

Intervertebral disc herniation, spinal nociceptive signaling and pro-inflammatory mediators

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Master thesis

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Abstract

Lumbar disc herniation may affect the spinal nerve roots through mechanical pressure, but may also induce a local inflammatory response.

Therefore, in an animal model mimicking the clinical situation after intervertebral disc herniation, the spinal nociceptive signaling and the gene expression changes in nucleus pulposus (NP) and dorsal root ganglion (DRG) tissue were studied. In addition, the effect of minocycline on the spinal nociceptive signaling and on the changes in gene expression in NP tissue was investigated.

Electrophysiological recordings showed that NP applied onto the dorsal nerve roots of female Lewis rats induced a significant increase in spinal nociceptive signaling. Minocycline, when applied together with NP, attenuated this increase, without having any persistent effect on nociceptive activity by itself.

Furthermore, qPCR analysis of the NP tissue exposed to the dorsal nerve roots showed an increase in the gene expression of IL-1 β , Csf1 and CD68. The upregulation of IL-1 β and Csf1 suggests that NP has a pro-inflammatory effect, underlying the pro-nociceptive process after disc herniation. In addition, the upregulation of CD68 indicates phagocytic activation of NP cells following contact with the nerve roots.

We also demonstrated an upregulation of FKN and its receptor CX3CR1 in NP tissue. This upregulation indicates a new mechanism for NP in the induction and/or maintenance of pain hypersensitivity.

In the DRG, after NP was exposed to the dorsal nerve roots, the gene expression of TNF α , FKN and CX3CR1 was also upregulated. It is likely that this upregulation affects the excitability of primary afferent nerve fibers. This could be related to the positive feedback loop involving satellite glial cells and neurons.

Minocycline inhibited the increase in gene expression of IL-1 β , Csf1, CD68, FKN and CX3CR1 in NP tissue, demonstrating an inhibitory effect on these cells, possibly through MAPK p38 inhibition.

The present study suggests that disc herniation increases the excitability in nociceptive pathways, possibly through a mechanism involving both NP cells and satellite glial cells.

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Abbreviations

A	adenine
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor
ANOVA	analysis of variance
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Bp	base pair
C	cytosine
CD68	Cluster of Differentiation 68
cDNA	complementary DNA
CNS	central nervous system
CREB	cAMP response element-binding protein
Csf1	colony stimulating factor-1
C_t	threshold cycle
CTSS	cathepsin S
CX3CR1	CX3 chemokine receptor 1 / fractalkine receptor
DLPT	dorsolateral pontine tegmentum
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ERK	extracellular signal-regulated kinase
FKN	fractalkine

G	guanine
IFN- γ	interferon- γ
IL	interleukin
IP3	inositol triphosphate
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
mRNA	messenger RNA
NF- κ B	nuclear factor- κ B
NGF	nerve growth factor
NK1R	neurokinin 1 receptor
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
NP	nucleus pulposus
PAG	periaqueductal grey
PB	parabrachial area
PLC	phospholipase C
PNS	peripheral nervous system
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity number
rmANOVA	repeated measures ANOVA
RNA	ribonucleic acid

X

RVM	rostral ventromedial medulla
SEM	standard error of the mean
SP	substance P
T	thymine
TNF	tumor necrosis factor
VGCC	voltage-gated calcium channel

1 Introduction

1.1 Pain

According to the definition provided by the International Association for the Study of Pain: “*pain* is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. The neuronal signaling evoked by noxious stimuli, in contrast, is called *nociception* and is not the same as pain. Pain is always psychological, and is therefore under contextual, cognitive and emotional regulation. Although pain often follows activity in nociceptive pathways, it can occur in the absence of such signaling. *Allodynia* is the phenomenon of experiencing pain in response to normally non-painful stimuli. *Hyperalgesia* on the other hand is an enhancement of pain experienced after normally painful stimuli.

1.2 Nociception

Under normal – healthy – circumstances, nociceptive signaling is initiated by the activation of high threshold sensory nerve fibers by noxious thermal, chemical or mechanical stimuli. Axon diameter on nociceptors varies from thin unmyelinated C-fibers to medium-sized A δ -fibers with a thin myelin sheath. The speed of conduction is determined by axon diameter and degree of myelination, and is ~2 m/s for C-fibers and up to 30 m/s for A δ fibers.

These afferent nociceptive nerve fibers synapse with spinal nerves in laminae I, II and V of the dorsal horn. The nociceptive signal is carried across the spinal synapse by the presynaptic release of the neurotransmitters glutamate (Kangrga and Randic, 1991) and substance P (SP) (Kantner et al., 1985). They depolarize the postsynaptic membrane by binding to their receptors α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), metabotropic glutamate receptor (mGluR) and neurokinin 1 receptor (NK1R).

Spinal neurons fall under three different categories defined by their axon projections: interneurons, propriospinal neurons and projection neurons. Interneurons are the most abundant class of neurons in the dorsal horn, are neither sensory nor motor neurons and are contained in their entirety within the grey matter of the spinal cord. These neurons form connections between other neurons and are involved in the local modulation of signals.

Propriospinal neurons on the other hand are located in the white columns of the spinal cord and connect different spinal levels. Lastly, the axons of projection neurons are much longer, they cross the midline and they signal to higher brain centers.

Ascending nociceptive and wide dynamic range neurons primarily innervate the parabrachial area (PB), the periaqueductal grey (PAG), the thalamus and the hypothalamus. From there the signal is relayed to brain centers involved in the pain experience: amygdala, insular cortex, anterior cingulate cortex, somatosensory cortex and frontal lobe. These, in turn, signal back to the PAG, through which most of the descending modulating control is routed (figure 1.1).

1.3 Descending modulation

Descending neurons in the PAG, rostral ventromedial medulla (RVM) and the dorsolateral pontine tegmentum (DLPT) can be divided into three categories: on-cells, off-cells and neutral cells. On-cells enhance nociceptive signaling when activated, while off-cells have the opposite effect (Fields et al., 1983). The role of neutral cells remains unclear. Opioid signaling in PAG, RVM or DLPT leads to the inactivation of on-cells, the activation of off-cells, and ultimately a decrease in the nociceptive signaling in the spinal dorsal horn (Jones and Gebhart, 1988; Heinricher et al., 1994).

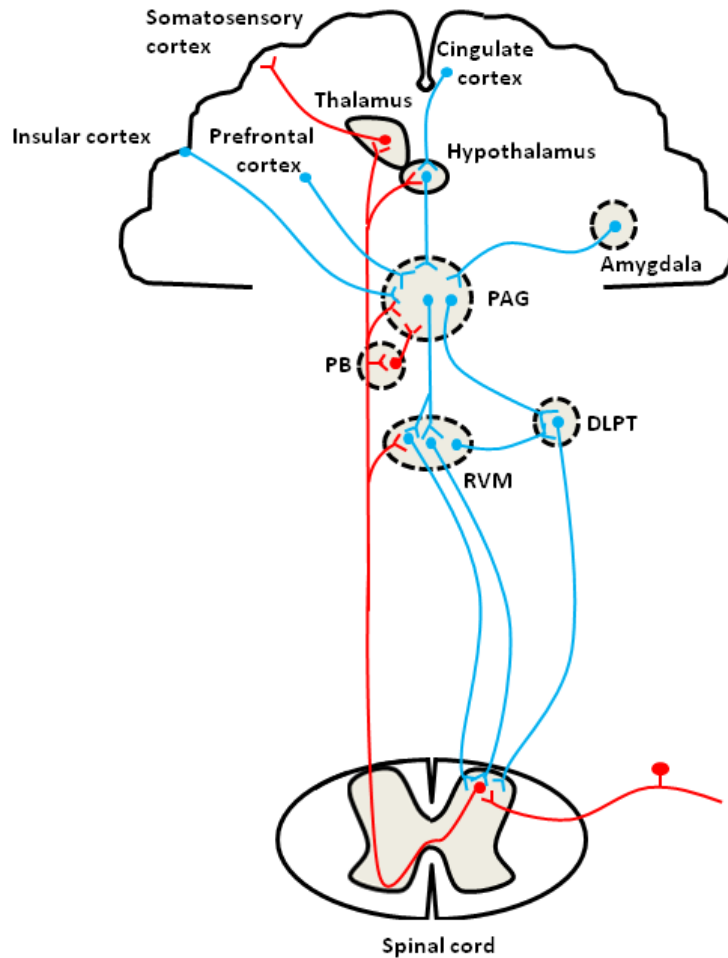


Figure 1.1 A simplified overview of nociceptive signaling and modulation. Afferent and ascending fibers are shown in red, while fibers involved in descending modulation are shown in blue. A peripheral stimulus initiates a nociceptive signal, which is carried through the dorsal horn to the rostral ventromedial medulla (RVM), the parabrachial nucleus (PB), the periaqueductal grey (PGA), thalamus and hypothalamus. From there the signaling is relayed to other brain centers involved in the experience of pain and the initiation of descending modulation. The amygdala, cingulate cortex, insular cortex, prefrontal cortex and hypothalamus communicate to PAG, which relays descending control through either RVM or dorsolateral pontine tegmentum (DLPT) to the dorsal horn.

1.4 Sensitization

Sensitization is the phenomenon of increased nociceptive responsiveness to suprathreshold stimulation, and/or the recruitment of a nociceptive response following normally subthreshold stimulation – hyperalgesia and allodynia, respectively. Sensitization can turn acute pain after injury into a chronic pain state.

1.4.1 Peripheral sensitization

Peripheral sensitization refers to increased responsiveness of nociceptors in the periphery to supra- and/or subthreshold input. This is often caused by tissue damage and subsequent release of cytokines, histamine, adenosine triphosphate (ATP) and nerve growth factor (NGF). Such substances affect nociceptive signaling by directly or indirectly influencing ion channels and receptors. Peripheral sensitization is restricted to the site of injury, since only local nociceptors are affected.

1.4.2 Central sensitization

Central sensitization is characterized by an increased responsiveness within the central nervous system (CNS) to nociceptive input from primary afferents. Central sensitization often affects a large area of the body because of converging pathways of afferent pain signaling. This persistent state of heightened sensitivity may involve changes within the spinal cord, brain stem and subcortical areas.

Following mild noxious stimuli, only glutamate is released from the presynaptic membrane. Glutamate binds and opens AMPARs on the postsynaptic membrane. AMPARs are highly permeable to Na^+ , and the result is a short-lasting (<10 ms) depolarization. Glutamate also binds the non-selective cation-channel N-methyl-D-aspartate receptor (NMDAR), but a magnesium ion, held in place by the negative membrane potential, blocks this channel.

Strong noxious stimuli, on the other hand, lead to the presynaptic release of SP in addition to glutamate. SP binds to NK1Rs on the postsynaptic membrane and activates phospholipase C (PLC), which in turn leads to Ca^{2+} release from intracellular stores through inositol triphosphate (IP3) signaling (Drdla and Sandkuhler, 2008). The result is a long-lasting (>100 ms) depolarization that removes the magnesium-block from NMDARs, thereby permitting influx of cations, notably Ca^{2+} . In addition, voltage-gated calcium channels (VGCCs), affected by the depolarized membrane potential, further permit Ca^{2+} influx.

