Cellular mechanisms involved in the induction and maintenance of long-term potentiation (LTP) in the spinal dorsal horn

by

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To my dear family

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Abbreviations and explanations

AIP	([Ala^{9}]-Autocamtide 2-related inhibitory peptide trifluoroacetate salt
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
Arc	Activity-regulated-cytoskeleton-associated protein
ATP	Adenosine triphosphate
C	Cytosine
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	Central nervous system
COX2	Cyclooxygenase 2
CRE	cAMP response element
CREB	cAMP response element-binding protein
Ct	Threshold cycle
DAG	Diacylglycerol
ERK	Extracellular signal-regulated kinase
G	Guanine
GDNF	Glial cell line-derived neurotrophic factor
GFRα1	GDNF family receptor α1
Glu	Glutamate
HFS	High-frequency stimulation
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
LC	Locus coeruleus
LFS	Low-frequency stimulatuion
LRGs	Late-response genes
LTP	Long-term potentiation
mGluR	Metabotrophic glutamate receptor

NCAM	Neuronal cell adhesion molecule
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
NMDA-2B	N-methyl-D-aspartate containing the 2B subunit
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	N-methyl-D-aspartate (NMDA) receptor
NS	Nociceptive specific
P2X	Purinergic 2X
p38 MAPK	p38 mitogen-activated protein kinase
PAG	Periaqueductal grey
PB	Parabrachial
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RT-PCR	Reverse transcriptase- polymerase chain reaction
RVM	Rostroventromedial medulla
SC	Subcoereleus
SHT	Spinohypothalamic tract
SMT	Spinomesencephalic tract
SP	Substance P
SRT	Spinoreticular tract
STT	Spinothalamic tract
Tm	Melting temperature
TNFα	Tumor necrosis factor α
WDR	Wide dynamic range

Abstract

The cellular processes leading to spinal long-term potentiation (LTP) are regarded as underlying mechanisms of sensitization in the dorsal horn.

In this study, spinal LTP was induced by high-frequency stimulation (HFS) conditioning of the sciatic nerve. Electrophysiological extracellular recordings from nociceptive single dorsal horn neurons were used in combination with quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) to examine the mechanisms for induction and maintenance of spinal LTP.

Spinal administration of the N-methyl-D-aspartate-2B (NMDA-2B) receptor antagonist Ro 25-6981 showed an antinociceptive effect on spinal dorsal horn neuronal activity and clearly attenuated the magnitude of spinal LTP. Moreover, induction of LTP after HFS conditioning was not observed following pre-treatment of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor AIP. A transient increase in the expression of the gene for the transcription factor Zif268 was observed in the spinal cord 120 minutes after HFS conditioning. Further, the expression of the genes for interleukin-1β (IL-1β), glial cell line-derived neurotrophic factor (GDNF) and inducible nitric oxide synthase (iNOS) increased significantly in the ipsilateral dorsal horn 360 minutes after HFS conditioning.

These data demonstrate that activation of the spinal NMDA-2B receptor and the intracellular CaMKII enzyme may be important for the induction of spinal LTP. Moreover our results indicate that increased gene expression of Zif268, IL-1β, GDNF and iNOS following HFS might be associated with the maintenance of spinal LTP.

List of original papers

This project has resulted in the following papers that will be referred to in the text by their roman numbering I-IV:

Paper I. Pedersen, L. M. and Gjerstad, J. (2008) Spinal cord long-term potentiation (LTP) is attenuated by the NMDA-2B receptor antagonist Ro 25-6981. *Acta Physiologica,* 192 (3): 421-427.

Paper II. Pedersen, L. M., Lien, G. F., Bollerud, I. and Gjerstad, J. (2005) Induction of long-term potentiation in single nociceptive dorsal horn neurons is blocked by the CaMKII inhibitor AIP. *Brain Research*, 1041:66-71.

Paper III. Gjerstad, J. Lien, G. F., Pedersen, L. M., Valen, E. C. and Mollerup, S. (2005) Changes in gene expression of Zif, c-fos and cyclooxygenase-2 associated with spinal long-term potentiation. *Neuroreport*, 16 (13): 1477-1481.

Paper IV. Pedersen, L. M., Jacobsen, L. M., Mollerup, S. and Gjerstad, J. (2009) Spinal cord long-term potentiation (LTP) is associated with increased spinal gene expression of IL-1β, GDNF and iNOS. *European Journal of Pain,* In press.

1. INTRODUCTION

1.1. Pain versus nociception

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage, for review see (Loeser and Treede, 2008). According to this definition, pain is a subjective experience. The pain processing is complex and do not only include the perception of sensory signals, but also cognitive analysis and associated emotional responses. Whether or not a particular stimulus will be perceived as painful depends not only on the nature of the stimulus, but also on memories, emotions and the context within it is experienced.

