniated human NP cells in culture increase their production of MMPs, NO, IL-6 and PGE_2 in response to stimulation of IL1β (Kang et al., 1997). High mRNA expression of TNF, IL1α, and IL8 has also been found in herniated human lumbar intervertebral disc specimens (Ahn et al., 2002), whereas high levels of IL6, IL8 and PGE_2 protein have been detected in disc specimens from patients with low back pain (Burke et al., 2002). Also, NO has been implicated in the NP-induced effects on rat spinal nerve roots (Brisby et al., 2000). These mediators may cause inflammatory responses in the surrounding tissue.

Another contributor to spinal hyperexcitability and sensitization could be activated glial cells. Glial cells are not involved in the normal nociceptive transmission of acute pain, but they can be activated in response to stimuli such as inflammation or nerve injury. Glial cells can be activated by glutamate and substance P released from primary afferents, in addition to ATP, BK, PG, and local and circulating proinflammatory cytokines. Upon activation, spinal microglia and astrocytes release a variety of neuroexcitatory and pronociceptive substances such as more glutamate, ATP, reactive oxygen species, PG, and nerve growth factors. Thus, consequently, glia may potentiate the nociceptive transmission. In addition, as immunocompetent cells,

activated microglia also release proinflammatory cytokines such as IL1, IL6, TNF and COX2. They also enhance the release of substance P and excitatory amino acids from the primary afferents, further increasing the excitability of nearby neurons. Microglia seems to be the first to be activated, which then recruits the astrocytes (Tanga et al., 2004). Furthermore, intraspinal injection of ATP-activated microglia has been shown to induce mechanical allodynia, indicating that microglial activation is sufficient to induce sensitization (Tsuda et al., 2003). Together, all of these factors contribute to the enhancement of nociceptive transmission. We find it reasonable to believe that glial cells also play a role in the observed spinal hyperexcitability after NP application.

In both inflammation and neuropathy, structural and functional changes in the afferents may occur. Substance P is normally expressed in high-threshold Aδ- and C-fibres, but previous studies indicate that in neuropathic pain, this neuropeptide may also be released by low-threshold Aβ fibres in the spinal cord (Malcangio et al., 2000). As a consequence, the Aβ fibres acquire the phenotype of C-fibres, i.e. a phenotypic switch. Recruitment of Aβ fibres that may start to express substance P and BDNF and thereby start transmitting nociceptive input, is thought to be one of the long-term contributors of sensitization. Moreover, after peripheral nerve injury, structural reorganization may change the connectivity of C-fibres and A-fibres, in which central A-fibre terminals sprout from their deeper laminar location into lamina II (Mannion et al., 1996; Woolf et al., 1992). In addition, mechanoreceptors can make connections with interneurons in the spinal cord, and influence the transmission of nociceptors. Peripheral nerve injury may also reduce the amount of inhibitory control of dorsal horn neurons, i.e. disinhibition, through reduction and downregulation of GABA and its receptors, or through loss of inhibitory interneurons in lamina II. This affects the circuitry in the spinal cord, and consequently contributes to the inflammatory hypersensitivity and increased excitability. Whether or not such structural reorganizations take place in the spinal cord after NP application, is uncertain. Still, as NP has been shown to have both inflammatory and damaging effects on neuronal tissue, it is tempting to speculate that these changes may be established in the spinal cord tissue after disc herniation.

Our results indicate that NP in itself has direct effect on nerve roots, and mediates spinal hyperexcitability. It is possible that these changes are brought about by activated microglia, sprouting, disinhibition, and inflammatory responses elicited by local or circulating cytokines. In fact, IL1α, IL1β and TNF have been reported to cross the blood brain barrier, and are thus able to directly affect CNS function (Banks et al., 1995). Thus it is believed that NP may have both inflammatory and neuropathic effects.

6.2 Gene expression in NP tissue

In this study, we demonstrated that the gene expression of the proinflammatory cytokines IL1α, IL1β and TNF was upregulated in NP tissue that had been exposed to the spinal dorsal nerve roots for 180 minutes. We observed no change in gene expression of the enzyme MMP1. The exact mechanisms by which the upregulation affects the activity in the dorsal horn neurons are unknown, but it appears to be rather complex, with several factors working in concert.

Proinflammatory cytokines may directly or indirectly contribute to the pathogenesis of disc herniation, and cause radicular pain by affecting the nerve roots. The role of TNF in generating pain in experimental disc herniation has also been supported by behavioural studies in rats (Olmarker et al., 2003).