This may lead to the activation of Ca^{2+} dependent kinases. Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) contributes to the induction of heightened neuronal excitability by affecting AMPARs in two ways; phosphorylation of the mGluR subunit increases channel conductance (Derkach et al., 1999), and AMPAR trafficking, i.e. vesicles containing

AMPA receptors fusing with the postsynaptic membrane, introduces additional AMPARs to the synapse (Hayashi et al., 2000).

Protein kinase C (PKC), another Ca^{2+} -dependent kinase, phosphorylates mGluRs on the same amino acid residue as CaMKII (Barria et al., 1997). In addition, phosphorylation of NMDAR by PKC enhances receptor function by increasing channel opening rate (Lan et al., 2001). Hence, both CaMKII and PKC induce a short-term increase in synaptic efficacy.

Prolonged depolarization may induce transcriptional changes. Increased Ca^{2+} concentration leads to the phosphorylation of extracellular signal-regulated kinase (ERK). Activated ERK is translocated to the nucleus where it phosphorylates the transcription factor cAMP response element-binding protein (CREB) (Impey et al., 1998). CREB causes changes in gene transcription, which may lead to a more persistent increase in synaptic efficacy (Song et al., 2005). Genes containing a cAMP response element in the upstream regulatory sequence include NK1R (Seybold et al., 2003), interleukin-1 β (IL-1 β) (Chandra et al., 1995) and tumor necrosis factor alpha (TNF α) (Tsai et al., 1996).

Hence, central sensitization may involve both phosphorylation of existing protein as well as changes in gene transcription (figure 1.2).

1.4.3 Cytokines

Cytokines are small signaling molecules, i.e. proteins, performing paracrine, autocrine and endocrine signaling. Several subgroups of cytokines are involved in the establishment of an inflammation.

Interleukins are a family of cytokines, play a pivotal role in immune communication, and are known to both have anti- and pro-inflammatory effects. IL-1 α and IL-1 β are strongly involved in the generation and maintenance of neuropathic pain (Samad et al., 2001; Wolf et al., 2006). The binding of these interleukins to IL-1 receptor type 1 or type 2 leads to the activation of the transcription factors nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) p38. The involvement of IL-1 β in central sensitization is confirmed by the observation that intrathecal administration of this cytokine leads to both allodynia and hyperalgesia in rats (Malcangio et al., 1996; Oprea and Kress, 2000; Obreja et al., 2002).

Moreover, the expression of other cytokines involved in neuropathic pain may be controlled by IL-1 β signaling. Notably, TNF α and interferon- γ (IFN- γ) are both upregulated by IL-1 β .

The TNF family of cytokines comprises signaling proteins capable of inducing apoptosis. TNF α has also been implicated in enhancing neuronal excitability and propagating inflammatory states (Sorkin et al., 1997; Zhang et al., 2002). As a result, TNF α is the target of many experimental drug treatments (Sfikakis, 2010).

The family name interferon stems from the ability to prevent protein synthesis within virally infected cells, and thereby “interfering” with viral proliferation. IFN-1 γ is known to activate microglia and macrophages (Tsuda et al., 2009). In addition, by causing phosphorylation of AMPARs in neurons, IFN- γ has a direct effect on nociceptive signaling.

Chemokines, named after their ability to induce chemotaxis in nearby cells, are less studied in regard to neuropathic pain. Fractalkine (FKN) is a chemokine, affects the migration and activation of microglia in the CNS, and has been recognized as a key player in neuropathic pain (Milligan et al., 2004).

Recently, colony stimulating factor-1 has been implicated in central sensitization following disc herniation (Egeland et al., 2013). Colony stimulating factors cause proliferation and differentiation of hemopoietic stem cells. Csf1 in particular is known to stimulate macrophages, but has also displayed a role in microglial activation (Sawada et al., 1990).

1.5 Vertebral column

The vertebral column forms a protective housing around the spinal cord. In humans, it consists of 33 vertebrae – 7 cervical, 12 thoracic, 5 lumbar, 5 sacral and 4 coccygeal – separated by intervertebral discs. These discs form “joints” which bind the individual vertebrae together to form the vertebral column, and allow slight movement between the vertebrae. Nucleus pulposus is a gel-like substance, exerting hydraulic pressure within each disc. Contained within a protective layer of annulus fibrosus, it prevents the vertebral column from being damaged during compression and contortion.

1.5.1 Nucleus pulposus

Nucleus pulposus contains chondrocyte-like cells and gets its gel-like properties from its high contents of water, proteoglycans and collagen. Recent discoveries have shown that the cells found in the nucleus pulposus tissue can be induced into becoming phagocytic, allowing for the removal of apoptotic cells within the vertebral disc (Nerlich et al., 2002; Jones et al., 2008; Chen et al., 2013). Their phagocytic potential could imply more features in common with macrophages and microglial cells. Their ability to release pro-inflammatory cytokines has already been well established (Takahashi et al., 1996; Cuellar et al., 2013). The discovery that colony stimulating factor 1 is upregulated following contact between nucleus pulposus and neural tissue (Egeland et al., 2013) could also imply a role for these cells in macrophage recruitment.

1.5.2 Spinal disc herniation

Spinal disc herniation is a medical condition in which a tear in the annulus fibrosus allows the soft nucleus pulposus tissue to leak out of the disc. This condition is often accompanied by low back pain and sciatica. Initially it was thought that mechanical pressure created by the herniation was the sole cause behind these symptoms. Recently it has been recognized that the establishment of this medical condition also has an inflammatory aspect, and that nucleus pulposus is capable of initiating this inflammation through the release of pro-inflammatory cytokines (McCarron et al., 1987; Kang et al., 1997) (figure 1.2). Local application of nucleus pulposus onto the DRG or dorsal nerve roots of an animal model has been proven to be sufficient to induce a change in nociceptive signaling even without mechanical pressure applied (Olmarker et al., 1993). IL-1 β and TNF α are key players in the establishment of an inflammation, are released by nucleus pulposus and are both implicated in the generation of allodynia and hyperalgesia following disc herniation.

1.6 Glia

Glial cells are non-neuronal cells within the central nervous system. Different types are defined by their specialized function. Macroglia are mainly responsible for the maintenance of homeostasis, myelin formation and support and insulation for neurons. Microglia, on the other hand, are macrophage-like cells, capable of phagocytosis and migration. Being the primary

defense system of the CNS, it is essential that microglia are extremely sensitive to pathological changes.

Following activation, microglia synthesize and release pro-inflammatory mediators, including IL- β and TNF- α (figure 1.2). These mediators further activate microglia as well as some classes of macroglia, like astrocytes.

Both astrocytes and microglia have been implicated in the establishment and maintenance of central sensitization (Coyle, 1998; Zhuang et al., 2005; Ikeda et al., 2012). Moreover, their inhibition has been shown to prevent development and maintenance of allodynia and hyperalgesia in different models of neuropathic pain (Ledeboer et al., 2005). For review, see (Mika et al., 2013).

Microglia can be activated by many mediators of inflammation: INF- γ , ATP, matrix metalloproteinase 9, excitatory amino acids, nitric oxide, prostaglandins, IL-1 β , TNF α and Csf1. Yet it seems that most of the pathways converge on MAPK p38. Its phosphorylation may be considered an indication of activation of microglia. MAPK p38 is also a key component in the activation of macrophages, which are similar to microglia in many ways.

In 2007 Clark et al. published findings implicating a new mediator in microglial activation (Clark et al., 2007). A substrate for cleavage by the proteolytic enzyme cathepsin S was identified as the membrane tethered protein fractalkine. This membrane protein is primarily expressed by neurons in the CNS, and in its full length functions as an adhesion molecule. Cleavage by CTSS releases the extracellular chemokine-domain from the membrane, which then can bind its receptor CX3CR1. This receptor is located on microglia, and binding by fractalkine activates these cells through phosphorylation of MAPK p38 (Zhuang et al., 2007) (figure 1.2). CTSS is selectively expressed by antigen presenting cells (Shi et al., 1994), including macrophages (Shi et al., 1992) and microglia (Liuzzo et al., 1999a; Liuzzo et al., 1999b).

Satellite glial cells (SGCs) are glial cells located in the dorsal root ganglion, responsible for controlling the environment around the somas of neurons. Recently, the presence of CX3CR1 on SGCs and also a role for these cells in the induction of neuropathic pain has been established (Souza et al., 2013) (Figure 1.2).