Nociception is defined as the neuronal processes of encoding and processing noxious stimuli, for review see (Loeser and Treede, 2008). Hence, nociception, which refers to the object of sensory physiology including activity in specialized receptors, i.e. nociceptors, and specialized pathways activated by stimulation of the nociceptors, must be distinguished from the subjective phenomenon pain. Nociception is the core of many pain states, but pain may also occur without nociception and vice versa.

1.2. Adaptive and maladaptive pain

Pain is extremely important for all animals and essential for their survival. Generally, pain function as a protective mechanism warning the organism of potential or actual tissue damage. Acute nociceptive pain that is evoked by some sort of noxious stimuli has a clear biological function and normally causes the individual to escape from or remove the painful stimuli in time to prevent tissue damage, for review see (Kavaliers, 1988).

The pain system has the ability to increase its sensitivity and reduce the threshold following exposure to inflammation or an injurious stimulus. The increased pain sensitivity for stimulation in the area of damaged tissue (primary hyperalgesia) and adjacent undamaged tissue (central hyperalgesia) is caused by sensitization of peripheral nociceptors innervating the area or sensitization centrally in the spinal cord, respectively. In addition, pain in response to non-nociceptive stimulus (allodynia) may occur. The development of

hyperalgesia is a necessary process encouraging immobilization to optimize the healing process. Like acute nociceptive pain evoked by noxious stimuli, this increased pain sensation following minor injuries and inflammation has a clear adaptive function.

Normally the pain is temporary and disappears after tissue healing, but sometimes the pain and the pain hypersensitivity exceeds the healing process and may become long-lasting or chronic. The maintenance of pain following a healing process does not seem to serve a biological purpose and might result in unnecessary suffering for the individual. Under these circumstances, the pain sensation has a maladaptive dysfunction.

1.3. Nociceptive processing and plasticity

In most cases our perception of pain starts with activation of peripheral nociceptors, located on primary afferent nerve endings. The primary afferent nerve fibers conduct nociceptive information into the dorsal horn of the spinal cord where it is integrated with information from other sensory systems or segmental- or supraspinal regulation. From the spinal cord the nociceptive signals are conducted via neurons projecting to important areas in the brainstem and thalamus and further to sub-cortical and cortical areas where the perception of pain occurs.

Nociceptive processing is subject to extensive plasticity at all levels of the pain system from the nociceptors in the periphery and centrally where connections between neurons are made. The plasticity of nociceptive processing is important for the development of different pain states. Multiple neurotransmitters, neuromodulators, receptors and structural changes are involved in the nociceptive transmission and contribute to the system's plastic properties. Recently, recruitment of spinal glial cells has been shown to play a major role in plasticity and might influence on the development of hyperalgesia and allodynia (Meller et al., 1994; Watkins et al., 1997; Ma and Zhao, 2002; Ying et al., 2006).

The mechanisms of plasticity in the nociceptive system are not fully revealed. Hence, there is a need for more knowledge about the cellular mechanisms responsible for peripheral and central neuronal plasticity important for development of pain hypersensitivity. In this thesis

components of central neuronal plasticity, which might be important for the development of pain hypersensitivity are investigated.

1.4. The nociceptive signaling- and modulatory system

1.4.1. Primary afferent nerve fibers

The primary afferent nerve fibers respond to a variety of sensory modalities including mechanical, thermal and chemical stimuli. Thickly myelinated Aβ-fibers with low activation threshold receptors are responsible for conveying tactile information, whereas thinly myelinated Aδ-fibers and polymodal nociceptors of the unmyelinated C-fibers respond to more intense mechanical, chemical and thermal stimuli. These fibertypes conduct action potentials from the periphery to the central terminals at different velocities, with the Aβ-fibers being the fastest and the C-fibers being the slowest, for review see (D'Mello and Dickenson, 2008).

Nociceptors are free peripheral nerve endings innervating most of the tissues of the body including skin, muscles, joints and viscera, for review see (Willis and Westlund, 1997). In general, the nociceptive information is conducted from the periphery via the dorsal root ganglion into the grey matter of the spinal dorsal horn through primary afferent Aδ- and C-fibers. Two important transmitters released from the primary afferent fibers following peripheral noxious stimuli are glutamate (Glu) (Kangrga and Randic, 1991) and substance P (SP) (Kantner et al., 1985). Activation of Aδ-fibers leads to a sensation of fast, sharp and pricking pain in contrast to activation of the C-fibers that gives a sensation of slow burning and aching pain (Konietzny et al., 1981; Ochoa and Torebjork, 1989). In the spinal cord, neurotransmitters and neuromodulators released from the primary afferent nerve fibers may activate populations of both second order neurons and glial cells.

1.4.2. The spinal dorsal horn

The spinal cord consists of a butterfly-shaped core of grey matter containing cell bodies surrounded of a white matter primarily consisting of myelinated axons. In 1954, Rexed demonstrated that the grey matter of the spinal cord can be divided into ten distinct laminae

due to cellular architecture (Rexed, 1954). Physiological studies have since demonstrated an analogous functional laminar organization.

