Epiregulin (EREG) associated with disc herniation induces spontaneous activity in the pain pathways.

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Master Thesis

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June 2016
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Press: Reprosentralen, University of Oslo

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Acknowledgments

First and foremost, I would like thank my supervisor Johannes Gjerstad, for seeing the potential in me and for allowing me to work with such a fascinating field. Thanks for your excellent guidance through this project and always finding the time to help. Your knowledge and enthusiasm were highly appreciated.

Second, I would like to thank my co-supervisor Fred Haugen for excellent guidance with laboratory work and for always shearing your great ideas. Thanks to Aurora Moen, my co-supervisor, for always giving your time and help, and for always making me smile.

To all my supervisors thank you for making this thesis a memorable experience.

Thanks to Daniel Pitz Jacobsen and Aqsa Mahmood for excellent laboratory training and for always answering my questions. Thanks to Anna Lengfeld who helped me in the lab and performed three of the electrophysiological control experiments. To the rest of Work-related Musculoskeletal Disorders Group, thanks for letting me be part of such a wonderful work environment.

Thanks to all my fellow student at STAMI and UiO for your encouragement and inspiration. A special thanks my fellow students and good friends Marte, Heidrun and Malin for being my extra family here in Oslo. Thank you for always being there and making me laugh. Your love and hugs were extremely appreciated.

Lastly I would like to thank my parents and my sister for always believing in me. Your interest in my work and allowing me to share it with you were greatly appreciated. Thanks for always making me believe that this would possible and for making me feel better during stressful times. Thanks to my entire family for your constant support and for always cheering me on.

Mette Kongstorp
Oslo, May 2016
Abstract

Low back pain and sciatica after disc herniation may be caused by mechanical compression of the nerve roots, but also by the release of pro-inflammatory agents and growth factors from the nucleus pulposus (NP) of the herniated disc. In the present study the functional changes in nociceptive signaling due to disc herniation were investigated.

NP tissue was harvested from tail vertebrae and incubated in cell medium to examine if NP cells were able to secrete epiregulin (EREG), a member of the epidermal growth factor (EGF) family. Extracellular single-cell recordings in the spinal dorsal horn (DH) were performed to investigate the effect of EREG on neuronal excitability. The expression of EREG and its receptors were examined by qPCR in NP tissue, DH of the spinal cord, and the dorsal root ganglions (DRG).

The present data demonstrated that EREG may be released after disc herniation from NP tissue. Directly administration of EREG onto the spinal dorsal nerve roots induced a decrease in responsiveness to electrical stimuli, but a pronounced increase in spontaneous activity in nociceptive neurons. A significant up-regulation of the gene encoding EREG was observed in the DH, when NP tissue was exposed to the spinal dorsal nerve roots. The EREG receptors EGFR and HER4 were up-regulated in NP tissue and DH tissue, whereas HER3 was up-regulated in DRGs.

Taken together, our findings suggest that EREG signaling through its receptors may induce sensory abnormalities and pain hypersensitivity following a disc herniation.
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## Abbreviations

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<tbody>
<tr>
<td>5-HT</td>
<td>Serotonergic/serotonin</td>
</tr>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus fibrosus</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CaMKII</td>
<td>( \text{Ca}^{2+} ) calmodulin dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid type 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLIA</td>
<td>Chemiluminescence immunoassay</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>DH</td>
<td>Dorsal horn</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EREG</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor (also used in animals, often called ErbB)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK1</td>
<td>Neurokinin 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulin</td>
</tr>
<tr>
<td>NS</td>
<td>Nociceptive specific</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>PB</td>
<td>Parabrachial nucleus</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinostiol-dependent kinase 1</td>
</tr>
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XII
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RAF</td>
<td>Proto-oncogene serine/threonine-protein kinase</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TE</td>
<td>Trisethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tyrosine receptor kinase A</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential cation channel, subfamily V, member 1</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Pain versus nociception

The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Pain is a result of a complex interaction between sensory, emotional, cognitive and contextual factors. Hence, the experience of pain is always subjective. Nociception is not the same as pain and is defined by IASP as “the neural process of encoding noxious stimuli”. Pain often occurs when the nociceptive pathways are activated, however pain may occur in the absence of nociceptive signaling. Allodynia is the phenomenon where the experience of pain occurs to stimuli that are normally non-painful. The phenomenon where enhanced pain experience occurs after a normally painful stimuli is referred to as hyperalgesia.

1.2 Nociceptive signaling

Under normal and healthy conditions pain is initiated by activation of nociceptors. These nociceptors are located on primary afferent nerve endings and are specialized high-threshold sensory receptors. Nociceptors are polymodal and detect noxious thermal, chemical and mechanical stimuli. They are located on myelinated Aδ-fibers and unmyelinated C-fibers. Aδ-fibers have a conductance velocity of 5-30m/s and are responsible for the “fast pain”. Since C-fibers are unmyelinated they convey nociceptive signals slower (conductance velocity 0.2-2m/s) and are responsible for the “slow pain”. After activation the noxious signals are conducted along Aδ- and C- axons from the periphery into the dorsal horn (DH) of the spinal cord.

In the DH the afferent nociceptive nerve fibers synapse with spinal nerves located in laminae I, II and V. The signal is then conveyed across the synapse by presynaptic release of glutamate, substance P (SP) and other neuropeptides (Kangrga and Randic 1991; Kantner et al., 1985). Glutamate binds to their receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and metabotropic glutamate receptor (mGluR), whereas SP bind to neurokinin 1 (NK1) receptor, which results in a depolarization of the postsynaptic membrane.
Spinal neurons may be classified into three broad classes depending on their projection: interneurons, propriospinal neurons and projecting neurons. Interneurons are neither sensory nor motor neurons. They are involved in local processing and modulation by making connections with other neurons, and may have inhibitory or excitatory effects. Interneurons make up the majority of neurons in the DH, and are contained within the grey matter of the spinal cord. The propriospinal neurons, on the other hand, integrate several spinal levels; they have the soma in one spinal segment and an axon terminating in another segment. These neurons, like interneurons, are involved in processing and modulation of signaling in other neurons in the spinal cord. The projecting neurons have much longer axons. These neurons convey signals from the DH, with input from primary sensory neurons, along the anterolateral white matter to higher brain centers. See figure 1.1. For a review see (Todd 2010).

These projecting neurons comprise of nociceptive specific (NS) neurons and wide dynamic range (WDR) neurons. NS neurons only receive and process noxious input and are mostly located superficially in the DH. The WDR neurons are located deeper in the DH and have the ability to respond to both noxious and innocuous input.

The nociceptive signal is processed by various areas of the brain and as a result the perception of pain may occur. Information about location and intensity of the stimuli is conveyed via the thalamus to primary- and secondary somatosensory cortex, thus providing the sensory-discriminative aspects of the pain. On the other hand, the affective-motivational aspects of pain are transmitted to the parabrachial nucleus (PB), hypothalamus, periaqueductal grey (PAG), amygdala, insula and cingulate cortex (figure 1.1). These areas are responsible for the unpleasant feeling, fear and anxiety following a painful stimulus. For a review see (Gauriau and Bernard 2002).

### 1.2.1 Descending modulation

The ascending pathways are under control of a complex descending system, which modulates and control nociceptive transmission in the spinal cord. The main regions that are involved in these descending modulations are PAG, pons and rostral ventromedial medulla (RVM). These brain areas modulate neuronal transmission in the DH, by controlling activity in the serotonergic (5-HT), noradrenergic and enkephalinergic descending projections. (Marlier et al., 1991; Rajaoefetra et al., 1992; Reddy et al., 1990). PAG seems to be particular important in this system. Studies of single cells in the lumbar spinal cord demonstrate that activation of
PAG increase the activity in descending inhibitory pathways (Jones and Gebhart 1988). PAG receives input not only from ascending pathways, but also from other brain centers like the prefrontal cortex, the anterior cingulated cortex, amygdala, insula, and hypothalamus. Information received and processed in PAG is transmitted to the RVM (figure 1.1). RMV consist of on-cells and off-cells that connect with ascending nociceptive neurons in lamina I and II. On-cells enhance nociceptive signaling, while off-cells inhibit nociceptive signaling (Fields et al., 1983). Hence, nociceptive signaling in the spinal cord is highly controlled by supraspinal systems. For a review see (Gjerstad 2007).
Figure 1.1 An overview of nociceptive signaling and modulation. Afferent and ascending fibers are shown in red, while descending fibers are shown in blue. Nociceptive input from the periphery is conducted along A\(\delta\) – and C-fibers into the dorsal horn (DH) of the spinal cord. Projecting neurons in the spinal cord convey the signals along the anterolateral system to the brainstem and supraspinal centers. The sensory-discriminative aspects of pain are conveyed via the thalamus to the somatosensory cortex. The parabrachial nucleus, hypothalamus, periaqueductal grey (PAG), amygdala, insula and cingulate cortex is involved in the affective-motivational aspects of pain. A complex descending system modulates the activity in the spinal cord and thereby the ascending pathways. DRG: dorsal root ganglion, RVM: rostral ventromedial medulla, SP: substance P. (Gjerstad 2007)

1.3 Sensitization

Acute pain has a clear adaptive function; it functions as a warning for events that are capable of producing injury, and the ability to react to painful stimuli is essential for survival. Chronic pain is pain that persists or progress over a long period of time. Long-lasting pain have no known profound biological advantage, it just causes suffering. Acute pain may become chronic through peripheral and/or central sensitization after injury.
Injury, inflammation and nerve damage may cause sensitization and enhanced nociceptive signaling. Sensitization are defined as an increased responsiveness of neurons to their normal input or recruitment of a response to normally subthreshold inputs (Loeser and Treede 2008). It includes a drop in threshold and an increase in suprathreshold response – allodynia and hyperalgesia, respectively. In addition, spontaneous discharges and increases in receptive field size may also occur.