Previous studies have suggested that certain gene variants encoding MMP1 may contribute to the development of degenerative disc disease (Jacobsen et al.). Moreover, in intervertebral discs it has been reported that the cytokine IL1 may change the balance between degrading enzymes and matrix proteins ensuring the matrix homeostasis. This results in increased expression of the degrading enzymes, and a decrease in the expression of the matrix proteins (Le Maitre et al., 2005). Similarly, *in vitro* studies of NP tissue show that 48 hours treatment with TNF may result in a decreased expression of aggrecan and collagen, but an increased expression of MMPs, including the MMP1 (Séguin et al., 2005). These findings as well as similar observations by other studies suggest that TNF may contribute to the degenerative disc changes by acting partly on MMP1. However, in our time-span of

180 minutes, we did not find any upregulation of MMP1 in NP tissue exposed to the nerve roots compared to NP from controls.

Other *in vivo* studies have shown that both TNF and IL1β contribute to the upregulation of NGF, which plays a major role in the production of inflammatory pain hypersensitivity and hyperalgesia (Safieh-Garabedian et al., 1995; Woolf et al., 1997). This is supported by *in vitro* studies demonstrating that both IL1β and TNF from intervertebral disc cells stimulate the production of NGF (Abe et al., 2007). NGF can promote ingrowth of nerve fibres into the intervertebral discs (Freemont et al., 2002), i.e. sprouting, but NGF has also been implicated in sensitization and inflammatory hyperalgesia. Also, NGF has been shown to increase the excitability of nociceptive sensory neurons (Zhang et al., 2002), and sensitize sensory neurons to capsaicin (Chuang et al., 2001; Shu and Mendell, 1999), causing thermal hyperalgesia. Thus, IL1β and TNF may also act indirectly via NGF to contribute the increased excitability observed in the present study.

Previous data show that local application of TNF has a more pronounced effect on reducing nerve conduction velocity than IL1β. Thus, it has been suggested that TNF is an "early player" in the pathophysiology (Aoki et al., 2002). This crucial and early role of TNF is supported by other studies. For instance, cytokines are known to be able to induce both their own production, and that of other cytokines. Specifically, TNF may activate cascades of cytokine release, leading to inflammatory hyperalgesia (Cunha et al., 1992). In fact, TNF has been shown to be an inducer of IL1 (Cunha et al., 1992; Dinarello et al., 1986), while IL1 has been shown to be an inducer of IL1 itself (Dinarello et al., 1987). TNF has also been shown to induce both Il1β and NGF (Woolf et al., 1997).

Several studies have demonstrated that cytokines can affect neuronal activity directly. In hippocampal pyramidal cells, TNF seems to increase excitatory responses by increasing the surface expression of AMPA receptors, while decreasing surface expression of $GABA_A$ receptors (Stellwagen et al., 2005). Moreover, it has been shown that TNF enhances the conductivity of AMPA receptors (De et al., 2003). Interestingly, TNF might also insert itself into cell membranes forming a voltage-

gated cation channel, increasing Na^+ influx in the cells in response to H^+ (Baldwin et al., 1996; Kagan et al., 1992).

Hence, TNF may affect primary afferent nerve fibres. For example, application of TNF onto nociceptive peripheral afferents may induce ectopic firing, suggesting that local release of TNF could be a key mediator in neuropathic pain states after peripheral nerve injury (Sorkin et al., 1997). Excitatory effect of cytokines has also been seen in the nociceptive neurons of the spinal cord. Patch-clamp recordings of lamina II neurons in spinal cord slices showed that the proinflammatory cytokines IL1β and TNF enhanced the excitatory synaptic transmission and potentiated AMPA- and NMDA-induced currents. In fact, IL1β has been demonstrated to increase the function of the NMDA receptor through activation of the kinase Src, thereby enhancing the calcium current into the cells (Viviani et al., 2003). In addition, it has been shown that IL1β and IL6 inhibited the inhibitory synaptic transmission, and suppressed GABA- and glycine-induced currents. Spinal injection of TNF, IL1β and IL6 can also induce heat hyperalgesia (Kawasaki et al., 2008). Altogether, this correlates well with our findings of an upregulation of IL1α, IL1β and TNF, and the NP-induced spinal hyperexcitability.

TNF, IL1β and IL6 may induce phosphorylation of the transcription factor CREB (Kawasaki et al., 2008), which is necessary in the maintenance of long-term synaptic plasticity (Bourtchuladze et al., 1994; Dash et al., 1990; Davis et al., 1996). In fact, IL1β has been shown to be upregulated in spinal dorsal horn tissue 6 hours after HFS conditioning (Pedersen et al., 2010). This indicates that this interleukin is upregulated over time, and suggests that IL1β may be involved in the maintenance of spinal hyperexcitability.

Moreover, IL1β, via release of substance P, act on neurons in the spinal cord and may lead to transcription of COX2, which again leads to PG production, contributing to inflammatory pain hypersensitivity (Inoue et al., 1999; Samad et al., 2001). In cultured DRG cells, NO has been shown to potentiate the IL1β-induced increase in COX2 expression (Morioka et al., 2002).