The dorsal horn of the spinal cord includes laminae I-VI. Laminae VIII and IX comprise the ventral horn of the spinal cord, lamina VII is the transition between the dorsal and the ventral horn and lamina X is the grey matter around the central canal (Figure 1A). The central terminals of the primary afferent fibers terminate in the dorsal horn of the spinal cord. Aβ-fibers predominantly innervate laminae III-VI, whereas the high threshold nociceptive Aδ- and C-fibers terminate predominantly superficially in laminae I and II, with a smaller number reaching deeper into lamina V (Light and Perl, 1979; Sugiura et al., 1986), for review see (Todd, 2002) (Figure 1B).

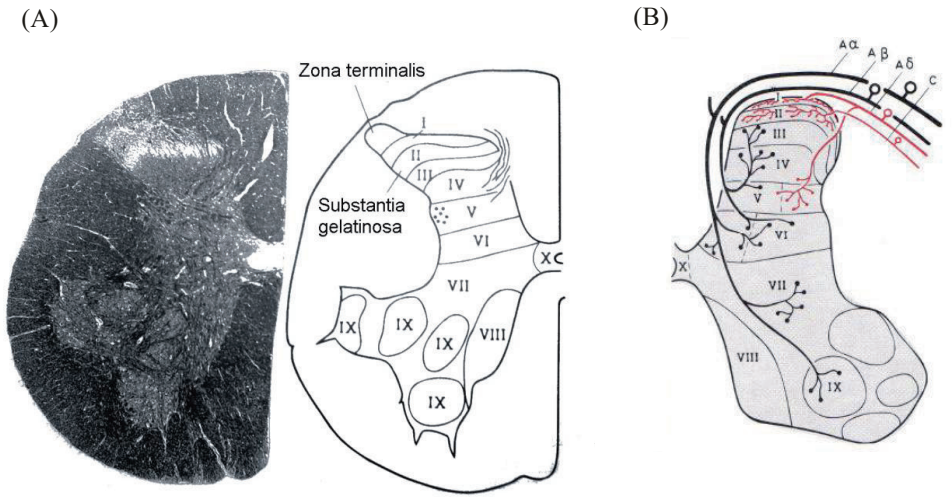


Figure 1. Cross section of the spinal cord showing; (A) the localization of the spinal cord laminae I-X and (B) the central termination of the different primary afferents. Adapted from (Brodal, 2007a; b).

The spinal cord itself contains various neuronal cell types, which make direct or indirect connections with primary afferent fibers. Based on the projection of their axons, dorsal horn neurons can be divided into three general classes; interneurons, propriospinal neurons and projection neurons. Interneurons are local intrinsic dorsal horn neurons, which send their axons for only a short distance within the spinal cord. They act as local relays in spinal processing and comprise both excitatory and inhibitory interneurons. Propriospinal neurons are larger and send axons across segments of the spinal cord and are therefore involved with information transfer across spinal cord segments and reflex responses. Projection neurons send axons to supraspinal centers along ascending pathways and are responsible for the transfer of sensory information from the spinal cord to the brain, for review see (Sandkuhler, 1996; Willis and Westlund, 1997). Several lines of evidence have demonstrated that changes in synaptic efficacy in between the primary afferent neurons and the projection neurons may be crucial for the activity in the ascending pathways (Svendsen et al., 1997; Sandkuhler and Liu, 1998; Rygh et al., 1999; Gjerstad et al., 2001; Ikeda et al., 2003).

The spinal dorsal horn sensory projection neurons are in turn divided into different classes based on their synaptic input, laminar location and projection site. Low-threshold mechano-receptive cells are excited by Aβ-afferent fibers and respond to innocuous stimuli. These cells are found in all laminae, but primarily in laminae III and IV. The nociceptive specific (NS) cells respond exclusively to noxious stimuli, such as intense mechanical stimulation, chemicals and temperatures above 45 ^{o}C. These nociceptive specific cells are present in lamina I, but also in laminae IV and V and synapse with Aδ- and C-fibers only. Convergent multireceptive cells, termed wide-dynamic range (WDR) cells, receive input from all types of sensory fibers; Aβ-, Aδ- and C-fibers, and therefore respond to the full range of stimulation, from light touch to noxious pinch, heat and chemicals. The WDR cells respond in a graded manner to peripheral stimuli with increasing response to increasing stimulus intensity. They are located widespread in the dorsal horn. However, the highest density of these neurons is within the deeper laminae of the dorsal horn, for review see (D'Mello and Dickenson, 2008).

Essential to and modulating these neuronal spinal cord cell types are the non-neuronal glial cells. Glial cells have generally been considered primarily to subserve housekeeping and

supportive roles in the nervous system, but there is evidence showing that glial cells also influence on nociceptive transmission (Ma and Zhao, 2002). Glial cells in the spinal cord consist of oligodendrocytes, astrocytes and microglia and are the most abundant cell type in the central nervous system (CNS). There are 3-5 more glial cells than neurons in the CNS, for review see (Hansson, 2006). Oligodendrocytes are cells responsible for myelin sheath formation and until today there are few experiments investigating its contributions to the regulation of nociceptiv transmission. Therefore, only astrocytes and microglia are discussed in this thesis.