### 1.3.1 Peripheral sensitization

Peripheral sensitization represents a reduction in nociceptor activation threshold and an amplification in the responsiveness of the peripheral nociceptive neurons (Latremoliere and Woolf 2009). This hypersensitivity is often due to tissue damage and changes in the chemical environment surrounding the nerve fibers. Damaged and inflamed tissue release cytokines, histamine, adenosine triphosphate (ATP), SP, bradykinin (BK), prostaglandin (PG), 5-HT, nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). Such substances may directly or indirectly influence ion channels and receptors, thereby increasing their response and sensitizing the high-threshold. Since only local nociceptors are affected, peripheral sensitization is restricted to the site of injury. For a review see (Julius and Basbaum 2001; Latremoliere and Woolf 2009).

Binding of NGF to its receptors may affect transcription of various peptides, like SP, and high-threshold nociceptors like the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) (Zhang et al., 2005). Moreover, NGF induces up-regulation of BDNF in the dorsal root ganglion (DRG). BDNF is then transported along afferent axons and released in the DH where it may phosphorylate non-selective cation-channel N-methyl-D-aspartate (NMDA) receptors located on the postsynaptic membrane. For a review see (Coutaux et al., 2005).

### 1.3.2 Central sensitization

Central sensitization is defined by IASP as “an increased responsiveness of nociceptive neurons in the central nervous system (CNS) to their normal or subthreshold afferent input”. In central sensitization, responses to sensory stimuli may be enhanced without increased excitability of primary afferent neurons. Hence, a persistent state of increased sensitivity may involve changes within the spinal cord and supraspinal areas.
Mechanisms of central sensitization

Following a mild noxious stimulus, glutamate is released from the presynaptic membrane. Glutamate binds to AMPA receptors localized on the postsynaptic membrane, which leads to opening of the ion channel. AMPA receptors are highly permeable to $\text{Na}^+$ and as a result the postsynaptic membrane is depolarized for a short time. In addition, glutamate have the ability to bind to NMDA receptor, however this ion channels is blocked by a magnesium ion.

Strong noxious stimuli results in a release of SP in addition to glutamate from the presynaptic membrane. SP binds to NK1 receptors on the postsynaptic membrane, which results in activation of phospholipase C (PLC). When PLC is activated, signaling through inositol triphosphate (IP3) induces release of $\text{Ca}^{2+}$ from intracellular compartments (Drdla and Sandkuhler 2008). The intracellular release of $\text{Ca}^{2+}$ results in a long-lasting depolarization. This removes the $\text{Mg}^{2+}$ blockage from NMDA receptors, and induces additional influx of cations, leading to further depolarization of the membrane.

Long-lasting efficiency of transmission is called long-term potentiation (LTP). The distinct and long-lasting modulation in synaptic efficiency may be induced at primary afferent synapses with neurons in the superficial laminae of the DH. These changes can be physiologically relevant for the transmission and integration of long-lasting pain and other sensory information (Randic et al., 1993).

Since long-lasting and strong noxious stimuli increases the cytosolic $\text{Ca}^{2+}$ level, sensitization at a spinal level may be a result of activation of a variety of different $\text{Ca}^{2+}$-dependent cellular responses. These responses includes activation of protein kinase C (PKC) (Lin et al., 1996), $\text{Ca}^{2+}$ calmodulin dependent kinase II (CaMKII) (Pedersen et al., 2005) and the mitogen-activated protein kinase (MAPK) called extracellular signal-regulated kinase (ERK) (Xin et al., 2006). This may alter neuronal excitability by inducing an up-regulation of ion channels in the membrane (Hayashi et al., 2000), alter conductance and channel opening (Derkach et al., 1999; Lan et al., 2001).
1.4 Disc herniation

![Illustration of the vertebral column](image)

**Figure 1.2. Illustration of the vertebral column.** The vertebral column consist of vertebrae that are separated by intervertebral discs. The intervertebral disc consists of the outer annulus fibrosus (AF) surrounding a gel-like nucleus pulposus (NP). The vertebral column serves as a protective housing for the spinal cord. During a disc herniation a tear forms in AF, resulting in leakage of NP. NP then puts mechanical pressure onto the dorsal nerve root.

1.4.1 The vertebral column

The vertebral column serves as a protective housing for the spinal cord. In humans the vertebral column consist of 7 cervical, 12 thoracic, 5 lumbar and 5 sacral vertebrae, whereas the rat has 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and 27-30 caudal tail vertebrae. These segments are separated by intervertebral discs, consisting of a though outer ring of fibrous cartilage, the annulus fibrosus (AF), which surrounds a gelatinous deformable nucleus pulposus (NP), see figure 1.2. Intervertebral discs allow small movement between vertebrae, making it possible for bending and torsion of the spine. NP being a gel-like substance acts as a cushion, transmitting load arising from body weight and muscle activity. Thereby preventing the vertebral column from being damaged during compression and contortion.

1.4.2 Nucleus pulposus

NP contains collagen fibers, which are organized randomly, and elastin fibers which are arranged radially (Urban and Roberts 2003). The gel-like structure is caused by water that is held between these collagen- and elastin fibers. NP consists mainly of proteoglycans, which provides an osmotic pressure that draws water into the disc. The most abundant cell type in NP is chondrocyte like cells, present at a low density. Under normal and healthy conditions
the NP is not vascularized and nutrition is supplied by diffusion. Thus, NP tissue is highly isolated from the rest of the body. Because of this isolation there are no immunological cells like circulating macrophages and monocytes present in NP (Nerlich et al., 2002). However, recent studies have shown that cells in NP have the ability to become phagocytic, allowing removal of apoptotic cells within the discs (Chen et al., 2013).

1.4.3 Degeneration and herniation of the intervertebral disc

During growth and skeletal maturation the boundary between the annulus and the nucleus of the disc becomes less obvious. NP generally becomes more fibrotic and less gel-like. The most significant biochemical change during disc degeneration is loss of proteoglycans (Lyons et al., 1981), and the content for fibronectin increases. These changes result in a lower osmotic pressure, making the disc less hydrostatically. Moreover, degeneration of the intervertebral disc makes the AF weaker and stiffer. Consequently, degeneration results in loss of elasticity and decreased load bearing ability. This induces an increased possibility for an annular tear. A tear in AF has been linked to ingrowths of blood vessels and nerve fibers (Nerlich et al., 2007; Ohtori et al., 2006). Most commonly, such tears are relatively small. However, more extensive tears may allow the inner NP to leak out into the spinal canal, resulting in a disc herniation (figure 1.2). For a review see (Urban and Roberts 2003).

Disc herniation is often accompanied by low back pain and sciatica. Originally it was thought that mechanical pressure caused by the herniated disc was the cause of the pain. However, it has been recognized that the leakage of NP into the spinal canal may cause inflammation through release of pro-inflammatory agents (McCarron et al., 1987; Olmarker et al., 1995). Animal studies have shown that application of NP onto DRG or dorsal nerve roots induce sensitization of the primary afferents or ascending pathways (Cuellar et al., 2005; Egeland et al., 2013; Takebayashi et al., 2001). This inflammatory aspect of the medical condition may contribute to low back pain and sciatica.

It has been shown that cytokines, as well as other mediators, are expressed in or around the herniated disc (figure 1.3), including interleukin (IL)-6, IL-1α and β, tumor necrosis factor (TNF) α, matrix metalloproteinases (MMP), nitric oxide (NO) and PG (Igarashi et al., 2000; Kang et al., 1996; Kawakami et al., 1997; Takahashi et al., 1996). These substances may be secreted from cells in the NP tissue, as studies performed on cultured NP tissue showed a increased production of inflammatory mediators (Kang et al., 1996). Other pro-inflammatory
agents may be released from attracted immune cells. Hence, the mechanisms underlying pain sensitization following disc herniation may involve release of a number of cytokines and other pro-inflammatory factors.

1.4.4 Inflammatory factors

Formation of tears and clefts in the disc during disc degeneration allows immune cell activation and infiltration of macrophages, neutrophils and T cells (Kokubo et al., 2008; Shamji et al., 2010). Following a disc herniation, NP tissue as well as the infiltrated immune cells releases pro-inflammatory agents.

Cytokines are small regulatory proteins that are involved in immune responses, inflammation and trauma. They perform paracrine, autocrine and endocrine signaling, and are primarily produced and released by immune cells and glia cells in CNS. However, many types of cells are able to synthesize and release cytokines in response to stimuli. Cytokines have the ability to induce or repress synthesis of themselves, as well as other proteins. Cytokines that are involved in the establishment of an inflammation are divided into several subgroups. For a review see (Dinarello 2000).

The IL family consists of small proteins that act as signaling molecules between immune cells. ILs are secreted from cells and carry out their action in communication among leukocytes by binding to their specific receptor. They play an important role in immune responses, and are known to have both anti- and pro-inflammatory effects. Previous studies have demonstrated that disc herniation induce elevated levels of several ILs like IL-1α and β, IL-6 and IL-8 (Pedersen et al., 2015; Takahashi et al., 1996).

IL-1 act through binding their receptors, IL-1 receptor type 1 or type 2, which activates the transcription factor nuclear factor-κB (NF-κB) and MAPK p38. It has been suggested that signaling through IL-1 receptors are involved in neuropathic pain, and increased nociceptive signaling in DRG neurons. Moreover, mice with impaired IL-1 signaling display markedly reduced neuropathic pain (Wolf et al., 2006). Activation of MAPK p38 in DRG neurons may be involved in the early phase of neuropathic pain (Jin et al., 2003). Hypersensitivity in nociceptive neurons due to high levels of ILs may be caused by an up-regulation of SP (Malcangio et al., 1996). Taken together, these findings indicate that ILs are involved in enhanced neuronal excitability and may cause hypersensitivity in nociceptive neurons.
The TNF family are known for its role in inflammatory pain, and includes signaling proteins that are able to induce apoptosis. Animal studies have shown that TNFα induces abnormal activity in nociceptive neurons (Sorkin et al., 1997). This aberrant activity may be caused by the ability of TNFα to induce release of IL-1β (Watkins et al., 1995).