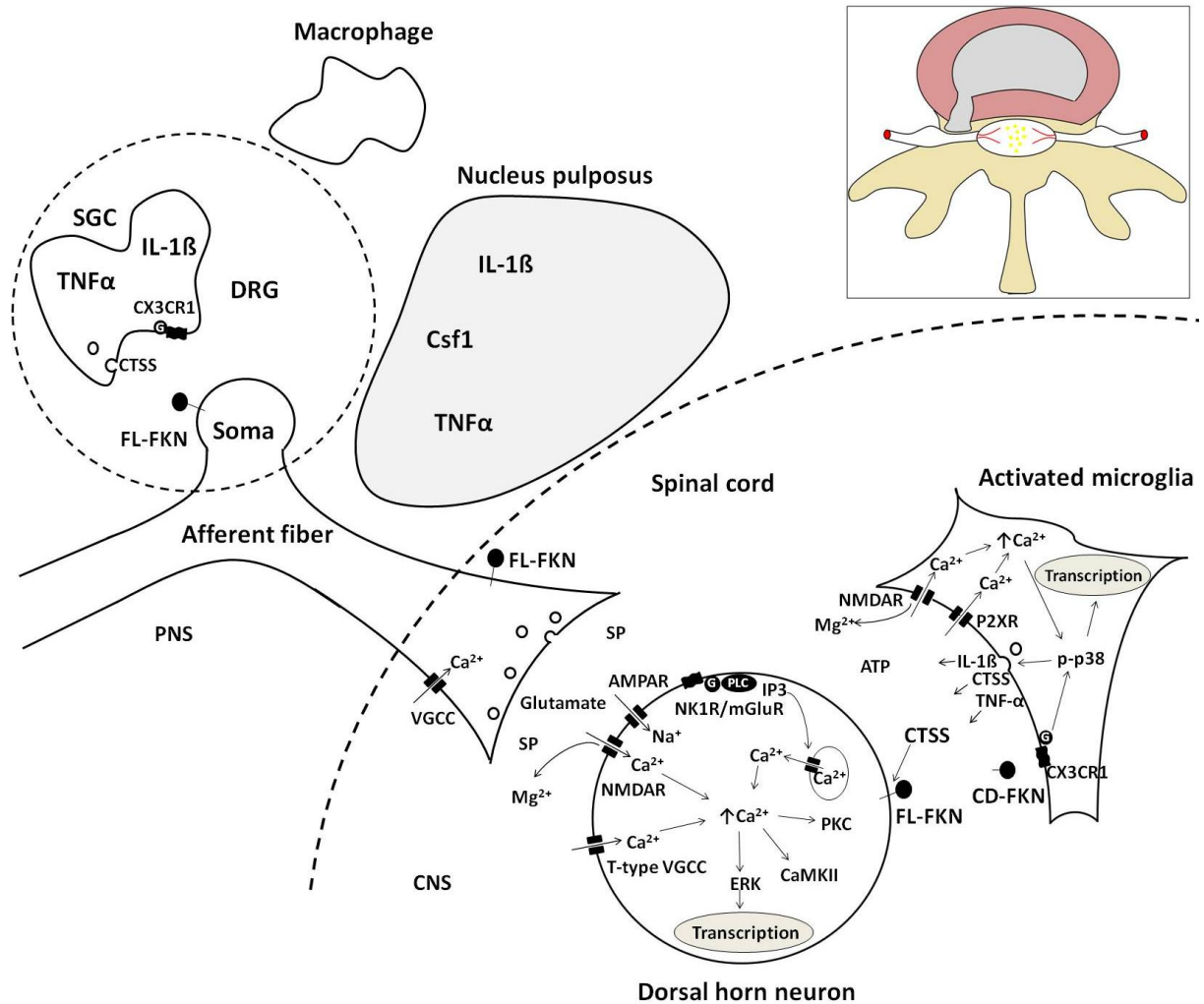


Figure 1.2 After disc herniation, nucleus pulposus comes in contact with the DRG and the dorsal nerve roots of an afferent nerve fiber. The primary afferent nerve fiber synapses with a dorsal horn neuron in the spinal cord. Presynaptic release of glutamate and SP depolarizes the postsynaptic membrane by binding to the receptors AMPAR, NK1R and mGluR. Depolarization opens additional membrane channels, like NMDAR and VGCCs. NP initiates an inflammation by releasing cytokines and mediators. This may affect the excitability of neurons as well as spinal microglia and satellite glial cells (SGCs). The activated microglia and SGCs contribute to the establishment and maintenance of this inflammation through further release of cytokines. In addition, CTSS is released and cleaves off the fractalkine chemokine domain (CD-FKN), which binds the receptor CX3CR1 on microglia and SGCs. This positive feedback loop keeps further activating microglia and SGCs. Insert: illustration of NP leaking out of an intervertebral disc and getting in contact with DRG and dorsal nerve roots.

1.7 Minocycline

The tetracycline family is a large group of broad-spectrum antibiotics. Their effect comes from their firm binding to the 30S subunit of the ribosome in bacteria, thereby inhibiting protein synthesis and bacterial proliferation. Minocycline is a tetracycline and is medically used to treat acne vulgaris (Strauss et al., 2007).

By mechanisms completely independent of its antibacterial properties, minocycline has been proven to also have anti-inflammatory effects. These include: inhibition of the inflammatory enzymes 5-lipoxygenase (Song et al., 2004) and inducible nitric oxide synthase (Amin et al., 1996), depression of oxygen radicals released from neutrophils (Gabler and Creamer, 1991), and – notably – inhibition of the phosphorylation of the MAPK p38 (Hua et al., 2005). The latter makes minocycline an inhibitor of activation for microglia, macrophages and macrophage-like cells. Minocycline administration in neuropathic pain models has implicated microglia as a key component in the generation of central sensitization, by alleviating allodynia and hyperalgesia (Piao et al., 2006).

1.8 Gene expression

Every cell in the body of an individual has the same genetic information. What distinguishes cell types from one another and gives them specific properties according to their location and role, is which genes are expressed, and to what extent. Some genes, like β -actin – an important protein in the cytoskeleton – are constitutively expressed in all cell types. Other genes are more tissue-specific and give these cells specialized properties.

Gene expression can also vary within the same cell type, depending on the conditions. Immune cells during an inflammation are good examples, as their activation triggers the increased synthesis of many pro-inflammatory mediators. Hence, the state of such an immune cell can be determined by studying its gene expression. IL-1 β and TNF α have previously been shown to be upregulated in NP tissue following disc herniation, and therefore serve the role as positive control in this work.

CD68 is sometimes falsely used as a macrophage-specific marker, even though it has been established that this is not the case. Instead, CD68 is a marker for phagocytosis and can be found in many cell types, like fibroblasts and activated epithelial cells (Kunisch et al., 2004).

2 Aims

The purpose of this study was to provide new knowledge about the relationship between gene expression changes and the development of long lasting pain following disc herniation. In an animal model mimicking the clinical situation after intervertebral disc herniation, the spinal nociceptive signaling was examined. The gene expression changes of several candidate genes in NP and DRG tissue were quantified. In addition, the effect of minocycline on the spinal nociceptive signaling and the changes in gene expression in NP tissue was examined. Four aims were defined:

1. Examine how NP from the herniated discs applied onto the dorsal nerve roots may induce increased nociceptive signaling in the spinal cord.
2. Study the effect of the microglia/macrophage inhibitor minocycline on the spinal nociceptive signaling when applied onto the dorsal nerve roots together with NP.
3. Examine changes in expression of the genes encoding IL-1 β , TNF- α , Csf1, CD68 CTSS, FKN and CX3CR1 in NP and DRG following application of NP onto the dorsal nerve roots.
4. Examine how the changes in gene expression in NP tissue may be affected by administration of minocycline together with NP onto the dorsal nerve roots.

3 Materials and methods

3.1 Animal handling

Female inbred Lewis rats weighing 180-220 g (Harlan Laboratories inc., UK) were housed in cages of four and four, with constant access to food and water. The temperature was 20-22 °C, the relative humidity was 45-55% and the air ventilation rate was 15 x the room volume per hour. All experiments were performed during the light period of an artificial 14h light/10h dark cycle. Immediately after each experiment the animals were euthanized. All animal experiments were approved by the Norwegian Animal Research Authority and were performed in conformity with the laws and regulations controlling experiments and procedures on live animals in Norway.

3.1.1 Anesthesia

The animals were sedated by a gas administration of isoflurane (Baxter International Inc., USA) in a gas chamber, and then anesthetized by intraperitoneal administration of 250 mg/ml urethane (Sigma-Aldrich Co., USA). The urethane administration consisted of an initial 0.5 ml dose, followed by 2-3 0.3 ml doses, to avoid a lethal overdose. Foot withdrawal, ear wriggling and eye reflexes were regularly checked between every 0.3 ml dose of urethane. Absence of these reflexes was considered an indication of sufficient anesthesia for surgery. Simplex (80% Vaseline and 20% paraffin) was applied to both eyes to prevent them from drying during surgery and electrophysiological recording. The body temperature of the animals was kept at 36 °C by a feedback heating pad connected to a homeothermic control unit (Harvard Apparatus Ltd. Kent, UK).

3.1.2 Animal surgery

To expose the sciatic nerve, a small incision was made above the pelvic girdle. A small piece of plastic film was then wrapped under the nerve to keep it separated from the surrounding tissue. A bipolar silver hook electrode was put in contact with the nerve fiber for electrical stimulation. The incision was held open by retractors.

A 5-10 mm wide laminectomy was performed, exposing the spinal cord and the dorsal roots on both sides between vertebrae TH13 and L1. Clamps, dorsal and caudal to the laminectomy-site, kept the vertebral column steady in a leveled state. Under a microscope, the dura mater and arachnoid mater were punctured by a cannula and removed from the spinal cord with tweezers.

Before DRG isolation, the animals were euthanized. The superior articular processes and transverse processes were removed on both sides of the spinal cord, exposing the ganglia. L3-L5, with dorsal roots synapsing spinal neurons between spinal cord segment Th13 and L1, were identified using the endpoint of the ribcage as reference. First, the nerve was cut distal to the ganglion. Then the dorsal nerve root was cut and the ganglion was isolated from the surrounding tissue before it was frozen on nitrogen.

3.2 Electrophysiology

A parylene coated tungsten microelectrode with impedance 2-4M Ω (Frederick Haer & Co, Bowdoinham, USA) was lowered into the spinal dorsal horn by a micromanipulator (Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar, Germany). A second electrode was placed subcutaneously and served as reference.

The signal was amplified by a headstage and an AC preamplifier, and subsequently filtered by a band pass filter (Digitimer Ltd, Hertfordshire, UK) with a half-amplitude cut-off of 500 – 1250 Hz, before it was digitalized by a CED 1401 μ interface. The software CED Spike 2, version 3.15 (Cambridge Electronic design, Cambridge, UK), was used for the sampling of data. Spinal cord segments L3-S1 were identified by light tapping on the left paw.

During the experiment an electrical test stimulus was applied to the sciatic nerve every 4th minute. The intensity of the test stimulus was regulated by a pulse buffer connected to a stimulus isolator unit (NeuroLog System, Digitimer Ltd, Hertfordshire, UK). The evoked signals were recorded 100-500 mm from the spinal cord surface (figure 3.1A).

Action potentials 0-50 ms after each test stimulus were defined as the A-fiber response, whereas action potentials 50-300 ms after each test stimulus were defined as the C-fiber response (figure 3.1B). The C-fiber threshold was defined as the minimum intensity required for eliciting a single spike in the 50-300 ms time interval. The intensity of the test stimulus

was kept at 1.5x pre-baseline C-fiber threshold. Only cells with a C-fiber baseline response of 5-20 spikes, and with no values diverging from the baseline mean by more than 20% were included in this study. Amplitude and shape of the spike were assessed to discriminate between signals from different cells, ensuring single-cell recording (figure 3.1C). The spinal cord was prevented from drying by routine topical applications of 0.25 μ l 0,9 % NaCl.