Astrocytes are in intimate contact with neurons and they communicate signals to each other via the extracellular space. The astrocytes thightly enwrap the vast majority of the synapses in the CNS and selectively modulate neuron to neuron synaptic communication. In addition, they make extensive contacts with endothelial cells from capillaries, for review see (Abbott et al., 2006), interact with microglia and are interconnected with one another by gap junctions forming networks of coupled astrocytes (Cornell-Bell et al., 1990; Blomstrand et al., 1999). Generally, astrocytes perform functions critical for optimal neuronal functioning, including regulation of the composition of the extracellular environment, as well as providing energy and metabolic precursors to neurons, for review see (Hansson, 2006; Watkins et al., 2007).

Microglia are small cells with a variable number of branching processes. They are considered to be the equivalent of macrophages in the periphery and perform immune surveillance under basal conditions. Resting microglia direct small processes toward blood vessels, other glial cells and neuronal elements and act as sensors to various stimuli that threaten physiological homeostasis. Activation of microglia may result in changes in morphology from a resting ramified shape into an active amoeboid shape, changes in gene expression, cell proliferation and function, for review see (Hansson and Ronnback, 2003; Watkins et al., 2007).

Both astrocytes and microglia are now known to play a key role in regulating synaptic transmission and participate in synaptic plasticity (Ma and Zhao, 2002), for review see (Araque et al., 1999). Glial cells are capable of responding to various neuronal factors, such as glutamate, SP and adenosine triphosphate (ATP) via their receptors and subsequently

change glial responsitivity leading to the release of chemical mediators, such as proinflammatory cytokines, ATP, nitric oxide (NO), excitatory amino acids, prostaglandins and nerve growth factors, that can act on neurons and alter their functions. Glial cells may in this way directly or indirectly participate in neuronal sensitization, for review see (De Leo et al., 2006; Watkins et al., 2007).

1.4.3. Ascending pathways

The output of sensory information from the dorsal horn to different structures in the brain is carried by spinal projection neurons along ascending pathways (Ikeda et al., 2003), for review see (Willis and Westlund, 1997; Gauriau and Bernard, 2002; Todd, 2002) (Figure 2). One of the major ascending pathways important for pain is the spinothalamic tract (STT) sending direct projections to different nuclei in thalamus. Most of the STT cells originate in the superficial laminae I, but some STT cells are also present in the deeper laminae III-VII and X (Giesler et al., 1979; Burstein et al., 1990). Moreover, the spinomesencephalic tract (SMT) and the spinoreticular tract (SRT) project to important homeostatic control regions in the brainstem and the mesencephalon, i.e. the parabrachial (PB) area, reticular formation, locus coeruleus (LC), subcoereleus (SC) and the midbrain periaqueductal grey (PAG) (Wiberg et al., 1987; Yezierski, 1988; Andrew et al., 2003). The SMT and the SRT originate in laminae I and V in the spinal cord. Some SMT are also located in lamina VII. The majority of the axons in the STT, SMT and the SRT cross to the contralateral side of the spinal cord before projecting to the supraspinal structures. In contrast, the spinohypothalamic tract (SHT) projects bilaterally to the hypothalamus and the ventral forebrain (Burstein et al., 1987). The SHT projections, originating in the laminae I, V, VII and X, may be important in autonomic, neuroendocrine and emotional aspects of pain.

Importantly, many projection neurons are involved in activation of descending modulatory systems, which in turn may control the sensory transmission in the dorsal horn.

1.4.4. Descending modulatory systems

Descending pathways from brainstem and midbrain structures are able to influence on nociceptive signaling in the dorsal horn of the spinal cord (Figure 2). Although some

descending projections are facilitatory, most descending projections are of inhibitory nature (Gjerstad et al., 2001). The midbrain PAG and the brainstem rostroventromedial medulla (RVM) are important areas in the supraspinal descending modulatory system. These structures control activity in the serotonergic, noradrenergic and enkephalinergic descending projections (Reddy et al., 1990; Marlier et al., 1991; Rajaofetra et al., 1992). PAG receives projections from both laminae I nociceptive neurons and laminae III-VI dorsal horn neurons (Keay et al., 1997). In addition, there are projections to the PAG from the reticular formation, LC, hypothalamus (Beitz, 1982), amygdala (Hopkins and Holstege, 1978), medial prefrontal areas, anterior cingulate cortex and insular cortex (Hardy and Leichnetz, 1981; Beitz, 1982). Thus, the nociceptive modulatory system is a complex network, which integrates information from other areas in the brain with information from the brainstem and the spinal dorsal horn. The descending modulatory system may therefore be controlled by complex cognitive and emotional processing (Matre et al., 2006). PAG projects minimally to the spinal dorsal horn, so the nociceptive-modulating action of the PAG on the spinal cord, is relayed largely through the RVM. Additional systems from the LC to the spinal cord also modulate spinal activity.