The interferon (IFN) family consists of proteins that are capable to prevent protein synthesis within virally infected cell. Studies have shown that intrathecal injections of IFNγ induced neuropathic pain-related behavior by activation of microglia and macrophages (Robertson et al., 1997; Tsuda et al., 2009). IFNγ may also alter neuron excitability directly by phosphorylation of AMPA receptors, increasing Ca\(^{2+}\) influx in nociceptive neurons (Mizuno et al., 2008).

The increased release of cytokines, following disc herniation, induces up-regulated levels of MMPs, NO, BKs and PG (Kang et al., 1997), which are known to play a part of inflammatory and neuropathic pain (Chapman and Dickenson 1992; Kawasaki et al., 2008; Lin et al., 1999). Taken together, disc herniation and the substantial release of cytokines may be involved in the development of chronic low back pain.

### 1.4.5 Growth factors

Growth factors are small proteins or steroid hormones that are capable of stimulating cellular growth, proliferation, healing and cellular differentiation. It has been suggested that growth factors may be able to alter nociceptive signaling. NGF and other neurotrophines may cause hyperalgesia through direct stimulation of the nociceptive terminals or by affecting the expression of genes that in turn alter nociceptive function.

NGF is known to be involved in the development of peripheral sensitization and may induce hyperalgesia by binding to the tyrosine receptor kinase A (TrkA) and the NGF receptor (Bonnington and McNaughton 2003). Previous studies indicate that NGF enhance neuronal excitability by inducing up-regulation of sodium channels in DRG neurons (Gould et al., 2000). Another growth factor known to be involved in hyperalgesia is neuregulin (NRG). Earlier studies suggest that NRG induces pain hypersensitivity through activation of microglia, which enhance inflammatory responses that may alter neuronal excitability (Calvo et al., 2010).
Recent studies done on patients with lumbar radicular pain following disc herniation showed a significant increase in serum levels of several growth factors. Patients with persistent pain 12 months after disc herniation disposed a higher level of the epidermal growth factor (EGF) and the transforming growth factor (TGF) β1, as well as many other cytokines (Moen et al., Accepted April 2016). This finding suggests that members of the EGF family and their receptors could be involved in pain hypersensitivity following disc herniation.
Figure 1.3 Illustration of potential mechanisms of nucleus pulposus (NP) leakage from the intervertebral disc may enhance nociceptive signaling. NP or recruited macrophages may release various pro-inflammatory cytokines, which are able to affect neurons and glia cells. This may lead to enhanced excitability in primary afferents, resulting in release of glutamate and substance P (SP). These neurotransmitters bind and activate α-amino-3-hydroksy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and neurokinin receptor 1 (NKR1), resulting in a depolarization of the postsynaptic membrane and release of Ca\(^{2+}\) from intracellular compartments through signaling by inositol triphosphate (IP3). These events leads to removal of the Mg\(^{2+}\) blockage of the N-methyl-D-aspartate (NMDA), followed by opening of the ion channel. High levels of Ca\(^{2+}\) also activate downstream kinases such as protein kinase C (PKC), extracellular signal-regulated kinase (ERK), calcium/calmodulin dependent kinase II (CaMKII) and protein kinase A (PKA). ERK may translocate to the nucleus where it stimulates transcription of a number of genes. SP, adenosine triphosphate (ATP), matrix metallocproteinases (MMP), prostaglandins (PG) and cytokines may also stimulate glia to synthesize and release additional cytokines. Cytokines may modulate synaptic transmission by acting on both pre- and postsynaptic neurons. BDNF: Brain-derived neurotrophic factor, DRG: dorsal root ganglion, IFNγ: interferon γ, IL: interleukin, NGF: nerve growth factor, NO: nitric oxide, TNFα: tumor necrosis factor α (Gjerstad 2007).
1.5 Epiregulin

Epiregulin (EREG) is a 46-amino acid protein and belongs to the EGF family. The EGF family consists of peptide hormones, which work in a paracrine or autocrine manner. EGF members have highly similar structural and functional characteristics. The gene encoding human EREG exhibit a 24-50% homology with the sequences of other EGF receptor ligands (Toyoda et al., 1995). Like all members, EREG is initially expressed as a transmembrane precursor, called proepiregulin. The precursor is cleaved within the extracellular region and released as the mature, soluble form.

EREG binds and activates the epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor (HER) 4. In addition EREG may stimulate signaling of HER2 and HER3 through ligand induced heterodimerization with a cognate receptor (Komurasaki et al., 1997). Binding of EREG to one of its receptors induces activation of a wide range of downstream signaling molecules. Previous studies have shown that EREG is more potent than EGF in activating mitogenesis, although EREG has lower binding affinity to HER receptors (Shelly et al., 1998). Ligands that bind HER receptors with a lower affinity hinder normal receptor down-regulation and degradation. This may be the explanation for the high mitogen activity shown in studies of EREG.

1.5.1 Signaling pathways

When EREG binds a receptor the receptor complex becomes active, resulting in phosphorylation of tyrosine residues on the intracellular domain (figure 1.4). This leads to further activation of intracellular signaling molecules. Binding of EREG may activate the MAPK pathway and the phosphoinositide 3-kinase (PI3K) pathway, which are the two main downstream signaling pathways for EREG.

Signaling through the MAPK pathway starts at the phosphorylated tyrosine residue on the receptor and ends by transcription of several genes in the nucleus. When the receptors are activated growth factor receptor-bound protein 2 (GRB2) binds to phosphorylated tyrosine residues. This binding activates GRB2 and induces recruitment and activation of a guanine nucleotide exchange factor called son of sevenless (SOS). SOS removes guanosine diphosphate (GDP) from members of the Ras subfamily, making it possible for Ras to bind guanosine-5'-triphosphate (GTP). When Ras members have bound GTP the signal moves...
from the plasma membrane and into the cytosol. Activated Ras members have the ability to phosphorylate kinases and thereby start a signaling cascade of mitogen-activated kinases. This kinase cascade includes the proto-oncogene serine/threonine-protein kinase (RAF), the mitogen-activated protein kinase kinase (MEK) and ERK. The signal is then conveyed into the nucleus where ERK activates different transcription factors (figure 1.4). In addition, ERK may alter translation of messenger ribonucleic acid (mRNA) into proteins.

Binding of EREG to its receptor may also activate the PI3K signaling pathway. PI3K catalyzes phosphorylation of the membrane bound phosphatidylinositol (PI), converting it to PIP3. PIP3 serves as a docking site for two serine/threonine kinases, Protein kinase B (PKB), also known as AKT, and the phosphatidylinositol-dependent kinase 1 (PDK1). Binding of AKT to PIP3 makes it possible for PDK1 and the mammalian target of rapamycin complex (mTOR) to phosphorylate and thereby activate AKT. Activated AKT then dissociates from the plasma membrane and further activates various target proteins in cytosol or nucleus (figure 1.4). An example is phosphorylation of the transcription factor cAMP response element binding protein (CREB), which is well known for its role in LTP and central sensitization.

In addition, these two signaling pathways may cross-talk. For example, inhibition of PI3K has shown to block the activation of ERK in sensory DRG neurons (Zhuang et al., 2004).
1.5.2 Neuronal excitability

Little is known about how EREG may affect neuronal excitability. However, intracellular signaling following activation of EREG receptors often results in transcriptional activity. This may induce an up-regulation of proteins that influence neuronal excitability.

Figure 1.4 Illustration of signaling pathways downstream for Epiregulin (EREG). Binding of EREG to its receptors may induce signaling in the mitogen-activated protein kinase (MAPK) signaling pathway. Growth factor receptor-bound protein 2 (GRB2) binds the activated receptor, which results in signaling in downstream signaling molecules. This leads to activation of a kinase cascade including proto-oncogene serine/threonine-protein kinase (RAF), mitogen-activated protein kinase kinase (MEK) and extracellular signaling-regulated kinase (ERK). ERK then translocates to the nucleus and activates transcription factors, and start transcription. EREG may also activate the phosphoinositide 3-kinase (PI3K) signaling pathway, leading to phosphorylation of protein kinase B (AKT) induced by phosphoinostiol-dependent kinase (PDK) 1 and mammalian target of rapamycin complex (mTOR). AKT then dissociates from the plasma membrane and activates various target proteins both in the cytosol and nucleus. GDP: Guanosine diphosphate, GTP: Guanosine-5’-triphosphate, P: phosphorylated, PI: phosphatidylioditol, SOS: Son of Sevenless, Tyr: tyrosine.
Activation of PI3K may activate the transcription factor CREB, which may induce transcription of genes that are involved in neuropathic pain (Song et al., 2005). Previous studies have shown that PI3K may regulate hyperalgesia through regulation of TRPV1 in DRG neurons. Moreover, inhibition of PI3K prevents heat hyperalgesia, by suppressing NGF-induced sensitization of TRPV1 (Zhuang et al., 2004).

EREG may also be involved in the development of inflammation. Previous studies have shown that EREG is the only growth factor present at an early stage of inflammation. EREG then triggers a temporal expression of other growth factors at a later stage (Harada et al., 2015). Moreover, removing EREG in a knockdown model, suppresses IL-6 which is considered as an inflammatory amplifier. It has also been shown that IL-6 induces EREG expression, suggesting that there is a positive-feedback loop mechanism between IL-6 and EREG signaling (Harada et al., 2015; Murakami et al., 2013).

Taken together, these findings suggest that EREG may have the ability to affect neuronal excitability, by either alter expression of genes involved in nociceptive signaling or by inducing an inflammatory response.

### 1.6 The HER receptor family

The HER family of receptor tyrosine kinases consists of four receptors; EGFR (HER1/ErbB1), HER 2 (Neu/ErbB2), HER3 (ErbB3), and HER4 (ErbB4). These receptors are known to regulate cellular growth, survival, proliferation and differentiation of fibroblasts and hepatocytes, reviewed in (Citri and Yarden 2006). The HER receptor family has a variety of ligands that all are members of the EGF family. Ligands that can bind the extracellular domain of the HER receptors include; EGF itself, TGF, EREG, heparin-binding EGF-like growth factor, and amphiregulin (Harris et al., 2003).