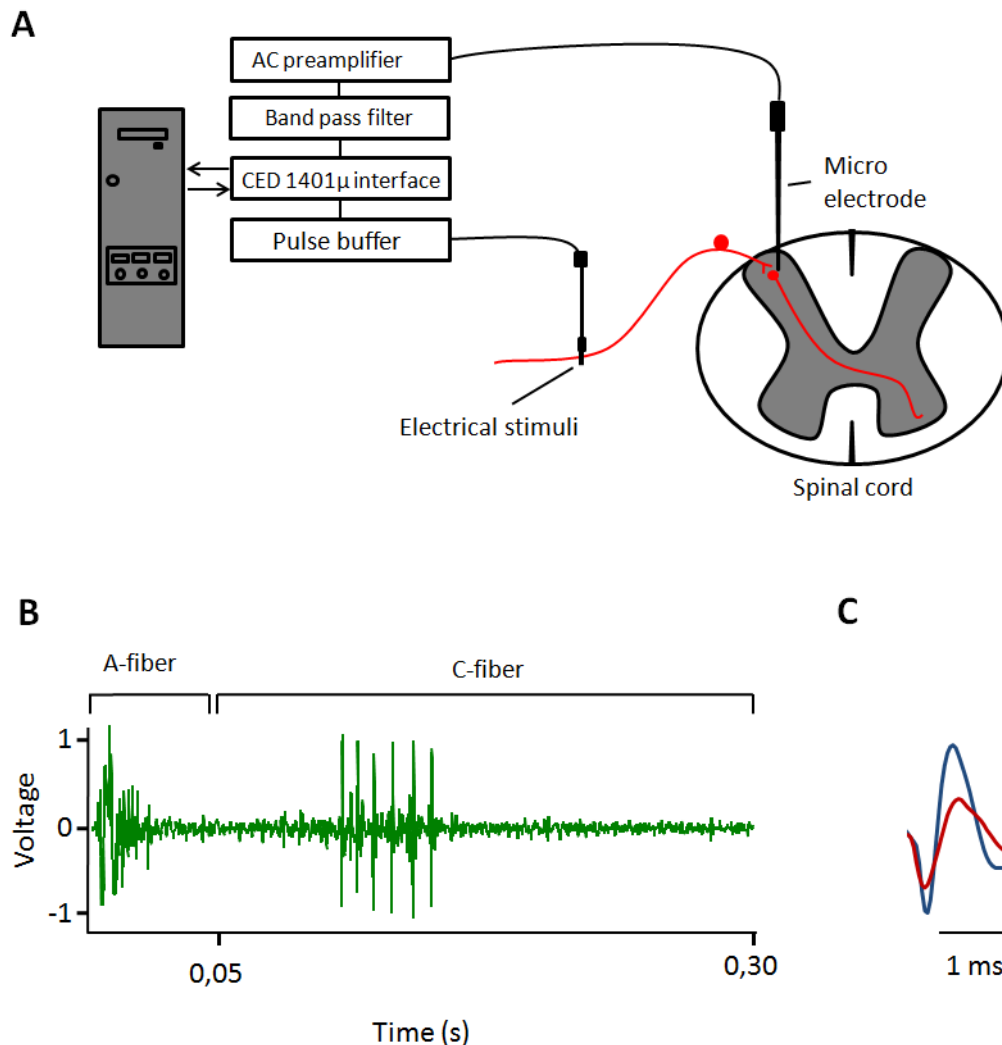


Figure 3.1 A) A schematic view of the experimental set-up during electrophysiological measurements. Electrical test stimuli were applied to the sciatic nerve and the evoked neuronal activity in the spinal cord was recorded by a microelectrode. The signals were amplified by an AC amplifier and filtered by a band pass filter before digitalization by the CED 1401 μ interface. B) An example of an extracellular recording after electrical test stimulation, showing the temporal difference between what we categorize as A-fiber and C-fiber response. C) Two action potentials, one in blue and one in red, demonstrating how amplitude and shape may be used to distinguish the neuronal responses from two different cells.

3.2.1 NP administration

NP was collected from 3-5 vertebrae of the tail from genetically identical donor rats. The collected NP tissue was applied onto the dorsal nerve roots L3 to L5.

3.2.2 Minocycline administration

Minocycline (M9511, Sigma-Aldrich) was dissolved in 0.9 % NaCl and diluted to a concentration of 5 µg/µl. At this concentration, minocycline was stored at -20°C until use. Two separate doses of 25 µl, one before, and one after NP were used in each experiment. The same procedure was performed during the control experiments, only without NP.

3.2.3 Experimental protocol

Four series of experiments – I, II, III, IV – were performed. I) NP was applied onto the dorsal nerve roots, II) NP was applied onto the dorsal nerve roots together with minocycline, III) minocycline was applied onto the dorsal nerve roots without NP and IV) sham operated rats served as vehicle control (figure 3.2).

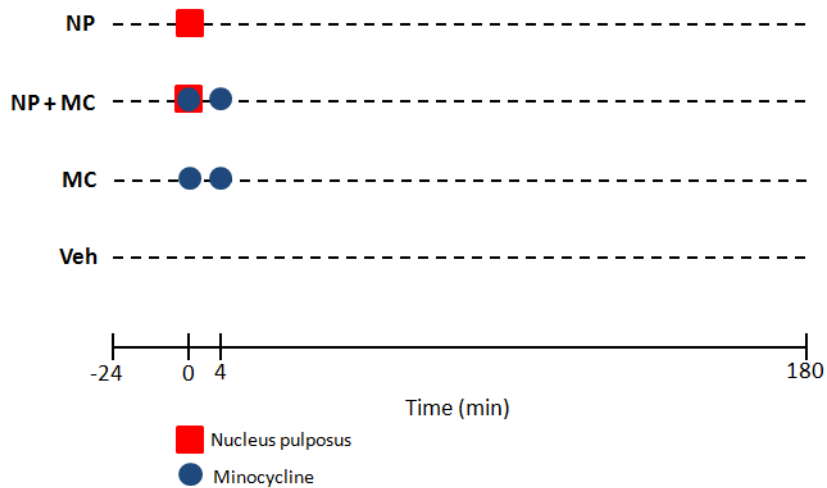


Figure 3.2 Overview of the four different experimental procedures for electrophysiological experiments. A red square indicates application of nucleus pulposus, a blue circle indicates spinal minocycline administration and a blue circle within a red square indicates both nucleus pulposus and minocycline conditioning.

3.3 Gene expression analysis

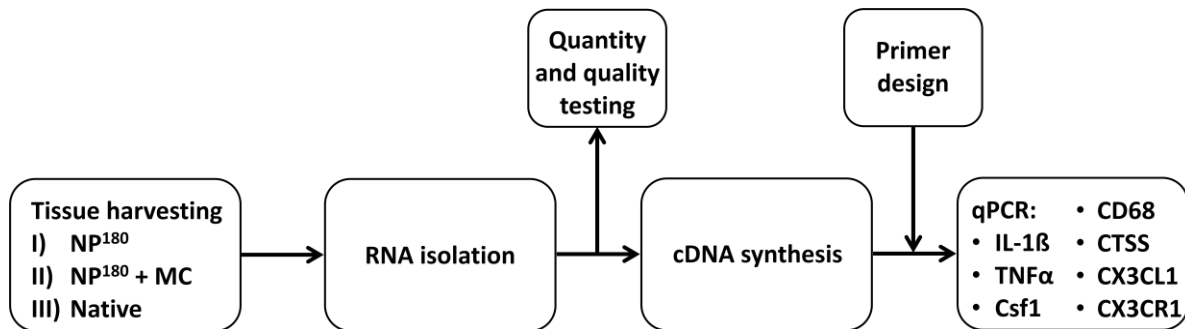


Figure 3.3 Overview of the procedural steps between NP tissue harvesting and gene expression analysis.

3.3.1 Harvesting NP tissue

NP was harvested after the experiments and immediately frozen on nitrogen. It was kept in a freezer at -80°C until use. Three series of NP experiments were performed: I) NP¹⁸⁰, II) NP¹⁸⁰+MC and III) NP^{native}. Both the NP¹⁸⁰ and the NP¹⁸⁰+MC groups were exposed to the dorsal nerve roots for 180 minutes, but the latter was also exposed to minocycline. The NP^{native} group was placed directly on nitrogen without being exposed to the dorsal nerve roots (figure 3.3, 3.4).

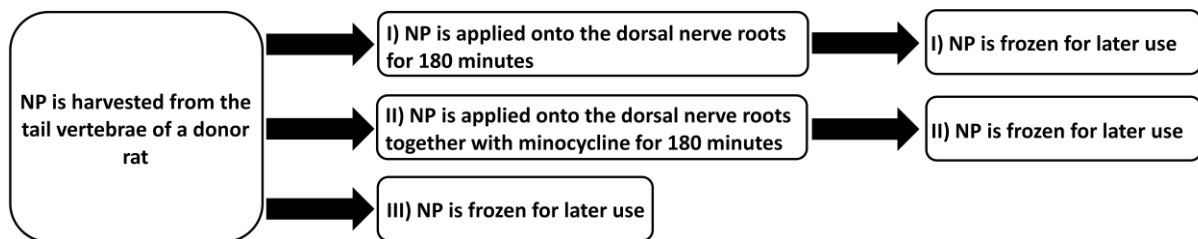


Figure 3.4 An overview of the three different experimental procedures before gene expression analysis.

3.3.2 DRG gene expression

Two series of DRG experiments were performed: native and NP. In both groups, a laminectomy was performed three hours before DRG isolation. In the NP group, NP tissue was applied onto the left dorsal nerve roots immediately after the laminectomy was performed. In both cases, both the left and the right dorsal root ganglia were isolated. L3, L4 and L5 were mixed together before gene expression analysis.

3.3.3 RNA isolation and cDNA synthesis

A mixer mill was used to homogenize NP and DRG tissue in isol-RNA lysis agent (5 PRIME), and cell debris was discarded. Chloroform was added, and the water phase isolated, leaving DNA, lipids and proteins behind. RNA was precipitated by isopropanol. The pellet was washed by 75% ethanol, dried, and then re-dissolved in ribonuclease (RNase) free water. RNA-concentrations were quantified by measuring optical densities with a spectrophotometer (Nanodrop 8000, Thermo Fisher Scientific inc.) and then diluted by RNase free water to a unifying concentration of 0.25 µg/µl. See appendix 1.

Two and two samples of total RNA were mixed and an RNA quantification and integrity analysis was performed using Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). An electropherogram and a RIN (RNA integrity number) value based on the on-chip gel electrophoresis were obtained. Two clear peaks on the electropherogram, corresponding to the 18S and the 28S ribosomal subunits, were considered the ideal result. A RIN value of >7 was defined as acceptable for the gene expression analysis. See appendix 2.

Reverse transcription of the RNA, resulting in cDNA was carried out using a first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). The reaction was performed in a Mastercycler nexus (Eppendorf, Hamburg, Germany) with the schedule: 42 °C for 60 minutes, 99 °C for 5 minutes and 4 °C for 5 minutes. After synthesis, the resulting cDNA was diluted to a concentration of 10 ng/µl in tris-ethylenediaminetetraacetic acid. See appendix 3.

3.3.4 qPCR

The expression of the candidate genes was determined using quantitative polymerase chain reaction (qPCR). Primers were designed using the software Primer Express 2.0, which allowed exclusion of candidate primers with a high number of internal complementation. To ensure specificity for cDNA, primers were designed to yield a product, which spanned an intron in the genomic DNA. A BLAST search was performed to check for identical sequences in other genes. β-actin was used as a reference gene, because of its constant high expression. Table 1 shows the primers used in this study.

Primer	Sequence 5'→3'	Bp	%GC	Tm °C
IL1β forward	CGT GGA GCT TCC AGG ATG AG	20	60.0	59.4
IL1β reverse	CGT CAT CAT CCC ACG AGT CA	20	50.0	59.1
TNFα forward	GCC ACC ACG CTC TTC TGT CTA	21	57.1	59.1
TNFα reverse	TGA GAG GGA GCC CAT TTG G	19	57.9	59.6
Csf1 forward	GGG AAT GGA CAC CTA CAG ATT TTG	24	45.8	59.6
Csf1 reverse	AAA TTT ATA TTC GAT CAG GCA TGCA	25	32.0	59.7
CD68 forward	CTCACAAAAAGGCTGCCACTCT	22	52.0	60.0
CD68 reverse	TTCCGGTGGTTGTAGGTGTCT	21	58.0	58.0
CTSS forward	CCACTGGGATCTCTGGAAGAAA	22	50.0	59.2
CTSS reverse	CGTAVGTCTTCTTCATTCTGATCTG	25	40.0	58.2
FKN forward	TTGCACAGCCCAGATCATTC	21	47.6	54.3
FKN reverse	CTGCGCTCTCAGATGTAGGAAA	22	50.0	55.7
CX3CR1	GTGGCCTTTGGGACCATCT	19	57.9	56.7
CX3CR1	CCACCAGACCGAACGTGAA	19	57.9	56.6
β-actin forward	CTA AGG CCA ACC GTG AAA AGA	21	47.6	58.0
β-actin reverse	ACA ACA CAG CCT GGA TGG CTA	21	52.4	59.2

Table 1 Primers used for qPCR analysis.