Thus, the spinal dorsal horn functions as a sensory filter that is controlled by the descending modulatory system from supraspinal structures (Figure 2). The nociceptive information reaching the brain may due to this regulation be conciderably changed compared to the intensity of the nociceptive peripheral stimuli.

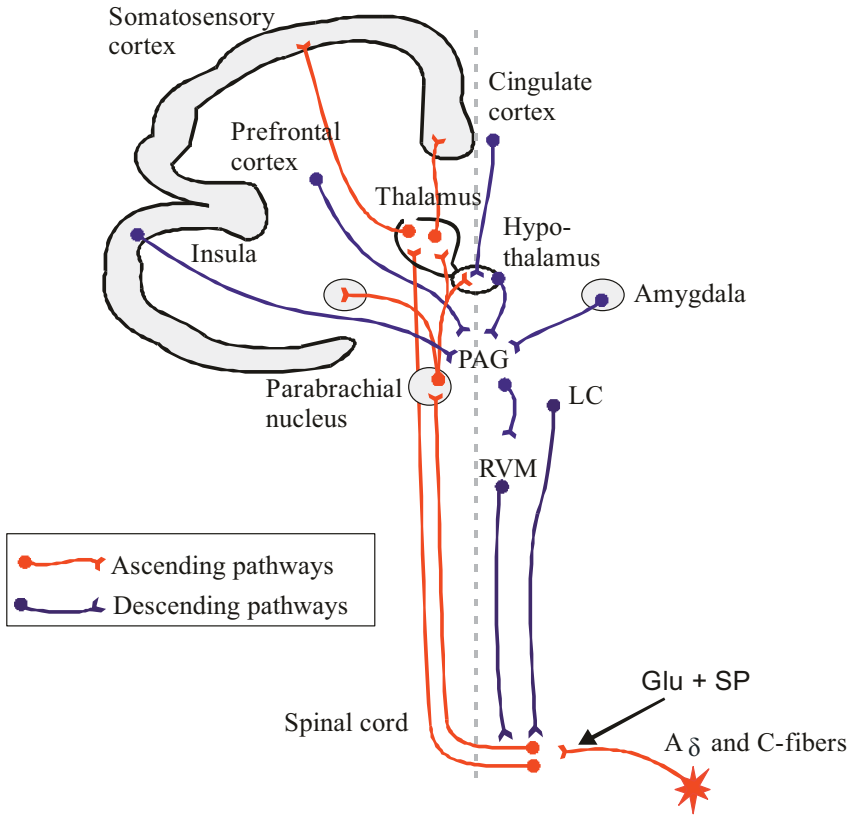


Figure 2. A simplified presentation of the nociceptive signaling- and modulatory system. Activation of peripheral nociceptors generates activity in Aδ- and C-fiber afferents, which conduct the nociceptive information into the spinal dorsal horn where glutamate (Glu) and substance P (SP) are released. From the spinal cord the nociceptive signals are transferred via neurons projecting to different areas in the brainstem, subcortical- and cortical areas. Pathways from the cortical areas, periaqueductal grey (PAG) and hypothalamus control, via a link in the rostroventral medulla (RVM), excitatory and inhibitory output from the brainstem to the spinal cord. Additional systems from the locus coeruleus (LC) to the spinal cord also modulate spinal activity. Adapted from (Gjerstad, 2007).

1.5. Synaptic plasticity and central sensitization in the spinal cord

An important feature of synaptic processing is that it is subject to diverse forms of use-dependent plasticity (Liu and Sandkuhler, 1995; Liu and Sandkuhler, 1997), for review see (Dubner and Ruda, 1992; Woolf, 1996). It has long been recognized that synaptic plasticity plays an important role in the development of sensitization of nociceptive neurons in the spinal cord and it is believed that central sensitization is critical for the development of different pain states where hypersensitivity in the CNS is likely to occur, for review see (Woolf and Salter, 2000).

Central sensitization of spinal nociceptive neurons has been defined to involve several phenomena including increased excitability of nociceptive neurons in the CNS to their normal afferent input and a reduction in the activation threshold, for review see (Loeser and Treede, 2008). In addition, central sensitization may also induce spontaneous discharges and a spread of the receptive field. On the behavioral level, central sensitization may manifest as secondary hyperalgesia (increased pain sensitivity to noxious stimulation) and/or allodynia (pain in response to non-nociceptive stimuli), for review see (Woolf, 1996).

Central sensitization may be the outcome of a variety of cellular and circuits changes that occur in the CNS, which may alter neuronal excitability either for a short or prolonged period. In addition to an increase in synaptic strength (Randic et al., 1993; Liu and Sandkuhler, 1995), central sensitization may also involve other mechanisms not directly affecting the synapses. These mechanisms may include loss of inhibitory interneurons or structural reorganization (Woolf et al., 1992), for review see (Woolf and Doubell, 1994). Central sensitization needs to be differentiated from peripheral sensitization, which is characterized by increased excitability of peripheral nerve terminals. In central sensitization, responses to sensory stimulation may be enhanced in the spinal cord and/or the brain without any change in the excitability of primary afferent neurons, for review see (Willis, 2002).