It is unclear whether the cytokines found in NP originates from the intervertebral disc cells themselves, or from inflammatory cells that infiltrate the NP. As stated

above, glial cells have also been found to be important for the increased excitability in central sensitization. Upon activation, spinal microglia and astrocytes can release substances that act on neurons in the nociceptive pathways, subsequently leading to hyperalgesia and allodynia (Watkins and Maier, 2003). Consequently, glial cells may contribute to increased excitability due to the release of proinflammatory cytokines, including the IL1α, IL1β and TNF, which we have shown to be upregulated in NP tissue when exposed to the spinal dorsal nerve roots.

7 Conclusion

I) Extracellular field potential recordings in the rat spinal dorsal horn following noxious electrical HFS conditioning of the sciatic nerve, showed a persistent increase in the spinal C-fibre responses. This spinal hyperexcitability of nociceptive neurons outlasted the experimental time period of 180 minutes. Thus, our results confirm that this protocol is a reliable method for inducing LTP in the spinal cord and for studying sensitization.

II) Extracellular single cell recordings in the rat spinal dorsal horn following NP application onto spinal dorsal nerve roots, demonstrated a persistent increase in the spinal C-fibre responses, also outlasting the experimental time period of 180 minutes. Hence, the NP conditioning caused a clear spinal nociceptive hyperexcitability. This shows that the described methodological approach with NP conditioning can be used for studying sensitization following intervertebral disc herniation. Moreover, it suggests that NP in contact with the dorsal nerve roots may have pro-nociceptive or inflammatory actions leading to sensitization, i.e. an LTP-like phenomenon, in the nociceptive pathways.

III) The gene expression of the proinflammatory cytokines IL1α, IL1β and TNF was found to be significantly increased in the NP tissue 180 minutes after NP was applied onto the spinal dorsal nerve roots. These results suggest that an increase of these cytokines may contribute to low back pain and sciatica pathogenesis. The increased gene expression of these cytokines may involve activation of various receptors and intracellular pathways underlying the maintenance of the spinal LTP-like phenomenon. However, the exact details of how these cytokines affect the nerve roots remain to be investigated. No significant changes were seen in the gene expression of MMP1, thus MMP1 does not seem to have a direct influence on the observed increased efficacy of spinal nociceptive signalling.

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Appendices

Appendix I Procedure for RNA isolation

1. The spinal cord tissue was transferred to a pre-cooled 2.0 ml PCR clean eppendorf tube and 0.8 ml Isol-RNA Lysis Reagent (5PRIME) was added.

2. 3 sterile metal balls were added to each sample, and the tissue was homogenized by aid of a mixer mill (frequency: 30, time: 4 x 30 seconds).

3. The sample was incubated for 5 min at room temperature.

4. The sample was centrifuged at 12 000 g for 5 min at 4 °C. The supernatant was transferred to a new eppendorf tube.

5. 0.2 ml chloroform was added. The sample was shaken vigorously by hand for 15 sec and incubated for 3 min at room temperature.

6. The sample was centrifuged at 12 000 g for 15 min at 4 °C.

7. The water phase was transferred to a new Eppendorf tube. 0.5 ml isopropanol was added. The content was mixed well and incubated for 10 min at room temperature.

8. The sample was centrifuged at 12 000 g for 15 min at 4 °C.

9. The supernatant was removed and the RNA pellet was washed with 1 ml 75 % EtOH, mixed and vortexed.

10. The sample was centrifuged at 12 000 g for 5 min at 4 °C.

11. The supernatant was removed. The pellet was dried for 15-30 min at room temperature, dissolved in 10 µl RNase free water and kept on ice.

12. The sample was incubated for 10 min at 65 °C, placed on ice, spun, placed back on ice and mixed by a pipette.

13. The sample was then frozen and stored at -80 °C.

14. A 70x dilution was made to establish RNA concentration: 1 µl sample + 70 µl TE buffer were mixed and vortexed. The RNA concentration was estimated from the optical density of the solution at 260nm and 280 nm. Slit = 0.5 nm.

15. The sample was diluted to 0.5 µg/µl by adding ((10 µl x concentration µg/µl) / 0.5 µg/µl)) – 10 µl =) x.x µl RNase free water.

16. The sample was stored at -80 °C.

TE-buffer

RNase free water was added 0.5 M EDTA (pH 8) to a final concentration of 0.1 mM and 1M Tris-HCl (pH 8) to a final concentration of 10 mM.

Appendix II Evaluation of RNA quality

Procedure for evaluation of RNA quality by on-chip electrophoresis using "Agilent RNA 6000 Nano Kit" (Agilent Technologies, Waldbronn, Germany)

The reagents were equilibrated to room temperature for 30 minutes before use.