The qPCR was performed by a StepOnePlus qPCR machine (Applied Biosystems, California, USA) with the following program: 90°C for 2 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds. The results were viewed in StepOne Software v2.3. Perfecta SYBR green fast mix (Quanta Bioscience, Gaithersburg, MD, USA) was used for the qPCR. SYBR green dye, ROX, dNTP and Taq polymerase were included in the mix. SYBR green dye, which emits fluorescence at 520nm when it is incorporated in a double stranded DNA molecule, was used to quantify the amount of PCR product. The melting curve was analyzed for each well (figure 3.5B).

The ROX dye, which does not require ds-DNA for fluorescence, was used to normalize the SYBR green dye, thereby correcting for any discrepancies in volume between the different wells. The normalized fluorescence in each well was plotted against the number of cycles performed to create an amplification plot.

To set up a standard curve, a fourfold dilution series was created for both the candidate gene and β -actin, using a mix of 3 samples. Using this standard curve, the software defined a threshold value of fluorescence. The intercept between this threshold value and the amplification plot of a given sample was defined as the C_t value of that sample. The quantity was extrapolated by the C_t value for each sample and the standard curve set up by the dilution series (figure 3.5A). See appendix 4.

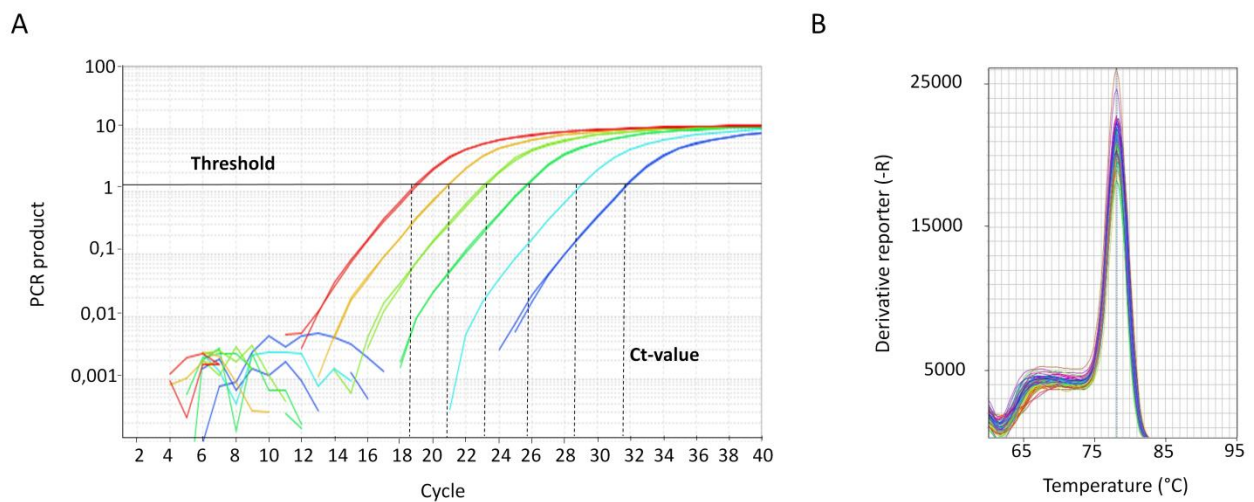


Figure 3.5 A) An example of a qPCR amplification plot. The software defines a threshold-value of PCR product (fluorescence). The cycle at which each well reached this threshold determined the C_t -value of that sample. A fourfold dilution series was set up, giving amplification plots sequentially two cycles apart. B) Example of melting curves used to verify that the measured fluorescence in the samples was a result of a quantitative increase of the desired product only, and not that of a byproduct.

3.4 Statistics

All statistical analyses were performed in SPSS 21 (IBM SPSS inc. Chicago, USA), and all graphs created in Sigma plot 10.0 (Scientific Computing). Data are given by examples and by means \pm standard error of the mean (SEM). A p-value below 0.05 was considered significant.

3.4.1 Electrophysiology

The mean of the first six measurements comprising baseline was defined as 100%, and all other measurements throughout the experiments were given values as percent of baseline. Pre-conditioning recordings were converted to two baseline values (each comprising 3

recordings), and the post-conditioning recordings were converted to 9 measurements (each comprising 5 recordings).

The four series of experiments – NP, NP+MC, MC, veh – were compared using a repeated measures analysis of variance (rmANOVA). When sphericity was violated, a Greenhouse-Geisser correction was applied. In addition, the mean values between 60 and 180 minutes of the different groups were compared using a one-way analysis of variance with a Tukey's post hoc test.

3.4.2 Gene expression

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 using the native group values. For NP gene expression, group means were then compared by a one-way ANOVA analysis with a Tukey's post hoc test. DRG expressions were compared using Student's t-test.

4 Results

4.1 Electrophysiology

Application of NP onto the dorsal nerve roots induced an increase in the number of spikes observed by single cell recordings. In six out of seven cells, we observed an increase in C-fiber response already after 12 minutes. However, following application of NP together with minocycline, only a short-lasting decrease in C-fiber response, 4-20 minutes after application, and no increase in the later part of the experiments, was observed. Representative examples of recordings made after conditioning with NP and NP together with minocycline are shown (figure 4.1).

The C-fiber response 60-180 minutes after NP conditioning was $136.1 \% \pm 13.9$ of baseline, whereas the C-fiber response of the vehicle control in the same time interval was $91.7 \% \pm 7.4$ of baseline (figure 4.2A, D, E). However, NP failed to induce a similar increase when applied onto the dorsal nerve roots together with minocycline. The C-fiber response measured after conditioning with NP alone was significantly higher than that measured after conditioning with NP together with minocycline. Furthermore, 60-180 minutes after NP and minocycline administration, the C-fiber response was $99.9 \% \pm 6.5$ of baseline, similar to the C-fiber response measured for the minocycline control, which was $92.0\% \pm 8.6$ of baseline (figure 4.2B, C, E).

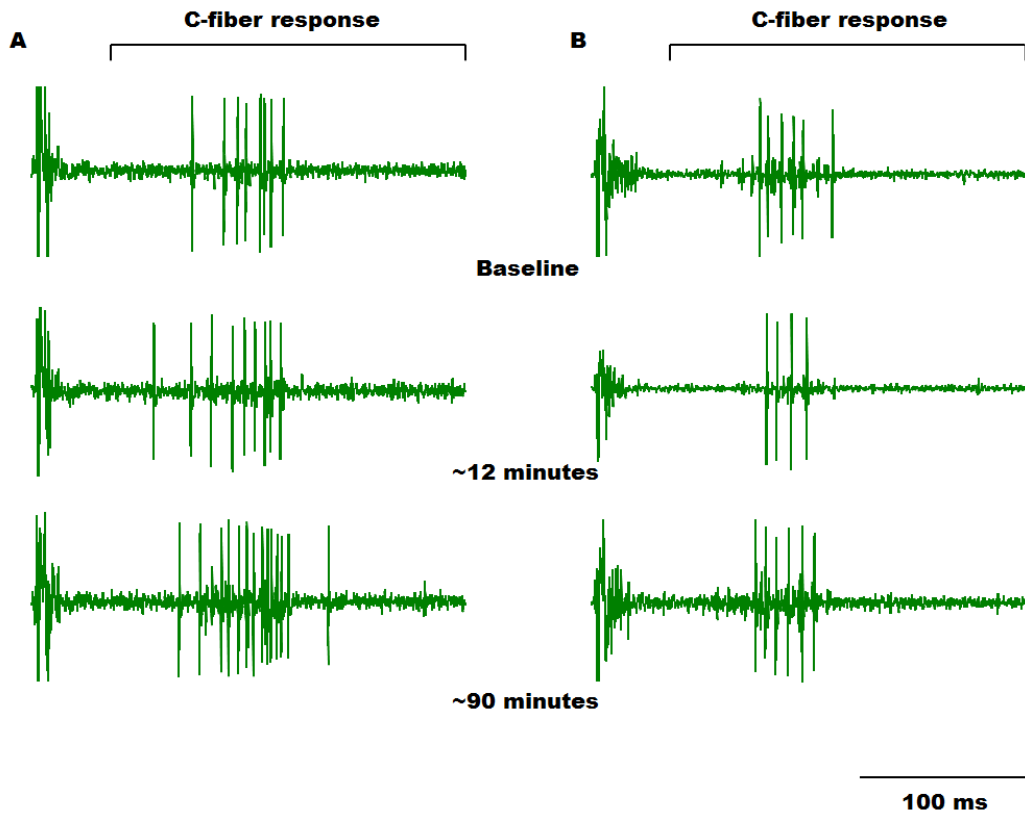


Figure 4.1 Examples of single cell recordings at baseline, ~12 minutes after, and ~90 minutes after NP conditioning. A) NP alone: the response increased from 8 spikes at baseline to 9 spikes after 12 minutes and then to 14 spikes after 90 minutes. B) NP together with minocycline: a temporary drop in response from 6 spikes to 4 spikes is seen after 12 minutes. After 90 minutes the response is back at baseline level with 6 spikes.