Evidence for increased excitation (central sensitization) in the spinal cord following noxious afferent stimulation was first fully described in 1983 by Woolf (Woolf, 1983). In decerebrated rats with intact spinal and brainstem reflexes, Woolf showed that a heat injury to the lateral edge of the foot decreased the mechanical and heat withdrawal thresholds and

increased the amplitude of flexor reflexes both ipsi- and contralateral to the lesion. Following this study, a large number of published research reports have generated evidence for a central component in hypersensitive pain states, for review see (Coderre et al., 1993; Mannion and Woolf, 2000).

1.5.1. Long-term potentiation (LTP)

The discovery of use-dependent increase in synaptic strength, called long-term potentiation (LTP), in hippocampus initiated an extensive research in the field of neuroscience of synaptic plasticity (Bliss and Lømo, 1973). Brief high-frequency trains of electrical stimuli resulted in increased efficiency of transmission at the perforant path-granule cell synapse in the rabbit hippocampus that could last for hours (Bliss and Lømo, 1973).

Changes in synaptic strength has later been demonstrated in many other parts of the central nervous system (Randic et al., 1993; Liu and Sandkuhler, 1995; Rogan et al., 1997). Induction of spinal LTP was first demonstrated in vitro in spinal cord slices of the ventral horn (Pockett and Figurov, 1993). Later, induction of LTP has been shown both in vitro and in vivo in the dorsal horn using different techniques; field potential recordings in superficial dorsal horn neurons, single cell recordings in deep dorsal horn WDR neurons and patch-clamp techniques in identified superficial neurons in the spinal cord (Randic et al., 1993; Liu and Sandkuhler, 1995; Svendsen et al., 1997; Gjerstad et al., 2001; Azkue et al., 2003; Ikeda et al., 2003; Qu et al., 2009). Optical recording techniques have been utilized to monitor presynaptic activity in primary afferents (Ikeda and Murase 2004).

Previous studies have demonstrated that spinal LTP may be induced by various noxious stimuli. For example, brief electrical high-frequency stimulation (HFS) (about 100 Hz) conditioning of the sciatic nerve induces LTP in superficial and deep dorsal horn neurons (Liu and Sandkuhler, 1995; Svendsen et al., 1997; Gjerstad et al., 2001; Wallin et al., 2003; Ikeda et al., 2006). Recently, it has been shown that low-frequency stimulation (LFS) (2 Hz) conditioning of the dorsal root afferents successfully induced LTP in lamina I neurons projecting to the PAG (Ikeda et al., 2006). In deep dorsal horn neurons a frequency of 30 Hz induced a robust LTP, whereas LFS (3 Hz) conditioning induced a short-lasting spinal LTP (Haugan et al., 2008). Moreover, strong natural stimulation, such as inflammation, radiant

heating of the skin and tissue- and nerve injury has also been shown to induce LTP in the spinal dorsal horn (Sandkuhler and Liu, 1998; Rygh et al., 1999; Ikeda et al., 2006). However, some of these forms of low level afferent input may induce LTP only, if descending pathways are interrupted (Sandkuhler and Liu, 1998). Most of the experimental studies inducing spinal LTP are based on HFS conditioning of the sciatic nerve.

These studies have shown that the character, intensity and frequency of the peripheral conditioning stimuli are important for the time course and duration of spinal LTP. For instance, after a brief electrical or mild natural noxious stimulus LTP may last for only a few minutes, but may, however, last for up to 24 hours following repetitive trains of HFS applied to the sciatic nerve (Liu and Sandkuhler, 1997), for review see (Sandkuhler, 2007). Full expression of LTP may take only a few minutes after tetanic stimulation (Randic et al., 1993; Liu and Sandkuhler, 1995; Liu and Sandkuhler, 1997; Svendsen et al., 1998) or may require up to an hour after natural noxious stimulation (Sandkuhler and Liu, 1998). Moreover, the frequency of discharge might influence on which synapse that become potentiated (Ikeda et al., 2006).

Experimental data suggest that the expression of LTP is influenced by descending control and local inhibitory systems. In rats where the descending pathways were interrupted or weakened, HFS gave a greater LTP compared to intact rats (Svendsen et al., 1999a; Gjerstad et al., 2001), suggesting a tonic descending inhibition preventing LTP under physiological conditions. Moreover, spinal cord stimulation, increasing the activity in inhibitory systems, attenuated established spinal LTP in deep WDR neurons (Wallin et al., 2003). Interestingly, noxious sciatic stimulation, which induces spinal LTP, has also been shown to affect activity in the supraspinal pain modulating circuitry (Hjornevik et al., 2008; Hjornevik et al., 2009).