The receptors are composed of a large extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain preceding the tyrosine kinase domain and a C-terminal domain with several tyrosine residues. When a receptor binds one of its ligands two receptors come together and form a 2:2 ligand to receptor configuration. This dimerization leads to conformational changes that activates the tyrosine kinase domain in the intracellular domain of the receptors (Boni-Schnetzler and Pilch 1987). When the tyrosine kinase domain is activated a self phosphorylation occurs on the tyrosine residues. These tyrosine residues serve
as a docking site for various adaptor proteins or enzymes that initiates signaling cascades to produce a physiological outcome. For a review see (Citri and Yarden 2006).

It has been suggested that HER receptors affects molecules that are known to participate in pain processing. Studies on embryonic kidney cells demonstrated that activation of EGFR by EGF induces internalization of δ-opioid receptors, and that the effect was attenuated by inhibition of EGFR tyrosine kinase activity (Chen et al., 2008). Earlier studies indicated that EGFR may also transactivate β-adrenoceptors in cancerous cell lines (Liu et al., 2008). In addition, EGFR activity may affect cannabinoid type 1 (CB1) and TRPV1 receptors in corneal epithelial cells (Yang et al., 2010).

1.6.1 HERs role in the nervous system

In the CNS HERs are localized in neurons, astrocytes and microglia. HER receptors are thought to play an important role in the development of the CNS. Earlier studies have shown that mice lacking EGFR often do not survive to birth. However, mice that do survive are characterized by CNS defects and extensive neurodegeneration in the frontal cortex, olfactory bulb and thalamus, leading to a loss of extensive parts of the brain (Sibilia et al., 1998).

The HER family is also a part of the peripheral nervous system. During development of the DRG several members are present and may be important for a normal maturity for DRG cell, like neurons and satellite glia (Reinhard et al., 2009). In adult DRG HER receptors is evident in small, medium and large DRG neurons, suggesting that the function of some nociceptive neurons may be modified by several growth factors (Pearson and Carroll 2004).

1.6.2 Activation of microglia and astrocytes

Microglia are macrophage-like cells and are capable of phagocytosis and migration. When microglia are activated they change morphology, motility, proliferation, expression of specific cell surface molecules and release of cytokines and chemokines. Being the primary defense system in the CNS, it is essential that microglia are extremely sensitive to pathological changes. Microglia may be activated by inflammatory factors like IFNγ, ATP, MMP-9, excitatory amino acids, PGs, IL-1β, and TNFα. Following activation, microglia synthesizes and release pro-inflammatory mediators like IL-1β and TNFα. These agents further activate microglia, creating a positive feedback-loop.
Recent studies have shown that activated microglia highly express phosphorylated EGFR. Moreover, blocking EGFR activity induce a decrease in microglia activation and production of IL-1β and TNFα (Qu et al., 2012). These findings indicate that HER receptors may be involved in microglia activation.

Astrocytes are supportive glia cell compartments in neural tissue. Astrocytes have the ability to directly alter neuronal communication because they completely encapsulate synapses (Sofroniew and Vinters 2010). A wide range of molecules trigger activation of astrocytes; large polypeptide growth factors, cytokines like IL6, TNFα, and IFNγ, neurotransmitters, ATP, and NO. Active astrocytes may also participate in regulation of CNS inflammation.

Data exist that activation of EGFR triggers quiescent astrocytes into becoming hypertrophic, reactive astrocytes (Liu et al., 2006). In addition, astrocytes show an up-regulation of EGFR expression when they become active in the glaucomatous optic nerve (Liu and Neufeld 2004). This enhanced expression of EGFR in reactive astrocytes suggests that the EGFR signaling may regulate post-injury activities of astrocytes.

It has been implicated that both astrocyte- and microglial activity play a part of development and maintenance of inflammatory and neuropathic pain models (Coyle 1998; Fu et al., 1999). Activated glial cells may contribute to neuropathic pain by various mechanisms. They secrete a wide range of inflammatory mediators, which may alter nociceptive signaling in the DH. Activation of astrocytes is accompanied by ERK activation and a subsequent down-regulation of excitatory amino acid transporters; such as glutamate transporter 1, and glutamate-aspartate transporter. These changes lead to a decrease in glutamate uptake and an increase in excitatory synaptic transmission (Tawfik et al., 2006). For a review, see (Milligan and Watkins 2009).
2 Aims

The purpose of this study was to generate new knowledge about the mechanisms underlying sensory deficits and the development of long-lasting pain following disc herniation. By using an animal model mimicking the clinical situation after an intervertebral disc herniation, the effect of EREG released from NP tissue was examined. Changes in the spinal nociceptive signaling due to application of EREG were examined by electrophysiological extracellular single cell recordings. Changes in gene expression of EREG and its receptors due to application of NP tissue onto the dorsal nerve roots were examined by qPCR.

Three subgoals were defined:

I) Investigate if NP tissue may secrete EREG when taken out from the intervertebral disc.

II) Examine the effect of administration of EREG onto the dorsal nerve roots on nociceptive signaling in spinal neurons.

III) Study the expression of EREG and its receptors in NP, DH and DRG in native tissues, sham operated animals and in tissues harvested 180min after NP application onto the dorsal nerve roots.
3 Materials and methods

3.1 Animal handling

The animal experiments were approved by the Norwegian Animal Research Authority and were performed in confirmation to the laws and regulations controlling experiments and procedures on live animals in Norway. Adult inbred female Lewis rats (Harlan Laboratories Inc., UK) weighing 190-250g were used in all experiments. Upon arrival, the animals were housed at the National Institute of Occupational Health Norway, with free access to food and water. The air temperature was kept at 20-22°C, the relative humidity was 45-55% and the ventilation rate was 15 x the room volume per hour. All experiments were performed during the light period of an artificial 14 hour light and 10 hour dark cycle.

3.1.1 Animal surgery

The animals were sedated with isoflurane gas (Baxter International Inc., USA) for 2min, and anesthetized with intraperitoneal administration of 250mg/mL urethane (~2g/kg bodyweight, Sigma-Aldrich Co., USA). To avoid a lethal overdose, urethane was administrated by several small injections. Absence of eye reflexes and foot withdrawal to pinch was considered an indication of sufficient surgical anesthesia. Simplex (80% Vaseline and 20% paraffin) was applied to both eyes to prevent them from drying. The core temperature of the animals was maintained at 36/37°C by a feedback heating pad (homeothermic blanket control unit, Harvard Apparatus Ltd. Kent, UK).

A 5-10mm wide laminectomy was performed on vertebrates Th13-L1, to expose the spinal cord segments L3-S1. Th13 and L1 were identified using the endpoint of the ribcage as a reference. To fix the vertebral column, clamps were placed rostral and caudal to the laminectomy (Eriksen et al., 2012). The spinal meninges were removed using a cannula. A section of 8-10mm of the sciatic nerve was dissected free at the mid-thigh level and isolated from the surrounding tissue by a plastic film. The incision was held open by a retractor.

The animals were euthanized and the NP tissue was collected from 3-6 caudal (tail) vertebrae. The DH of the spinal cord segments L3-S1 and the ipsilateral L3-L5 DRGs was harvested and frozen on liquid nitrogen.
3.2 Protein expression

NP tissue harvested from 6 tail vertebrae was pooled and immediately split into 4 equal parts which were transferred to separate eppendorf tubes containing 300µL Ham'sF12 medium (Thermo Scientific, USA). After 2-, 60-, 120- and 180min in an incubator at 37°C with 5% CO₂ the tissue was centrifuged at 4000rpm for 5min and the supernatant was transferred to a new eppendorf tube. Both the supernatant (the conditioned medium) and the pellet (the NP tissue) were stored at -80°C.

3.2.1 Chemiluminescence immunoassay (CLIA)

A chemiluminescence immunoassay (CLIA) kit (SCB945Ra, Cloud-Clone Corp. USA) was used to measure EREG in NP tissue culture supernatants. A standard with an EREG concentration of 1000pg/mL was used to make a threefold dilution series. The standard dilution series and the samples were added on to a microtiter plate pre-coated with an antibody specific to EREG. A biotin-conjugated antibody, also specific to EREG, was added to each sample. Next, the plate was incubated and washed to remove any proteins not bound to the antibodies coated on the plate. Moreover, Avidin conjugated to Horseradish Peroxidase (HRP), which binds biotin, was added, before the plate was incubated and again washed. Finally, a mixture of substrates generating glow light emission kinetics, by the aid of HRP, was added to each well. For the CLIA principal see figure 3.1. For protocol see appendix 1.
Figure 3.1 CLIA principles. An illustration of the molecular mechanisms underlying the detection of epiregulin (EREG) by chemiluminescence. This set up gives an intensity of the emitted light that is proportional to the EREG level in the sample or standard. HRP: horseradish peroxidase.

To determine the amount of EREG present in the samples, the chemiluminescence signal was measured with a microplate luminometer (Modulus, Turner Biosystems Inc, USA). The standard dilution series was used to make a standard curve, giving a chemiluminescence value for the known concentrations. Unconditioned HamsF12 medium was used as blank in this experiment. The relative light unit (RLU) value for the medium was subtracted from the RLU value for each NP sample. The amount of EREG present in the samples was calculated by the equation from the standard curve, shown in figure 3.2.
3.3 Electrophysiology

3.3.1 Electrophysiological extracellular single cell recordings

A parylene coated tungsten microelectrode with impedance 2-4MΩ (Frederick Hear & Co, USA) was lowered into the left spinal DH by a micromanipulator (MP-285, Sutter Instrument, USA), whereas a reference electrode was placed subcutaneously. The recorded signals was amplified by a headstage and an AC preamplifier, and filtered by a band pass filter (Digitimer Ltd, UK) with a half-amplitude cut-off of 500 – 1250Hz, corresponding to a wave length of 0.8-2ms. The signal was digitalized by a CED 1401µ interface and displayed on a computer by the software CED spike 2 (4.15, Cambridge Electronic design, UK.). Spinal cord segments L3-S1 were identified by light tapping on the left paw and nociceptive neurons were identified by pinching in the receptive field on the left paw. For experimental setup see figure 3.3.