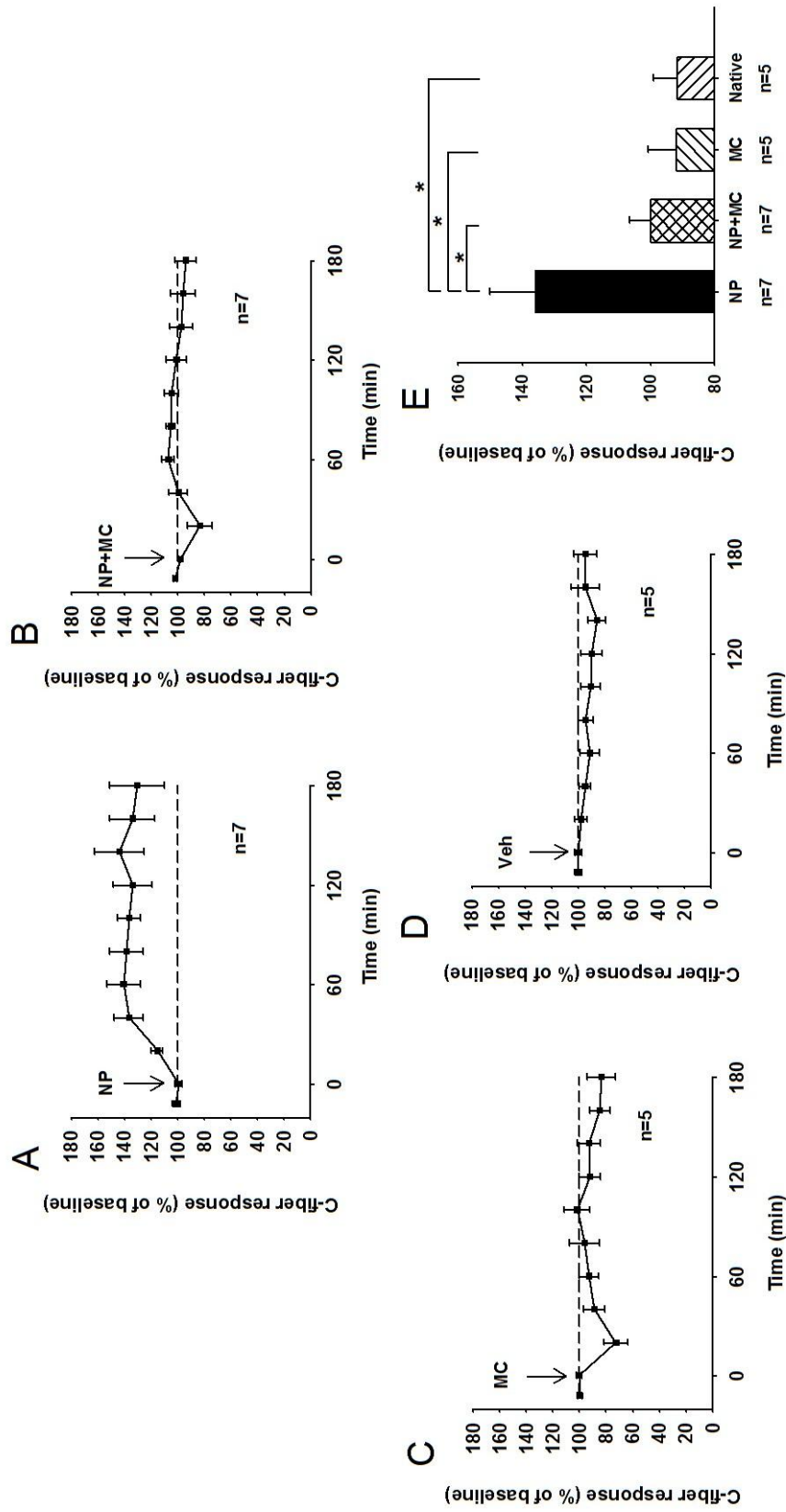


Figure 4.2 C-fiber response in percent of baseline after application of A) NP (NP), B) NP and minocycline (NP+MC), C) minocycline (MC), and D) Vehicle (Veh). E) The mean value 60 to 180 minutes after baseline in the four groups. $P=0.018$, rmANOVA, four groups; NP, NP+MC, MC, Veh. $*P<0.05$, one-way ANOVA, Tukey's post hoc test.

4.2 Gene expression

4.2.1 Nucleus pulposus

Application of NP onto the dorsal nerve roots increased the gene expression of several genes in the NP tissue. After 3 hours, a significant increase in the expression of IL-1 β , Csf1, CD68, FKN and the FKN receptor CX3CR1 was observed (fold expression: IL-1 β ; 13.47 ± 3.03 , Csf1; 3.13 ± 1.21 , CD68; 3.26 ± 0.26 , FKN; 7.32 ± 1.89 , CX3CR1; 7.32 ± 1.89). However, there were no significant changes in the expression of TNF α and CTSS (TNF α ; 2.89 ± 2.08 , CTSS; 2.19 ± 0.55). Moreover, no clear upregulation of the genes was observed after application of NP in the presence of minocycline, an inhibitor of microglia and macrophage activation (IL1 β ; 5.19 ± 2.08 , TNF α ; 1.55 ± 0.43 , Csf1; 0.92 ± 0.15 , CD68; 1.25 ± 0.25 , CTSS; 1.13 ± 0.52 , FKN; 2.01 ± 0.51 , CX3CR1; 1.95 ± 0.45) (figure 4.3).

4.2.2 Dorsal root ganglion

After 3 hours, NP applied onto the left dorsal nerve roots also induced an increase in the gene expressions of TNF α , FKN and the FKN receptor CX3CR1 in the ipsilateral DRG cells (fold expression ipsilateral DRG: TNF α ; 1.91 ± 0.56 , FKN; 1.88 ± 0.13 , CX3CR1; 1.86 ± 0.26 ; fold expression contralateral DRG: TNF α ; 1.47 ± 0.56 , FKN; 1.23 ± 0.27 , CX3CR1; 1.33 ± 0.47). Although NP was applied 5-15 mm proximal to the DRG, this seemed to have a clear effect on the DRG cells. However, the fold expressions of IL1- β , Csf1, CD68 and CTSS were not significantly affected (ipsilateral DRG: IL-1 β ; 1.08 ± 0.16 , Csf1; 1.03 ± 0.07 , CD68; 1.29 ± 0.18 , CTSS; 0.90 ± 0.14 ; contralateral DRG: IL-1 β ; 1.10 ± 0.28 , Csf1; 1.08 ± 0.09 , CD68; 1.27 ± 0.22 , CTSS; 1.26 ± 0.18) (figure 4.4).

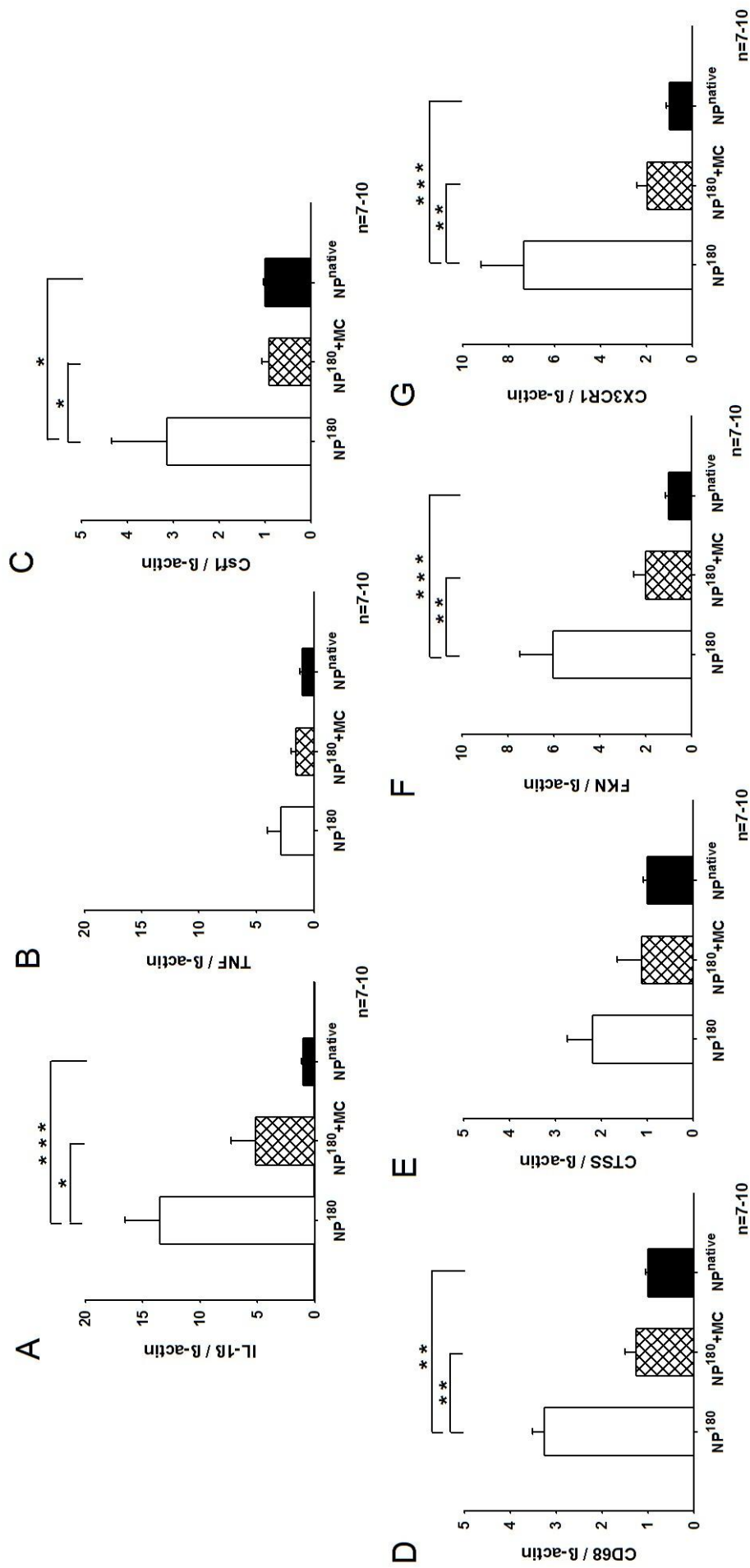


Figure 4.3 Fold expression of A) IL-1 β , B) TNF α , C) Csfl, D) CD68, E) CTSS, F) FKN and G) CX3CR1 in NP in the three groups: nucleus pulposus in contact with dorsal nerve roots for 180 minutes (NP¹⁸⁰), nucleus pulposus in contact with dorsal nerve roots for 180 minutes together with minocycline (NP¹⁸⁰+MC) and native nucleus pulposus (NP^{native}). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, Tukey's post hoc test