Spinal LTP has similarities with the phenomenon central sensitization and it has been suggested that spinal LTP is a form of central sensitization or vice versa, for review see (Sandkuhler, 2000; Willis, 2002). It is a well established experimental phenomenon within the spinal cord, for review see (Sandkuhler, 2000; 2007). Several mechanisms are involved in the induction and maintenance of spinal LTP.

1.5.2. Cellular mechanisms of spinal LTP

Previous data show that tetanical stimulation of the sciatic nerve leads to not only glutamate release from primary afferent neurons, but also increased extracellular SP level in the dorsal horn (Afrah et al., 2002).

It is now well established that co-release of glutamate and SP stimulates postsynaptic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-, neurokinin 1 (NK1)- and metabotrophic glutamate (mGlu) receptors, which in turn, because of the subsequent long-lasting postsynaptic depolarization, remove the Mg^{2+}-block of the N-methyl-D-aspartate (NMDA) receptors. Binding of glutamate to unblocked NMDA receptors triggers a substantial influx of Ca^{2+} into the postsynaptic neuron. In addition, a depolarization of postsynaptic neurons may lead to Ca^{2+}-influx through voltage-gated T-type Ca^{2+}-channels. Activation of both the mGluRI- and the NK1 receptors may trigger the phospholipase C (PLC)- inositol 1,4,5-triphosphate (IP_3) pathway, leading to release of Ca^{2+} from intracellular stores, which further increases the cytosolic Ca^{2+}-concentration. It has been shown that application of antagonists for the AMPA receptor (Svendsen et al., 1998), NK1 receptor (Liu and Sandkuhler, 1997), mGluRI (Azkue et al., 2003) and the NMDA receptor (Liu and Sandkuhler, 1995; Pockett, 1995; Svendsen et al., 1998; Ikeda et al., 2003) partly or fully prevent the induction of spinal LTP. Moreover, the NMDA receptor antagonist AP5 may reverse the potentiated C-fiber evoked response when it is given 1 hour after HFS (Svendsen et al., 1999b). In addition, evidence exists that induction of LTP also involves activation of the T-type Ca^{2+}-channels, PLC and the intracellular IP_3 receptors (Ikeda et al., 2003). Thus, these receptor systems seem to be important for the induction of spinal LTP (Figure 3).

As a consequence of the activation of these receptors, a significant rise in Ca^{2+}-level is induced in spinal dorsal horn neurons. A rise in postsynaptic Ca^{2+}-concentration is essential for LTP induction and the magnitude of Ca^{2+} is linearly correlated with the magnitude of LTP in vitro (Ikeda et al., 2003). Recent data have demonstrated that stimuli inducing LTP cause substantial rise in Ca^{2+} in lamina I neurons, not only in vitro, but also in intact animals (Ikeda et al., 2006).

Increased cytosolic Ca^{2+} may result in activation of a variety of different Ca^{2+}-dependent cellular responses. Previous data indicate that the Ca^{2+}-dependent enzymes protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) may be important for the induction of LTP of field potentials (Yang et al., 2004). Thus, spinal LTP may be associated with both phosphorylation of receptors and transcription factors, which may contribute to changes in the synaptic efficacy.

Furthermore, data suggest that the nitric oxide synthase (NOS) and NO are involved in LTP of C-fiber evoked field potentials induced by tetanic sciatic stimulation (Ikeda and Murase, 2004; Zhang et al., 2005b). The gaseous molecule NO, which is synthesized under the control of NOS, is a cell-permeable neuromodulator, and may diffuse freely in the extracellular space to excert its action on adjacent or distant cells.

A rise in cytosolic Ca^{2+}-level may also trigger changes in the transcriptional and translational levels in dorsal horn neurons. Recent data show that maintenance of LTP in the spinal dorsal horn may be inhibited by protein synthesis inhibitors anisomycin and cycloheximide (Hu et al., 2003). This indicates that the maintenance of LTP in the spinal cord may involve altered gene expression and protein synthesis. A primary event in this process may be induction of transcription factors, followed by altered expression of late-response genes (LRGs).

Prolonged activation of both extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB) following induction of spinal LTP of C-fiber evoked field potentials has been shown (Xin et al., 2006). Thus, it is suggested that the ERK/CREB pathway in the spinal dorsal horn is necessary for the induction and maintenance of LTP. Although ERK is involved in cytosolic cellular signaling, it can also be translocated to the nucleus where it activates CREB, which in turn stimulates transcription by binding to the regulatory cAMP response element (CRE). Actually, CREB binding sites have been found in the promoter region of a long list of genes. The ERK/CREB pathway may therefore have both short-term and long-term effects on the neuronal excitability.

Many transcription factors may be induced by the ERK-CREB pathway. Expression of the CRE-containing transcription factor Zif268 (Rygh et al., 2006; Haugan et al., 2008) and activity-regulated-cytoskeleton-associated protein (Arc) (Haugan et al., 2008) in the spinal dorsal horn have been shown to be associated with spinal LTP. Generally, the responses of the transcription factors are complex and may drive a chain of molecular events, including induction of other transcription factors as well as LRGs like growth factors, signaling enzymes and structural proteins that may lead to long-term changes in synaptic efficacy. Today, there are few studies examining the expression of specific genes or proteins in the spinal cord following HFS conditioning of the sciatic nerve.