A bipolar silver hook electrode (1.5mm distance between the hooks) was placed proximal to the main branches of the sciatic nerve for electrical stimulation. A pulse buffer connected to a stimulus isolator unit (NeuroLog System, Digitimer Ltd, UK) controlled the stimulus intensities. The C-fiber threshold was defined at the beginning of each experiment as the lowest stimulus intensity necessary to evoke a C-fiber response. Every 4th minute throughout the experiment, a single test stimuli (2ms rectangular pulse, 1.5 x C-fiber threshold) was applied to the sciatic nerve. Action potentials 0-50ms after each test stimulus were defined as A-fiber response, whereas action potentials 50-300ms after each test stimulus were defined as the C-fiber response (figure 3.3). The spontaneous activity was defined as action potentials fired between 300ms after a test stimulus and until the next test stimulus. The C-fiber response on each test stimulus, and the spontaneous activity, was quantified by counting the number of spikes. Six stable C-fiber responses, with variance less than 20%, served as a baseline for the subsequent experiments. Only cells with a baseline C-fiber response between 5-20 spikes were included. To ensure single cell recordings, shape and amplitude of the spike were assessed to discriminate between signals from different cells.
Recombinant EREG produced in E-Coli (Cloud-Clone Corp. USA) was dissolved in 0.9% NaCl, and diluted to a concentration of 50µg/mL. The solution of EREG was then stored at -80°C until use. One dose of 50µL EREG was administrated on the dorsal nerve roots in each experiment. The signal was recorded for 180min after EREG administration (n=6). Sham operated rats i.e. no conditioning served as control (n=6).
3.4 Investigation of gene expression

![Figure 3.4 Overview of the procedural steps between tissue harvesting and gene expression analysis.](image)

Total ribonucleic acid (RNA) was isolated from tissue and tested. The RNA samples were then converted to complementary deoxyribonucleic acid (cDNA), and the expression level was examined by quantitative polymerase chain reaction (qPCR).

3.4.1 Gene expression in NP

NP tissue was harvested from 3-6 tail vertebrae and three series of experiments were performed: I) native II) control and III) 3 hours. In the native group, NP was frozen directly after harvesting. In the control and 3 hours group, NP tissue was harvested from a genetically identical donor rat and then bisected. One piece was applied onto fat tissue for 180min (control), and the other piece was applied onto the dorsal nerve roots for 180min (3 hours). Following the experiments, the NP tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until later use.

3.4.2 Gene expression in the DH

The animals were euthanized and about 10mm of the spinal cord was harvested from the laminectomy site (Th13-L1). Then the ipsilateral DH (left) were rapidly isolated and frozen in liquid nitrogen. Like above, three series of DH experiments were performed: I) native II) control and III) 3 hours. In the native group, the tissue was harvested immediately after the laminectomy was performed. In the control group the DH was harvested 180min after laminectomy. In the 3 hours group, NP tissue was applied onto the left dorsal nerve roots. After 180min the NP tissue was removed and the DH was harvested.
3.4.3 Gene expression in DRG

Three series of experiments were performed: I) native, II) control and III) 3 hours. As for the DH tissue samples described above, the native DRG samples and the control DRG samples were harvested immediately- and 180min after laminectomy, respectively. In the 3 hours group, NP tissue was applied on the left dorsal nerve roots and DRG harvested after 180min. In all groups only DRGs from the left side were isolated. Samples from L3, L4 and L5 were pooled together before gene expression analysis.

3.4.4 RNA isolation

Isol-RNA Lysis Reagent (5PRIME) was added to each sample, and the tissue was homogenized by the aid of a mixer mill and three sterile metal balls (Retsch MM 301, Germany). After incubation and centrifugation the supernatant was collected, thereby leaving the non-soluble cell material behind. Chloroform was added to separate the solution into three different phases containing ribonucleic acid (RNA), deoxyribonucleic acid (DNA), proteins and lipids. The upper water phase containing RNA was transferred to a new eppendorf tube, isopropanol was added and the samples were centrifuged. The resulting RNA pellet was washed with 75% ethanol, dried and re-dissolved in ribonuclease (RNase) free water (Pedersen et al., 2010). For protocol see appendix 2.

RNA-concentrations were quantified using a NanoDrop 8000 Spectrophotometer (NanoDrop 8000 v2.2.1, Thermo Scientific, USA) and then diluted with RNase free water to a unifying concentration of 0.25μg/μL.

RNA quality was evaluated with on-chip electrophoresis using Aligent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Germany). Three and three samples of total RNA were mixed before testing. RNA was detected using florescence as the bioanalyzer separated the two ribosomal subunits S18 and S28. An electropherogram was generated and a RNA integrity number (RIN) value was calculated using an algorithm from Aglient Technologies. Two clear peaks, corresponding to the two ribosomal subunits (S18 and S28) indicate RNA of high quality (figure 3.5). A RIN-value above 7 was defined as acceptable for the gene expression analysis. For protocol see appendix 3.
3.4.5 cDNA synthesis

Reverse transcription of mRNA to complementary DNA (cDNA) was carried out using qScript cDNA synthesis Kit (Quanta Biosciences Inc., USA). A reaction mix containing a random primer, deoxynucleotides and 1µg of total RNA was made, and then MMLV reverse transcriptase and RNase inhibitor was added to convert the mRNA to cDNA. The reaction was performed by a Mastercycler nexus (Eppendorf, USA) following a program of 22°C for 2min, 42°C for 30min and 85°C for 5min. After ended cycles the samples were held at 4°C. The cDNA was diluted in tris ethylenediaminetetraacetic acid (TE)-buffer to a concentration of 10ng/µL and stored at -80°C. For protocol see appendix 4.

3.4.6 qPCR

The gene expression was analyzed by quantitative polymerase chain reaction (qPCR). Primers for the target genes were designed using Primer Express 3.0.1 (Applied Biosystems, USA). The primers were designed to span introns in the genomic DNA, to ensure specificity for the desirable mRNA. The software allowed us to exclude the primers with a high number of internal complementation. In addition, a basic local alignment search tool (BLAST) search was performed to check for identical sequences in other genes. β-actin (coding for actin protein that is a part of the cytoskeleton and levels of expression is therefore relatively constant) was used as a reference gene. The primers were delivered by Thermo Scientific (Germany). For primer sequences, see table 2.1
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<th>Tm(°C)</th>
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<td>59</td>
</tr>
<tr>
<td>EREG Reverse</td>
<td>TCA CAC CGC AGA CCA GTG TAG</td>
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<td>58</td>
</tr>
<tr>
<td>EGFR Forward</td>
<td>GGC CCA GAG AGA GTG ACT GTC T</td>
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<td>59</td>
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<tr>
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<tr>
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<td>β-Actin Reverse</td>
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Table 2.1 Rat specific primers used for qPCR. A: Adenosine, C: Cytosine, G: Guanine, T: Thymine, Bp: base pairs, Tm: melting temperature.

A qPCR reaction was set up with cDNA and a master mix with SYBR green dye, ROX, dNTP, Taq polymerase and primers. SYBR green dye, that emits fluorescence at 520nm when incorporated in a double stranded DNA molecule, was used to quantify the amount of PCR product for each cycle. The ROX dye was used as a passive reference signal to correct for differences in sample volume between the wells, normalizing the SYBR green dye. The normalized fluorescence in each well was plotted against the number of cycles performed to make an amplification plot (figure 3.6 A).

A fourfold dilution series was made, with a mix from four samples as a start concentration, making a standard curve (figure 3.6 B). The cDNA samples and the master mix were loaded into wells and the qPCR plate was sealed with a plastic film (Eriksen et al., 2012). For protocol see appendix 5.
The qPCR reaction was performed by a StepOnePlus qPCR machine (Applied Biosystems, USA), which was set up with the following schedule: 90°C for two min followed by 40 cycles at 95°C for 10s and 60°C for 30s. When the program was completed, a melting curve (figure 3.7) was established in a step-and-hold fashion up to 95°C. Using the standard curve the software, StepOne v2.3, defined a threshold value of fluorescence. At which cycle each sample reached this threshold determined the threshold cycle (Ct) value of the sample.

**Figure 3.6 qPCR on native tissue.** A) Amplification plot of a fourfold dilution series on β-actin run. The threshold-value is determined by the software, StepOne v2.3. At which cycle each sample reaches the threshold defines the cycle threshold (Ct) -value. A fourfold dilution series gives amplification plots sequentially two cycler apart. B) Example of standard curve from a dilution series on β-actin. A known Ct-value will give a quantity of cDNA in a sample.
3.5 Statistics

All statistical analyses were performed in SPSS 23 (IBM SPSS inc. USA), and all graphs created in Sigma plot 12.5 (Systat software Inc. USA). Data are given by examples and by means ± standard error of the mean (SEM). A p-value below 0.05 was considered significant.

3.5.1 Electrophysiology

The C-fiber response was recorded before and until 180min after conditioning. Six stable recordings were set as baseline, and defined as 100%, all other measurements throughout the experiment were given as percentage of baseline. Pre-condition recordings were converted to two baseline values, and the post-conditioning recordings were converted to 15 values (each comprising 3 recordings).

The effect of EREG on spinal nociceptive response was compared to the control group. The average response in baseline was subtracted from the average response between 0-180min. The effect of EREG on responsiveness to electrical stimuli in nociceptive neurons was analyzed by a two-tailed unpaired Student’s t-test.
Spontaneous activity was calculated as number of spikes without any mechanic or electronic stimulus. All spikes were counted between every electrical stimuli, and the average of 5s was calculated. The average of the baseline values was subtracted from the mean of all measurements between 0-180min. The effect of EREG on spontaneous activity in nociceptive neurons was analyzed by a two-tailed unpaired Student’s $t$-test.