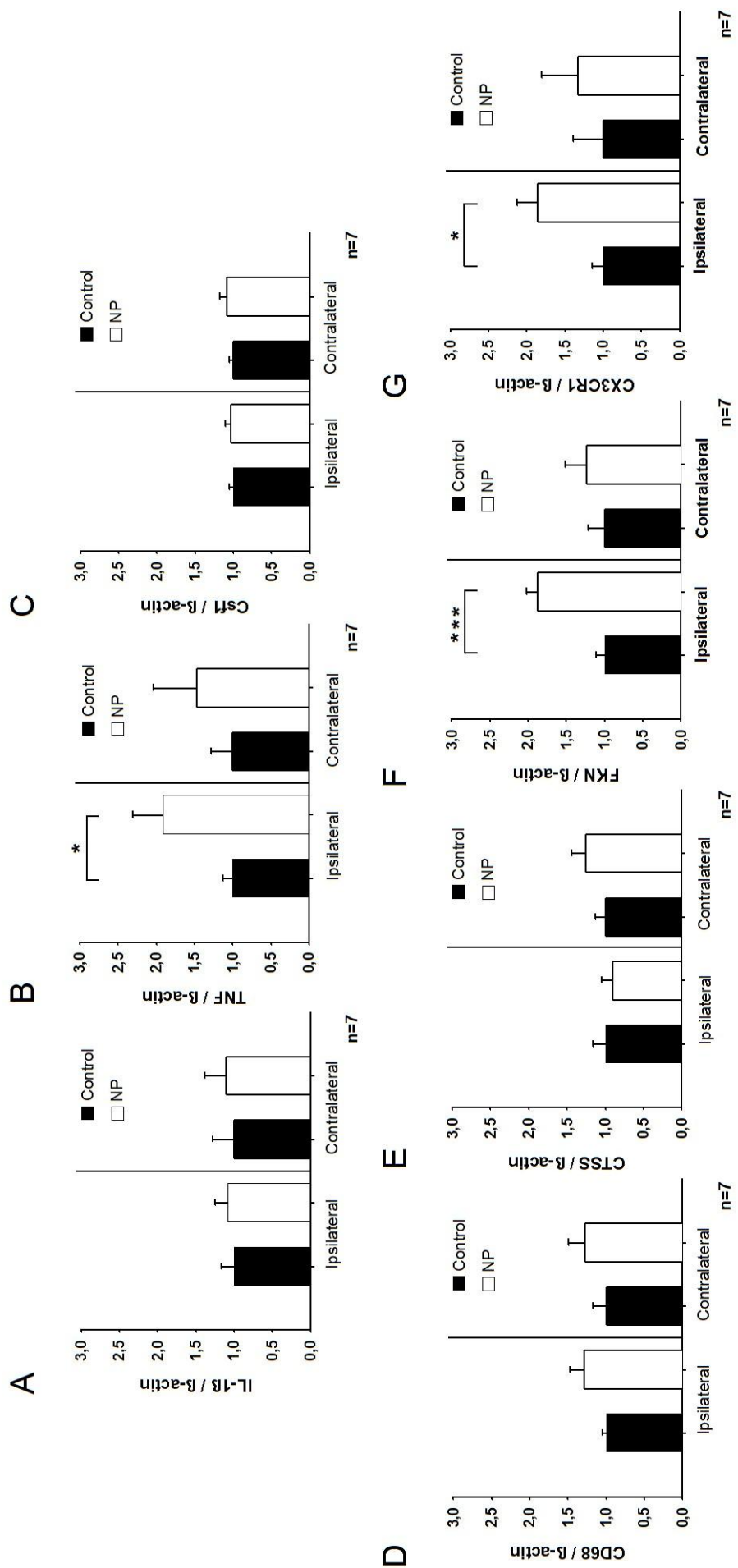


Figure 4.4 Fold expression of A) IL-1 β , B) TNF α , C) Csf1, D) CD68, E) CTSS, F) FKN and G) CX3CR1 in the ipsi- and contralateral DRG after NP application onto the dorsal nerve roots. *P<0.05, **P<0.01, ***P<0.001, students T-test.

5 Discussion of methods

5.1 Animal experiments

5.1.1 Rats

Lewis rats have a more pronounced inflammatory response than other strains (Popovich et al., 1997) and are frequently used to study inflammations. Hence, Lewis rats were used in the present study. Because allergens associated with rats are more prevalent in males, females were preferred. In vivo studies give unique insights into complex biological systems.

5.1.2 Anesthesia

Isoflurane is a halogenated ether widely used in animal research. Isoflurane was only used to ease the initial administration of urethane. There is no official exposure limit for isoflurane.

Urethane is water soluble and can be administered through several different routes. Intraperitoneal injection is an easy alternative, and it was therefore used in the present study. The dose was based on previous studies (Pedersen et al., 2010; Egeland et al., 2013). Urethane induces stable anesthesia, and compared to other anesthetics, has minimal effect on respiration or neuronal signaling in subcortical areas, the spinal cord or the periphery (Maggi and Meli, 1986). In high doses, however, urethane may affect several ion channels (Hara and Harris, 2002). Urethane is a known carcinogen and was therefore handled with gloves.

5.1.3 Electrophysiology

In our experiments, single-cell extracellular recordings were used to study spinal nociceptive activity. The nociceptive activity has, however, also previously been studied using patch-clamp (Ikeda et al., 2003) or field potential recordings (Eriksen et al., 2012). Patch-clamp is performed on slices of neuronal tissue, and can therefore not be used to mimic the clinical situation after disc herniation. In addition, ascending and descending pathways are not intact in this model. Field potential recordings, on the other hand, measure in vivo extracellular field potential, generated by the simultaneous activation of several neurons. However, if the position of the electrode changes during the experiment the results can be misleading. In

contrast, single-cell extracellular recording is a method that provides easily quantifiable recordings of neuronal activity in vivo. Studying spike amplitude and shape allows the researcher to distinguish between different cells, adding confidence to the results.

Performing single-cell recordings may be challenging. Minor vibrations may disturb the electrophysiological recording. Therefore, NP was harvested from a genetically identical donor rat, avoiding possible disturbances and minimizing the time between harvest and application onto the dorsal nerve roots. Easy access to the vertebral discs in the tail made it the preferred source of NP tissue. Although this model of disc herniation uses NP from a donor rat rather than autologous NP, the fact that the individuals are genetically identical still makes this a viable model.

Previous data show that minocycline is an inhibitor of microglial and macrophage activation (Yrjanheikki et al., 1998) and it is widely used for this purpose. In the present study, we used a concentration of 5 $\mu\text{g}/\mu\text{l}$ administered directly onto the dorsal nerve roots. Earlier experiments involving intrathecal administration of minocycline show that concentrations within the range 1-5 $\mu\text{g}/\mu\text{l}$ have an inhibitory effect on microglial activation (Ledeboer et al., 2005; Milligan et al., 2005).

5.2 Gene expression

qPCR was used for gene expression analysis, which allows fast and accurate quantification, and only requires small amounts of mRNA. Gene expression microarrays and northern blotting lack the accuracy of qPCR, while the RNase protection assay requires large amounts of RNA and is time consuming. Primer specificity was ensured by running a BLAST search.

To correct for sample to sample variations, an internal control was co-amplified together with target genes. β -actin has previously been shown to have a very stable gene expression independent of experimental intervention, and was therefore selected as the internal control for gene expression analysis (Pedersen et al., 2010).

It is important to note that qPCR only gives a measure of the mRNA concentration, and does not give any information about the actual protein level. Performing western blots or immunohistochemistry to stain the proteins of interest to complement these results would also be valuable.

The NP tissue used for gene expression analysis was first used in electrophysiological experiments to mimic disc herniation before being subject to qPCR analysis, thereby linking these two aspects of the study. DRG isolation was, however, performed in a parallel series of experiments. Ideally, DRG should have been isolated from rats following electrophysiological recordings. This could have given insight into the relationship between DRG gene expression, NP gene expression and nociceptive activity.

6 Discussion of Results

6.1 Disc herniation and nucleus pulposus

NP induced an increase in C-fiber response when applied onto the dorsal nerve roots. This increase in excitability of the nociceptive spinal neurons outlasted the duration of the experiments – 3 hours. The effect of NP on nociceptive signaling has previously been shown in both electrophysiological experiments (Cuellar et al., 2004) and behavioral animal studies (Olmarker et al., 2003). While traditionally thought to be a result solely of the mechanical pressure generated by NP leaking out from the herniated disc, it is now well established that pain hypersensitivity following disc herniation has an inflammatory aspect (McCarron et al., 1987; Olmarker et al., 1995). Several disc-related cytokines that may increase the activity in the nociceptive pathways have been studied (Aoki et al., 2002; Brisby et al., 2002).

Previous animal studies indicate that IL-1 β (Rothwell and Strijbos, 1995), TNF α (Olmarker and Larsson, 1998), and Csf1 (Egeland et al., 2013) may be upregulated in NP tissue exposed to the DRG and/or dorsal nerve roots. In particular, TNF α has been established as an important component of NP-induced inflammation (Aoki et al., 2002; Homma et al., 2002; Zhang et al., 2002). Inhibition of TNF α in disc herniation models has demonstrated pain alleviating effects (Olmarker et al., 2003; Cuellar et al., 2004). However, the observation that inhibition of several cytokines at the same time has a greater effect on nociceptive signaling than blockade of only TNF α (Olmarker and Larsson, 1998) demonstrates the importance of the interplay between different pro-inflammatory mediators in the development of pain hypersensitivity.

In the present study, only a minor increase in the expression of TNF α was observed. However, in accordance with our previous studies, a clear upregulation of IL-1 β and Csf1 was demonstrated in NP tissue after contact with dorsal nerve roots (Egeland et al., 2013). The importance of IL-1 β in the development of central sensitization has been shown in several previous studies. IL-1 β may have direct effects on AMPA- and NMDA-induced currents, on the phosphorylation of transcription factors, and on the presynaptic release of SP (Malcangio et al., 1996; Kawasaki et al., 2008). The upregulation of Csf1 in NP following experimental disc herniation has also recently been established. This molecule may induce recruitment of nearby macrophages. In addition, Csf1 may activate microglia (Sawada et al., 1990; Chitu and

Stanley, 2006). Hence, it is tempting to speculate that upregulation of Csf1 may act as a link between herniated NP and the activation of microglial cells.

We also demonstrated an upregulation of CD68, FKN and CX3CR1 in NP tissue exposed to the dorsal nerve roots. CD68 has traditionally been used as a marker of macrophages and monocytes. However, more recent data show that CD68 may be expressed by several different cells, including fibroblasts and activated epithelial cells (Kunisch et al., 2004). In light of this discovery, its expression is now used as a marker of phagocytic cells. The presence of CD68 positive cells in NP and the phagocytic potential of these cells has been reported (Nerlich et al., 2002; Jones et al., 2008; Chen et al., 2013). Hence, the upregulation of CD68 in the present study could indicate some sort of activation of, and an increased phagocytic potential for NP cells following disc herniation.

Phagocytosis is characterized by internalization of particles by cells, creating a phagosome, i.e. a vesicle. After fusion with lysosomes, the particles within the phagosomes are digested. The detection of pathogen-associated molecular patterns initiates phagocytosis by immune cells through the activation of NK- κ B. Phagocytosis is also important for the removal of cell debris following cell death. Apoptosis by disc cells has been shown previously (Lotz et al., 1998; Ha et al., 2006), and because the environment within the intervertebral discs is isolated from the immune system, it makes sense that the NP cells themselves are capable of phagocytosis (Jones et al., 2008; Chen et al., 2013).

CTSS, FKN and CX3CR1 are components of the newly discovered positive feedback loop between activated glial cells and neurons (Clark et al., 2007). CTSS, expressed and released exclusively by antigen presenting cells (Shi et al., 1994), cleaves off the chemokine domain of the full length membrane tethered protein FKN. FKN activates microglial and satellite glial cells by binding to the receptor CX3CR1. The upregulation of FKN on neurons and astroglia has been linked to the induction of mechanical allodynia and thermal hyperalgesia in a behavioral study, also using NP transplantation as a model of disc herniation (Park et al., 2011). The fact that CTSS, FKN and CX3CR1 are all expressed by NP, and especially the fact that FKN and CX3CR1 are upregulated in the present study, implies a new mechanism for NP in the generation of neuropathic pain following herniation.

Minocycline is a tetracycline antibiotic, preventing bacterial proliferation by halting protein synthesis. However, minocycline also inhibits the activation of microglial cells and

macrophages. Medically it is used to treat skin infections (Rogers and Perkins, 2006) and acne vulgaris (Strauss et al., 2007). Although it might be an effective treatment, its use is restricted by many adverse side effects (Fanning and Gump, 1976).