1. 550 µl of the RNA 6000 Nano gel matrix was transferred to a spin filter and centrifuged at 1500 g for 10 min at room temperature. An aliquot of 65 µl of the filtered gel was transferred to a 0.5 ml microfuge tube.
2. The RNA 6000 Nano dye concentrate was vortexed for 10 sec and spun down. 1 µl of the dye was added to the filtered gel. The solution was vortexed well and centrifuged at 13 000 g for 10 min at room temperature.
3. The RNA samples were diluted to a final concentration of 300 ng/µl and heat denatured at 70 °C for 2 min.
4. 350 µl of RNase Away was loaded to a microchip and run for 1 min on the Bioanalyzer for decontamination of the electrodes. The procedure was repeated with 350 µl RNase-free water for 10 sec.
5. 9 µl of the gel-dye mix was loaded to the well marked ^G on a new RNA 6000 Nano microchip.
6. The microchip was mounted on the chip priming station. The priming station was closed and pressure was applied to the microchip for 30 sec by a plunger.
7. 9 µl of the gel-dye mix were loaded to the wells marked G.
8. 5 µl of the RNA 6000 Nano marker were loaded to all 12 test-wells and to the ladder-well.
9. The standard ladder was heat denatured at 70 °C for 2 min. 1 µl of the ladder was loaded to the well marked with the ladder.
10. 1 µl of the samples were loaded to the test-wells.

11. The microchip was vortexed at 2000 rpm for 1 min, and then run on the Bioanalyzer.

12. After the Bioanalyzer had completed the analysis-program, 350 µl of RNase-free water was loaded to a microchip and run for 10 sec on the Bioanalyzer for decontamination of the electrodes.

Appendix III cDNA synthesis

Procedure for cDNA synthesis using "First Strand cDNA Synthesis Kit for RT-PCR (AMV)" (Roche Diagnostics, Mannheim, Germany)

All reagents and samples were kept on ice unless specified otherwise.

1. 1.5 µg of RNA was mixed with water to a total volume of 4.5 µl in 0.5 ml Eppendorf tubes.
2. Mixture 1 was prepared:

Reagent:	volume/sample
Random Primer p(dN)	1.5 µl
Deoxynucleotide Mix	1.5 µl
Total	3.0 µl

3. 3 µl of mixture 1 was added to each sample. The tubes were vortexed and spun down.
4. The tubes were incubated at 65 °C for 15 min, and then put directly on ice.
5. Mixture 2 was prepared:

Reagent	volume/sample
10 x Reaction Buffer	1.50 µl
25 mM $MgCl_2$	3.00 µl
RNase Inhibitor 50 U/ µl	0.68 µl
AMV Reverse Transcriptase	0.53 µl
Sterile water	1.80 µl
Total	7.51 µl

6. 7.5 µl of mixture 2 was added to each tube. The tubes were vortexed and spun down.
7. The reverse transcription reaction was run on the PCR machine at the following program: 42 °C for 60 min, 99 °C for 5 min and 4 °C for 5 min.
8. Each sample was added 135 µl of TE-buffer to a final concentration of 10 ng/µl, mixed and spun down.
9. The samples were stored at – 80 °C.

Appendix IV: Quantitative PCR, qPCR

Procedure for qPCR analysis of IL1α, IL1β, TNF and MMP1 gene expression

All reagents and samples were kept on ice unless specified otherwise.

1. A master mix was prepared:

Reagent	volume/sample
ddH_2O	5.22 µl
Perfecta SYBR Green FastMix	10.0 µl
Primer forward (25 pmol/ µl)	0.21 µl
Primer reverse (25 pmol/ µl)	0.21 µl
Total	15.64 µl

2. The cDNA samples used for β-actin analysis were diluted: 1 µl cDNA (10 ng/µl) + 9 µl RNase free water.

3. 8 µl from three different cDNA samples (10 ng/µl) were mixed to give a stock cDNA solution. A dilution series used to generate a standard curve for each gene was prepared.

4.

Dilution series nr	cDNA	RNase free water
1	4.35 µl	undiluted
2	6 µl	+ 18µl
3	6 µl from nr 2	+ 18 µl
4	6 µl from nr 3	+ 18 µl
5	6 µl from nr 4	+ 18 µl
6	6 µl from nr 5	+ 18 µl

5. 15.65 µl master mix was loaded to each well on a 96 well plate.
6. 4.35 µl ddH_2O were added to the non-template control (NTC) wells.
7. 4.35 µl sample cDNA or pre-diluted samples for β-actin analysis or dilution series samples were transferred to the PCR-plate in two parallels and mixed well.
8. The PCR plate was sealed with a plastic film and spun down at 2500 rpm. A rubber mat was placed on top of the PCR plate.
9. The qPCR reaction was run at the following schedule: 90 °C for 2 min followed by 40 cycles of 95 °C for 10 sec and finally 60 °C for 30 sec.