### 3.5.2 Gene expression

For gene expression analysis, relative quantities were given by comparing the Ct-value with the standard curve. Fold change values were defined by the quantity mean for each sample divided by the quantity of the mean reference gene $\beta$-actin. All values were normalized to the mean of the native group. The effect of NP tissue in contact with the dorsal nerve roots were analyzed by comparing groups means by a one-way ANOVA analysis, with a Tukey’s post hoc test.
4 Results

4.1 Protein expression

Pro-inflammatory agents released from NP tissue following a disc herniation may alter neuronal excitability. To examine this process, NP tissue was harvested and incubated in cell medium.

The protein analysis (CLIA) performed on medium conditioned with NP tissue showed that EREG was released from NP tissue in a time-dependent fashion. The concentration of EREG in the conditioned medium was highest at 2min. Then a drop was observed within the first hour, followed by a small increase in EREG over the next two hours (see figure 4.1).
Figure 4.1 Protein expression of epiregulin (EREG). Illustration of experimental setup for the protein analysis. Nucleus pulposus (NP) was harvested from rats and incubated in cell medium. The samples were centrifuged and the protein secreted was detected with chemiluminescence immunoassay (CLIA) and a luminometer. Secretion of EREG from NP tissue removed from the disc (in medium).
4.2 Electrophysiology

Only WDR neurons were included in this study. The C-fiber response (number of spikes) was defined according to the latencies. EREG was administrated onto the dorsal nerve roots and the C-fiber response, i.e. the electrically evoked nociceptive response, was examined. In the control group the C-fiber response was stable throughout the whole experiment of 180min. In contrast, the C-fiber response decreased to 30-40% of baseline, 180min after 50µL EREG (50µg/mL) was applied onto the dorsal nerve roots (figure 4.2).

To examine the effect of EREG on spontaneous activity in the same nociceptive neurons, the number of spikes between each electrical stimulus, i.e. the spontaneous activity, was calculated. The control group was stable at ~3 spikes per 5s in baseline and for 180min after vehicle (0.9% NaCl). In contrast, 50µL EREG (50µg/mL) increased the neuronal activity from an average of ~3 spikes per 5s in baseline to ~35 spikes per 5s 180min after EREG administration (figure 4.3).

Although a decrease in responsiveness in nociceptive neurons to electrical stimuli was observed, EREG induced a pronounced increase in spontaneous activity. The C-fiber response decreased with 7.08 ± 2.61 spikes per 300ms (p=0.031, Student’s t-test), whereas the spontaneous activity increased with 21.06 ± 8.14 spikes per 5s (p=0.048, Student’s t-test) see figure 4.4.
Figure 4.2 Electrically evoked C-fiber responses. A) Example of single cell recordings at baseline and 180min after administration of epiregulin (EREG). B) C-fiber response in percent of baseline after administered EREG or vehicle (veh). The response decreased to 30-40 % of baseline 180min after EREG was administered.
Figure 4.3 Spontaneous activity in nociceptive neurons. A) The spontaneous activity in nociceptive neurons was stable for 180 min in the control group. B) The spontaneous activity in nociceptive cells increased after application of epiregulin (EREG) onto the dorsal nerve roots. Veh: vehicle.
Figure 4.4 The neuronal activity after administration of vehicle or epiregulin (EREG) (average 0-180 min) relative to baseline. A) The number of spikes per electrical stimulus decreased after EREG was administrated compared to the control group where no changes was observed. B) After EREG was administrated the spontaneous activity in the nociceptive neurons increased. *P<0.05, **P<0.01, ***P<0.001, Student’s t-test.
4.3 Gene expression

To study the role of EREG, in NP, DH and DRG tissues the gene expression was analyzed by qPCR.

The Ct-values were used to estimate the expression of the genes of interest in native tissues. A lower Ct-value was considered as a higher expression of the gene (table 4.1).

<table>
<thead>
<tr>
<th></th>
<th>EREG</th>
<th>EGFR</th>
<th>HER 2</th>
<th>HER 3</th>
<th>HER 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus pulposus</td>
<td>28.18 ± 0.54</td>
<td>23.36 ± 0.26</td>
<td>24.57 ± 0.26</td>
<td>27.89 ± 0.60</td>
<td>32.98 ± 0.39</td>
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<td>Dorsal horn</td>
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<td>27.81 ± 0.52</td>
<td>25.51 ± 0.23</td>
<td>19.70 ± 0.38</td>
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<tr>
<td>Dorsal root ganglion</td>
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<td>24.94 ± 0.13</td>
<td>21.28 ± 0.42</td>
<td>23.31 ± 0.26</td>
<td></td>
</tr>
</tbody>
</table>

n=6-7

Table 4.1 Ct-values. Ct-values in native tissue, indicating the level of expression.

4.3.1 Changes in gene expression of EREG and its receptors

The ligand

A nonsignificant increase in expression of EREG was observed in NP tissue exposed to the dorsal nerve roots for 180min (fold expression 3.09 ± 1.05). Application of NP tissue onto the dorsal nerve roots however induced a significant up-regulation of EREG in the DH (fold expression 26.03 ± 5.82). No significant changes in EREG gene expression were found in DRG. See figure 4.5.
The receptors

NP tissue: NP tissue in contact with the dorsal nerve roots for 180min induced a significant up-regulation of EGFR and HER4 in NP tissue (fold expression: EGFR; 7.04 ± 2.53, HER4; 4.90 ± 0.98) see figure 4.6 and 4.9.

DH tissue: We also observed a significant up-regulation of EGFR and HER4 in the DH 180min after NP was applied onto the dorsal nerve roots (fold expression EGFR; 2.07 ± 0.44, HER4; 2.27 ± 0.27) see figure 4.6 and 4.9.

DRG tissue: In the DRG tissue we found a significant up-regulation of HER3 (fold expression 4.84 ± 1.25) see figure 4.8. No significant changes in gene expression were found for HER2, see figure 4.7.
Figure 4.5 Gene expression (qPCR) of epiregulin (EREG). Fold expression (normalized to mean of native) of EREG in nucleus pulposus (NP), dorsal horn (DH) and dorsal root ganglion (DRG) in the three groups: native tissue (native), NP in contact with fat tissue for 180min (control) and NP in contact with the dorsal nerve roots for 180min (3 hours). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, Tukey’s post hoc test.
**Figure 4.6 Gene expression (qPCR) of EREG receptor epidermal growth factor receptor (EGFR).** Fold expression (normalized to mean of native) of EGFR in nucleus pulposus (NP), dorsal horn (DH) and dorsal root ganglion (DRG) in the three groups; native tissue (native), NP in contact with fat tissue for 180min (control) and NP in contact with the dorsal nerve roots for 180min (3 hours). NP in contact with the dorsal nerve roots induced a $7.04 \pm 2.52$ fold up-regulation of EGFR in NP, in addition it induced a $2.07 \pm 0.44$ fold up-regulation of EGFR in the DH. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, one-way ANOVA, Tukey’s post hoc test.
Figure 4.7 Gene expression (qPCR) of EREG receptor human epidermal growth factor receptor 2 (HER2). Fold expression (normalized to mean of native) of EGFR in nucleus pulposus (NP), dorsal horn (DH) and dorsal root ganglion (DRG) in the three groups; native tissue (native), NP in contact with fat tissue for 180min (control) and NP in contact with the dorsal nerve roots for 180min (3 hours). No significant changes was found in the gene expression of HER2.*P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, Tukey’s post hoc test.
Figure 4.8 Gene expression (qPCR) of EREG receptor  human epidermal growth factor receptor (HER3). Fold expression (normalized to mean of native) of HER3 in nucleus pulposus (NP), dorsal horn (DH) and dorsal root ganglion (DRG) in the three groups; native tissue (native), NP in contact with fat tissue for 180min (control) and NP in contact with the dorsal nerve roots for 180min (3 hours). Application of NP tissue onto the dorsal nerve roots for 180min induced an significant up-regulation of the EREG receptor HER3 in DRG tissue. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, Tukey’s post hoc test.
Figure 4.9 Gene expression (qPCR) of EREG receptor human epidermal growth factor receptor (HER4). Fold expression (normalized to mean of native) of HER4 in nucleus pulposus (NP), dorsal horn (DH) and dorsal root ganglion (DRG) in the three groups; native tissue (native), NP in contact with fat tissue for 180 min (control) and NP in contact with the dorsal nerve roots for 180 min (3 hours). NP tissue in contact with the dorsal nerve roots induced a significant up-regulation of HER4 in both NP tissue and the dorsal horn. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA, Tukey’s post hoc test.
5 Discussion of methods

5.1 Animal experiments

In the present study we used an animal model mimicking the clinical situation after disc herniation.

5.1.1 Rat model

Lewis rats are inbred for 20 generations, and are used in studies were genetically identical animals are required, such as transplantation research. Additionally, Lewis rats are known to have a more pronounced inflammatory response, than other rat strains (Popovich et al., 1997), and are frequently used to study inflammation. EREG promote development of inflammation (Harada et al., 2015), therefore Lewis rats were used in the present study.

Female rats were selected in preference to male rats due to the lower risk for the researcher to develop allergies (Renstrom et al., 2001). However, previous studies indicate that pain threshold may vary in female rats because of the estrous cycle (Fillingim and Ness 2000). To avoid interference of the estrous cycle the experiments was done in a randomized order. Individual differences regarding the estrous cycle were therefore assumed not to influence the results at group level.