Inhibition of microglial activation with minocycline has demonstrated pain-alleviating effects in animal pain models (Ledeboer et al., 2005; Ikeda et al., 2012; Zhang et al., 2012). Through an unknown mechanism, minocycline performs this inhibition by preventing the phosphorylation of MAPK p38 (Hua et al., 2005). In the present study, minocycline attenuated the increase in nociceptive signaling induced by NP. IL-1 β , Csf1 and FKN – all upregulated in NP following contact with the dorsal nerve roots – are capable of activating microglial cells. Microglial cells have previously been implicated in the development of pain hypersensitivity (Coyle, 1998; Zhuang et al., 2005; Ikeda et al., 2007). Hence, it is likely that the induced pain hypersensitivity following experimental disc herniation may involve microglial activation. Furthermore, the pain-alleviating effects of minocycline may be associated with preventing MAPK p38 phosphorylation in microglia.

Minocycline also inhibited the increase in the gene expression of IL-1 β , Csf1, CD68, FKN and CX3CR1 in NP tissue, thereby implicating NP as another target for minocycline. Data from in vitro studies have demonstrated that inhibition of MAPK p38 in cytokine-activated human NP cells blunts production of factors associated with inflammation, pain, and disc matrix catabolism. (Studer et al., 2007). It is therefore possible that part of the observed alleviating effect of minocycline administration was caused by this drug acting on NP cells. The reduced gene expression in NP could also reduce the activity of microglia.

6.2 Dorsal root ganglion

The changes in gene expression were less pronounced in DRG than in NP. Still, a clear increase in the expression of TNF α , FKN and CX3CR1 was demonstrated. The changes in gene expression were only observed in the ipsilateral DRG. In our model, the contralateral DRG served as control. The relatively stable gene expression of the contralateral side indicates that the NP tissue has an effect directly on the left dorsal nerve roots.

Increased expression of FKN and its receptor CX3CR1 has previously been shown in the DRG in experimental neuropathic pain (Zhu et al., 2013). Also, TNF α production in satellite glial cells following FKN injection into the DRG has been demonstrated (Souza et al., 2013).

Both these studies support our findings in this model mimicking the clinical situation following disc herniation.

The L3-L5 ganglia are located 5-15mm lateral of the dorsal nerve roots, where NP was applied in the present study. This may suggest axoplasmic retrograde signaling within afferent nerve fibers. Our data may also suggest that the gene expression changes within the DRG are located in neurons and satellite glial cells. The fact that CD68 and CTSS did not show an increased gene expression in the DRG, which would have implicated immune cells, supports this assumption. Hence, our data support the previous observation that local application of NP onto the DRG may change the gene expression in the primary afferent nerve cells (Takahashi Sato et al., 2012). The upregulation of FKN in the somas of afferent nerve fibers could activate nearby satellite glial cells, thereby increasing the expression of TNF α and CX3CR1 in these cells.

Several lines of evidence suggest that neurotrophins perform axoplasmic retrograde signaling. The binding to trk receptors leads to the internalization of the receptor/ligand-complex within an endosome (Howe et al., 2001). This endosome travels on microtubules to the cell soma (Watson et al., 1999). In the soma, the activation of transcription factors – including CREB (Riccio et al., 1997) – may lead to gene expression changes. Several previous studies have reported neurotrophin signaling from NP tissue following spinal disc herniation (Abe et al., 2007; Purmessur et al., 2008; Navone et al., 2012), including NGF and brain-derived neurotrophic factor (BDNF). Hence, it is possible that the gene expression changes in the DRG cells in the present study were caused by neurotrophins released by NP cells (figure 6.1).

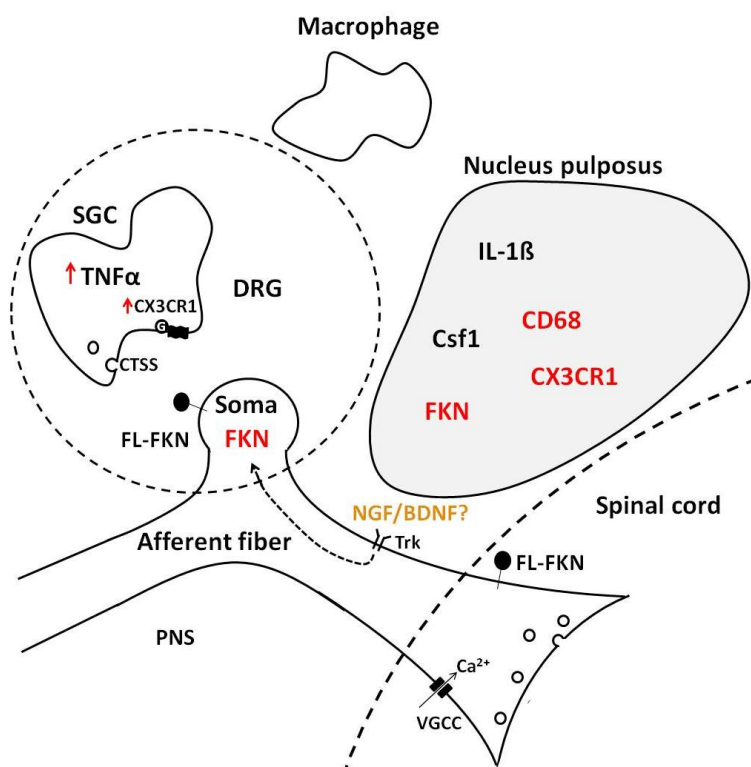


Figure 6.1 Following application of NP onto the dorsal nerve roots, the expression of IL1-β, Csf1, CD68, FKN and CX3CR1 was upregulated in NP tissue. IL-1β, Csf1 and CD68 upregulation suggests that NP may induce a local inflammatory process and phagocytic activity in NP tissue. An upregulation of FKN and its receptor CX3CR1 was also observed. NGF/BDNF signaling is illustrated as a suggested mechanism leading to FKN upregulation in the DRG. CX3CR1 and TNFα upregulation in the DRG is probably located within satellite glial cells (SGC). It is likely that the upregulation of FKN, CX3CR1 and TNFα may affect the excitability of primary afferent nerve fibers.

6.3 Further perspectives

The discovery that CX3CR1 is upregulated in NP tissue following contact with dorsal nerve roots suggests that FKN signaling may affect these cells. Previous studies have shown the effect of interleukins and TNFα on cultured NP cells. It would be interesting to investigate if FKN is capable of producing similar effects on NP cells.

The gene expression changes in DRG are located 5-15 mm lateral of the location of NP transplantation. Evidence exists that neurotrophins are capable of axoplasmic retrograde signaling. Hence, it would be interesting to investigate whether neurotrophin signaling may be involved in our model mimicking the clinical situation following disc herniation.

7 Conclusion

- 1) NP applied onto the dorsal nerve roots in our model mimicking the clinical situation after disc herniation caused a significant increase in nociceptive signaling after 3 hours. In accordance with previous studies, these results suggest that the release of pro-inflammatory molecules from NP may induce pain hypersensitivity following disc herniation.
- 2) Application of minocycline onto the dorsal nerve roots attenuated the effect of NP on the spinal nociceptive signaling. However, minocycline did not have any persistent effects on neuronal activity by itself. This suggests that minocycline inhibits the pro-nociceptive process induced by NP.
- 3) In NP tissue exposed to the dorsal nerve roots for 3 hours, the gene expression of IL-1 β , Csf1 and CD68 was upregulated. The upregulation of IL-1 β and Csf1 suggests that NP has a pro-inflammatory effect, underlying the pro-nociceptive process after disc herniation. In addition, the upregulation of CD68 indicates phagocytic activation of NP cells following contact with the nerve roots.

We also demonstrated an upregulation of FKN and its receptor CX3CR1 in NP tissue. The upregulation of FKN and CX3CR1 indicates a new mechanism for NP in the induction and/or maintenance of pain hypersensitivity.

In the DRG, after NP was exposed to the dorsal nerve roots, the gene expression of TNF α , FKN and CX3CR1 was upregulated. It is tempting to speculate that these gene expression changes in the DRG, located 5-15 mm lateral of the site of NP application, are a result of axoplasmic retrograde signaling within afferent nerve fibers.

- 4) Minocycline inhibited the increase in gene expression of IL-1 β , Csf1, CD68, FKN and CX3CR1 in NP tissue, demonstrating an inhibitory effect on these cells. These results show an anti-inflammatory effect of minocycline on our model mimicking the clinical situation following disc herniation, possibly by interfering with the phosphorylation of MAPK p38.

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Appendices

Appendix 1

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. The concentration of the samples was determined measuring optical densities with a Nanodrop 8000 spectrophotometer.
15. The sample was diluted to 0.25 µg/µl by adding $((10 \mu\text{l} \times \text{concentration } \mu\text{g}/\mu\text{l}) / 0.25 \mu\text{g}/\mu\text{l}) - 10 \mu\text{l} = x.x \mu\text{l}$ RNase free water.
16. The sample was stored at -80 °C.

Appendix 2

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

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 was 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 each samples was 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 3

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. 3 µg of RNA was mixed with water to a total volume of 6 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.
4. The tubes were incubated at 68 °C for 15 min, then put directly on ice
5. Mixture 5 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.
7. The reverse transcription reaction was run on the PCR machine with the following program: 42 °C for 60 min, 99°C for 5 min and 4 °C for 5 min.
8. Each sample was added 133.5 µl of TE-buffer to a final concentration of 10 ng/µl. The samples were then mixed and spun.
9. The samples were stored at -80 °C.

Appendix 4

Procedure for qPCR analysis

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

1. A master mix was prepared:

Reagent:	Volume/sample
ddH ₂ O	5.22 μ l
Perfecta SYBR Green Fast Mix	10.00 μ l
Primer forward (25 μ mol/ μ l)	0.21 μ l
Primer reverse (25 μ mol/ μ l)	0.21 μ l
Total	15.64 μ l

2. The cDNA samples used for β -actin analysis were diluted in the following way: 1 μ l cDNA (10 ng/ μ l) + 9 μ l RNase free water.
3. 8 μ l from three different cDNA samples (10ng/ μ l) were mixed to give a stock cDNA solution. A dilution series to generate a standard curve for each gene was prepared:

Dilution series nr	cDNA	Rnase free water	
1	24 μ l	Undiluted	100 ng
2	6 μ l	18 μ l	25 ng
3	6 μ l from nr 2	18 μ l	6,25 ng
4	6 μ l from nr 3	18 μ l	1,56 ng
5	6 μ l from nr 4	18 μ l	0,39 ng
6	6 μ l from nr 5	18 μ l	0,0975 ng

4. 15.65 μ l master mix was loaded into each well on a 96 well plate.
5. 4.35 μ l ddH₂O was added to the non-template control (NTC) wells.
6. 4.35 μ l sample cDNA, prediluted samples for β -actin analysis or dilution series samples was transferred to the PCR plate in two parallels and mixed well.
7. The PCR plate was sealed with a plastic film and spun at 2200 rpm.
8. The qPCR reaction was run with the following program: 90 °C for 2 min followed by 40 cycles of 95 °C for 10 sec and 60 °C for 30 sec. After completing the program, a melting curve was established in a step-and-hold fashion up to 95 °C.