5.1.2 Anesthesia

Urethane (ethyl carbamate NH2COOCH2CH3) is commonly used as a rodent anesthetic. It produces a long-lasting and steady surgical anesthesia that may last for as long as 24 hours (Field et al., 1993) with minimal effects on cardio-respiratory functions. Previous studies have shown that urethane, compared to other anesthetics, only has a modest effect on multiple neurotransmitter-gated ion channels (Hara and Harris 2002). However, in higher doses than required for surgical anesthesia, urethane may affect several ion channels. To get a sufficient surgical anesthesia and at the same time avoid unwanted effect on synaptic transmission and cardio-respiratory functions several small doses were applied. The maximum dose used in the present study was, as in previous studies, 2g/kg bodyweight (Egeland et al., 2013; Gjerstad et al., 2005; Pedersen et al., 2005).
5.2 Protein analysis

To investigate the ability of NP tissue to release EREG NP tissue was harvested from tail vertebra from genetically identical rats. Harvesting NP tissue from the tail is easy thereby shortening the time from the animal is euthanized to the tissue is harvested.

The NP samples were incubated in HamsF12 medium in an incubator with 37°C, 5% CO2 and the relative humidity was 95%. We used HamsF12 medium to keep the cells in a suitable environment for them to survive. HamsF12 medium was chosen because it is designed to cultivate a variety of cells and contains a mixture of nutrients that are essential for cell survival.

In the present study, a CLIA was used to investigate if NP tissue may release EREG. Other methods that may be used are enzyme-linked immunosorbent assay (ELISA), Western blot and nanodrop. These all have their advantages. However, using Western blot to investigate the concentration is not favorable, as this method only gives an idea of the concentration. Nanodrop is non-specific for different proteins and only measures of total protein in the sample. ELISA uses the same principals as CLIA, but CLIA uses chemiluminescent for detection whereas ELISA uses change in color.

We choose to use CLIA because immunoassays with luminescent provides low detection limits, and high specificity (Wang et al., 2012). In addition, CLIA and the standard curve with known concentrations provide a quantitative measure of concentration.

By using CLIA to investigate proteins in tissue culture supernatants some errors may occur. The cell medium often has a phenol red color whereas the standard solution is blank. This may give a misleading result since the color may interfere with the luminescent. To correct for this the RLU value from untreated cell medium was subtracted from each NP sample, removing the possibility of the background coloring to interfere with the results.
5.3 Electrophysiological extracellular single cell recordings.

In this study in vivo extracellular single cell recording was chosen to investigate spinal activity. We choose to use extracellular single cell recording because it provides an insight into how small proteins, such as EREG may effect a complex physiological system, such as nociceptive signaling. However, other electrophysiological techniques, such as in vitro patch-clamp (Ikeda et al., 2003) and in vivo field potential recordings (Jacobsen et al., 2010) have also previously been used to study the nociceptive activity in the DH.

Patch-clamp is performed on slices of neuron tissue or cultured cells. This provides better control of the extracellular environment, as well as the intracellular milieu. Other advantages compared to in vivo electrophysiological recording, is that the path-clamp method provides more stable long-term recordings. However, in vitro recordings lack the ability to examine neurons under descending control. In vivo studies provide a unique insight into complex biological systems, for example how immune responses and neuronal excitability interfere. Therefore an in vivo technique was chosen in this study to investigate spinal excitability.

In vivo extracellular recordings can be performed by either field potentials (Jacobsen et al., 2010), or single cell recordings (Pedersen et al., 2010). In the present study single cell recordings were preferred to allow observations of responsiveness to stimulation, post-discharge and spontaneous activity in nociceptive neurons. Single cell recordings were ensured by looking at the shape and the amplitude of the spikes. However, one cannot be absolutely sure that the recordings are of one cell, since spikes form two different cells may have similar shape and amplitude.

The cells were identified by pinching the receptive field. This may also activate other sensory nerve fibers in the dorsal horn, like touch and proprioceptive neurons. Thus the recordings of nociceptive neurons was based on the response to pinch and a clear C-fiber response. The C-fiber response was defined by the latency.
5.3.1 Administration of recombinant epiregulin.

During the electrophysiological experiment recombinant EREG was administrated onto the dorsal nerve roots. Recombinant proteins are produced by E.coli or other bacteria, which are transfected with viruses or plasmids containing the exact gene sequence for the desirable protein. The recombinant protein was dissolved in 0.9% NaCl and saline was therefore used in the control experiments.

5.4 Gene expression analysis

5.4.1 Application of nucleus pulposus onto the dorsal nerve roots

In the present study NP was applied onto the dorsal nerve roots to mimic the clinical situation after disc herniation. NP tissue was harvested from tail vertebrae from a genetically identical donor rat. Ideally, NP should be harvested from the same rat. However, since the rats are genetically identical, the observed changes in gene expression cannot be explained by a non-self immune response.

For the gene expression analysis sham operated animals were used as controls. One may argue that contralateral side could be used as controls instead. However, NP is a gel-like tissue and application onto only the dorsal nerve roots on the ipsilateral side would be difficult to achieve. In addition, NP tissue has the ability to release cytokines and other inflammatory agents. These secreted agents may be able to diffuse to the contralateral side and alter gene expression in the DH and the DRGs, giving a misleading result. Hence, sham operated animals, and application of NP onto fat tissue was preferred as a control.

5.4.2 Changes in gene expression on mRNA level.

To investigate changes in gene expression, qPCR was performed on cDNA made from isolated total RNA. It is important to note that changes in mRNA expression only suggest that there is a change in protein levels, as translation of mRNA into protein may be independently regulated.

Alternative methods to qPCR, like Northern blotting, in situ hybridization and RNA protecting assay, may be used to investigate changes in gene expression on mRNA level.
However, the main limitations of these methods are their low sensitivity, and the need for a big sample of RNA. qPCR on the other hand have a high sensitivity and only need small amount of RNA. In addition, qPCR is sequence specific, as the primers are designed for the genes of interest.

When using an unspecific fluorescence reporter, in this case SYBR green, the major limitation of this method is assumed to rise from human errors. One possible source of error would be poorly designed primers. Our primers were designed to span an exon–exon boundary to avoid amplification of genomic DNA. Moreover, a BLAST search was performed to assure that the primer sequence only was found in the target gene. To test this, the melting curve from the qPCR run was investigated. The presence of more than one peak would indicate off-target binding of the primers.

In the present study, β-actin was used as a reference gene, to correct for sample to sample variations, like differences in the amount of tissue, degradation of mRNA and cDNA and variations in efficiency of cDNA synthesis. In general the expression of the reference gene should not be influenced by the experimental procedure. Previous studies have shown small variations in the expression of β-actin following different experimental setups both in the DH and NP tissue (Egeland et al., 2013; Pedersen et al., 2010).
6 Discussion of results

6.1 Protein analysis

In the present study we demonstrated that EREG are secreted from cells found in NP tissue when taken out from its immune privileged environment. The protein analysis suggested a higher presence of EREG two minutes after harvesting, than 1-3 hours later.

When harvesting tissue, some cells may be damaged. Since EREG is released by cleavage from the membrane bound form, called proepiregulin, tearing of the cell membrane may result in release of proepiregulin. The antibodies detecting EREG in the protein analysis bind both EREG and proepiregulin. This may be one explanation of the high concentration of EREG observed at 2min. Moreover, binding of EREG to its receptors leads to internalization of the ligand-receptor complex resulting in lysosomal degradation, reviewed in (Citri and Yarden 2006). Thus the drop in concentration may also be related to lysosomal degradation.

Earlier studies have shown that EREG may be involved in the development of inflammatory responses. Hence, EREG released from NP tissue may induce inflammation and possibly enhanced nociceptive signaling. During inflammation circulating macrophages are also recruited. Previous studies indicate that EREG is physiologically critical for proper production of cytokines by macrophages (Shirasawa et al., 2004). EREG induces release of cytokines and other growth factors, which further enhance the inflammation (Harada et al., 2015; Murakami et al., 2013). Recruitment of circulating macrophages and release of pro-inflammatory agents may promote nociceptive signaling in primary afferent sensory neurons.

Microglia and astrocytes are supportive cells in the spinal cord, and may alter neuronal signaling once they become active (Hains and Waxman 2006). Previous studies have shown that EGFR signaling may lead to activation of microglia and astrocytes, thus enhancing their production of IL-1β and TNFα (Liu et al., 2006; Qu et al., 2012). Therefore it is possible that EREG released from NP may activate microglia and astrocytes and thereby alter nociceptive signaling.
6.2 Electrophysiological recordings

Previous studies have shown that application of NP onto the dorsal nerve roots affect excitability of DH neurons (Egeland et al., 2013). Moreover, NP in contact with DRG may induce spontaneous neural activity (Takebayashi et al., 2001).

In the present study we administrated EREG directly onto the dorsal nerve roots. The electrophysiological recordings showed that EREG induced a decreased C-fiber response, but a pronounced increase in spontaneously fired action potentials in nociceptive neurons. The decrease in C-fiber response and increase in spontaneous activity remained for more than 180min, indicating that EREG may induce long-term effects on nociceptive signaling. Our findings demonstrated that EREG have a dual effect on nociceptive neurons. Hence, EREG leaking from the herniated disc may be involved in sensory deficits and persistent pain often seen in patients with long-lasting pain following disc herniation.

Earlier studies indicate that NP tissue in contact with the dorsal nerve roots induces a significant reduction in nerve conduction velocity (Kayama et al., 1998; Olmarker et al., 1993). Moreover, epidural application of NP tissue may result in rapid increase in vascular permeability in spinal nerves (Byrod et al., 2000). These physiological changes may result in abnormal neural signaling and underlying sensory deficits. Decrease in nerve conduction may be an explanation for the reduced C-fiber response observed in the present study.

Moreover, pathophysiological changes observed after application of NP onto the dorsal nerve roots are thought to be caused by proteins on the surface of NP cells. Since NP tissue in intact discs have never been exposed to immune cells, these cells may be immunogenic. Hence, herniation may involve activation of a local immune response since NP tissue may be recognized as non-self (Kayama et al., 1998). Taken together, NP may have neurotoxic properties. Our findings suggest that EREG secreted from NP may be involved in the neurotoxic action induced by the herniated disc on nociceptive neurons.

Recent clinical studies on patient with neuropathic pain indicate that inhibition of EGFR may reduce neuropathic pain (Kersten et al., 2015). These finding support our observations that EREG promote spontaneous activity in nociceptive neurons.
Today very little is known about how EREG alters neuronal excitability. However, several studies indicate that downstream signaling molecules from EREG, like PI3K and ERK, are able to alter nociceptive signaling. Earlier studies performed on DRG neurons in rats suggests that PI3K, may sensitize primary afferent neurons by up-regulation of TRPV1, thus resulting in peripheral sensitization (Fang et al., 2015). Moreover, earlier studies have shown that signaling trough PI3K and ERK mediate inflammatory hyperalgesia in primary afferent neurons. In addition, inhibition of ERK eliminates the hypersensitivity seen following activation of PI3K (Zhuang et al., 2004). PI3K may also be involved in maintenance of spinal cord LTP. Previous studies have shown that inhibition PI3K activation reduced phosphorylation and activation of the NMDA receptor and decreased translocation of AMPA receptors to the plasma membrane in spinal neurons (Pezet et al., 2008).

Intracellular signaling following activation of the HER receptors may lead to activation of several transcription factors. CREB is a transcriptional factor that is closely linked to LTP, and can be activated by HER signaling. CREB alter gene expression by binding to the cAMP response element (CRE) promoter, which are located on so-called “pain-genes”, like NK1 receptor and BDNF (Hershey et al., 1991; Song et al., 2005). Hence, it is tempting to speculate that release of EREG from the herniated disc may activate various intracellular molecules and thereby alter gene expression of proteins that enhance neuronal excitability.

6.3 Gene expression analysis

In the present study we demonstrated a significant up-regulation of EREG in DH tissue, following application of NP tissue onto the dorsal nerve roots. This increased expression of EREG and the increased excitability in nociceptive neurons suggests that EREG may be involved in pathophysiology following a disc herniation.

EREG-HER signaling is important for cell differentiation, proliferation and growth. In the CNS HER receptors are expressed in neurons, astrocytes and microglia. Here we showed that application of NP tissue induces an up-regulation of EGFR and HER4 in NP itself as well as DH tissue. The up-regulation of these receptors indicates additional EREG-HER signaling, which may induce hyperexcitability in nociceptive neurons following disc herniation.
The up-regulation of EGFR and HER 4 in NP tissue indicates a positive feedback loop between HER activation and EREG expression. It is tempting to speculate that this self-supporting effect drives an inflammatory response forward, resulting in extensive release of pro-inflammatory agents like IL-1β and TNFα.

EREG that is released from NP tissue may bind receptors on neighboring astrocytes and microglia and thereby activate them. In the DH, the up-regulation of EGFR and HER4 may be due to heightened expression of these receptors in astrocytes and microglia. There is also a possibility that EREG can bind receptors localized on neurons and effect neuronal excitability directly.

Application of NP onto the dorsal nerve roots also induced an up-regulation of HER3 in DRG tissue. In accordance with our findings, previous studies on experimental spinal cord injury in rats have demonstrated that HER3 are up-regulated in DRG neurons (Mizobuchi et al., 2013). HER3 may be activated by both NRG and EREG, and signaling through NRG is important for remyelation and axonal regeneration in DRGs following nerve injury (Fricker et al., 2011). These findings suggest that HER3 may be important for regeneration of neurons and remyelation after injury or disc herniation.

In conclusion our finding support the hypothesis that EREG and signaling through HER receptors are involved in the physiological changes leading to sensory deficits and pain often seen following disc herniation.

6.4 Future perspectives

In this study we demonstrated that EREG have the ability to alter nociceptive signaling. However, through which mechanisms EREG affects neurons remains to be investigated.

It would be interesting to examine which signaling pathways that are activated in spinal- and/or DRG neurons following administration of EREG. Moreover, one could study whether the effect of EREG on neuronal excitability is due to a local inflammation or by a direct alternation of nociceptive neurons. In addition, it would be interesting to see if EREG can alter expression of genes that are directly linked to neuronal excitability, like neurotransmitters and ligand-gated ion channels.
7 Conclusions

I) Our data showed that NP taken out of its natural environment may release EREG. EREG may affect nearby cells by binding to its receptors.

II) Administration of EREG onto the dorsal nerve roots induced a decrease in C-fiber response. A loss in responsiveness to electrical stimuli applied onto the sciatic nerve was observed. Our findings suggest that EREG may be involved in sensory deficits in patients following a disc herniation.

III) Application of EREG onto the dorsal nerve roots induced a pronounced increase in spontaneous activity in nociceptive neurons in the dorsal horn. The increased excitability in nociceptive neurons lasted throughout the experiment of 180min. Hence, EREG may be important for the long-lasting pain often seen in patients following a disc herniation.

IV) NP tissue exposed to the dorsal nerve roots for 180min induced a significant up-regulation of EREG in the DH. This up-regulation suggests that NP tissue has a pro-inflammatory effect and may activate microglia and astrocytes in the spinal cord, in addition to recruiting circulating macrophages.

V) We demonstrated an up-regulation of EREG receptors in NP tissue, DH tissue and DRG tissue. These findings suggest that NP tissue enhance EREG signaling and may thereby strengthen pathophysiological changes following a disc herniation.
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Appendices

Appendix 1 Procedure for protein analysis using Chemiluminescence immunoassay (SCB945Ra, Cloud-Clone Corp. USA)

**Reagent preparation:**

1. All kit components were kept at room temperature.
2. The Standard solution was reconstituted with 1.0mL of Standard Diluent, to get a start concentration of 2000pg/mL. The solution was incubated for 10 min at room temperature and shaken gently.
3. 300μl of standard solution was diluted with 300μl standard Diluent to get a stock concentration of 1000pg/mL. The standard dilution series was prepared as described in the table below.

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<thead>
<tr>
<th>Dilution series</th>
<th>Standard</th>
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</tr>
</thead>
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<td></td>
<td>4.12pg/mL</td>
</tr>
<tr>
<td>6</td>
<td>300μL from nr 5 + 600μL</td>
<td></td>
<td>1.37pg/mL</td>
</tr>
<tr>
<td>7</td>
<td>Blank</td>
<td></td>
<td>0.00pg/mL</td>
</tr>
</tbody>
</table>

4. The stock Detection A and Detection B was spun down.
   120μl of Detection reagent A was mixed with 11.9mL of Assay Diluent A. 120μl Detection reagent B was mixed with 11.9mL of Assay Diluent B.
5. 300mL solution was prepared by diluting 10mL of the wash solution with 290mL distilled water.
6. The substrate working solution was prepared 15 min before assay by mixing 9900μl Substrat A with 100μl Substrat B.
Assay preparation:

1. 100μL of each dilution of standard, blank and samples was added to each well, and the plate was covered with a plate sealer and incubated for 2 hours at 37°C.
2. All liquid was removed from each well.
3. 100μl of the detection reagent A working solution was added to each well, and the plate was covered and incubated for 1 hour at 37°C.
4. All liquid was removed and the plate was washed by adding 50μl of washing solution to each well. The washing solution was removed completely by tapping the plate on an absorbent paper. The wash routine was repeated 3 times.
5. 100μl of Detection Reagent B working solution was added to each well and the plate was incubated for 30 min at 37°C.
6. The plate was then washed five times as described in step 4.
7. 100μl of Substrate working Solution was added to each well. The plate was covered with a plate sealer and incubated for 10 min at 37°C, and protected from light.
8. The chemiluminescence signal was measured.
Appendix 2 Procedure for RNA isolation

1. The tissue sample 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 3 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 4 Procedure for cDNA synthesis using “qScript cDNA synthesis kit” (Quanta Biosciences Inc., USA)

All reagents were thawed and kept on ice.

1. The RNA samples were diluted to get 1μg RNA in 15μL nuclease-free water in a 0.5 ml Eppendorf tubes.

2. 4μL qScript reaction mix (5X) and 1μL qScript reverse transcriptase was added to each sample.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>volume/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (1μg)</td>
<td>variable</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>variable</td>
</tr>
<tr>
<td>qScript reaction mix</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>qScript reverse transcriptase</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Total</td>
<td>20.0μL</td>
</tr>
</tbody>
</table>

3. The samples were vortexed and spun down for 10s.

4. The reverse transcription reaction was run on the PCR machine at the following program: 22 °C for 5min, 42 °C for 30 min and 85 °C for 5 min and held at 4°C after the program was finished.

5. Each sample was added 80 μl of TE-buffer, mixed and spun down.

6. The samples were stored at – 80 °C.
Appendix 5 Procedure for qPCR

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

1. A master mix was prepared:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>volume/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>5.58 µl</td>
</tr>
<tr>
<td>Perfecta SYBR Green FastMix</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Primer forward (25 pmol/µl)</td>
<td>0.21 µl</td>
</tr>
<tr>
<td>Primer reverse (25 pmol/µl)</td>
<td>0.21 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16.00 µl</strong></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Dilution series nr</th>
<th>cDNA</th>
<th>RNase free water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.35 µl</td>
<td>undiluted</td>
</tr>
<tr>
<td>2</td>
<td>6 µl</td>
<td>+ 18 µl</td>
</tr>
<tr>
<td>3</td>
<td>6 µl from nr 2</td>
<td>+ 18 µl</td>
</tr>
<tr>
<td>4</td>
<td>6 µl from nr 3</td>
<td>+ 18 µl</td>
</tr>
<tr>
<td>5</td>
<td>6 µl from nr 4</td>
<td>+ 18 µl</td>
</tr>
<tr>
<td>6</td>
<td>6 µl from nr 5</td>
<td>+ 18 µl</td>
</tr>
</tbody>
</table>

5. 16.00 µl master mix was loaded to each well on a 96 well plate.

6. 4.00 µl ddH₂O were added to the non-template control (NTC) wells.

7. 4.00 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.