Recently, recruitment of glial cells has shown to play a major role in plasticity (Ma and Zhao, 2002; Ikeda and Murase, 2004). Experimental data based on animals in anaesthesia have revealed that disruption of the function of glial cells by glial metabolic inhibitor may block spinal LTP (Ma and Zhao, 2002). Thus, it seems likely that induction of spinal LTP not only involves activation of neurons, but also activation of glial cells within the spinal cord (Ma and Zhao, 2002; Ikeda and Murase, 2004). However, the details on how the glial cells may influence on neuronal nociceptive transmission following induction of spinal LTP remains to be investigated.

In theory, SP released from neurons following tetanic stimulation (Afrah et al., 2002) may be a source of activation of glial cells via their receptors (Svensson et al., 2003; Liu et al., 2006; Werry et al., 2006). Upon activation, glial cells may activate, synthetize or release substances that directly or indirectly may enhance nociceptive processing and transmission in the spinal cord by increasing the release of neurotransmitters from presynaptic terminals and the excitability of nearby neurons. These substances may include the proinflammatory cytokine interleukin-1β (IL-1β), the trophic factor glial cell line-derived neurotrophic factor (GDNF), iNOS synthesized NO, p38 mitogen-activated protein kinase (p38 MAPK), cyclooxygenase 2 (COX2) synthetized prostaglandins and tumor necrosis factor α (TNFα) (Appel et al., 1997; Marcus et al., 2003; Raghavendra et al., 2004), for review see (Marchand et al., 2005; Watkins et al., 2007). Thus, glial cells may respond to various neuronal factors that subsequently may alter glial response and ultimately affect glial-neuronal interaction, for review see (De Leo et al., 2006).

Based on previous studies, it has been suggested that at least two different phases of LTP can be distinguished depending upon duration and signal transduction pathways involved, for review see (Sandkuhler, 2007). While the early- phase might involve activation and modification of existing receptors and proteins, it is suggested that the late- phase might involve changes in gene expression and de novo protein synthesis (Ma and Zhao, 2002; Azkue et al., 2003; Hu et al., 2003; Ikeda et al., 2003; Xin et al., 2006).

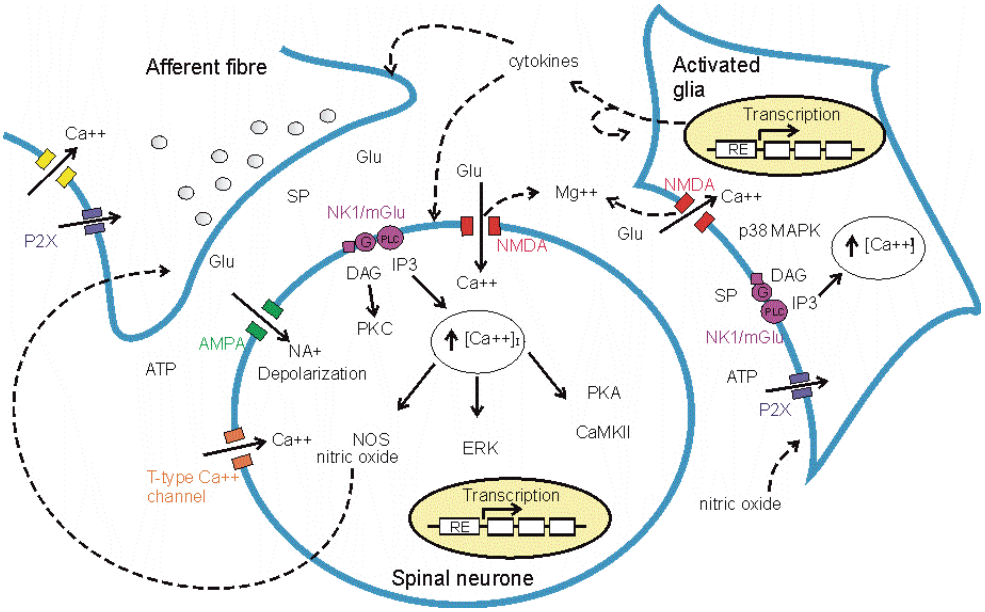


Figure 3. Diagram of a synapse consisting of a primary afferent nerve terminal, a spinal neuron, a glial cell and potential mechanisms involved in LTP and central sensitization. Co-release of glutamate (Glu) and substance P (SP) in the dorsal horn activates postsynaptic AMPA receptors, mGluR1 receptors, NK1 receptors and intracellular IP_3 receptors, which in turn because of subsequent long-lasting postsynaptic depolarization activates the T-type Ca^{2+}-channel and remove the Mg^{2+}-block of the NMDA receptors. This increases the cytosolic Ca^{2+}-level resulting in stimulation of NOS and the intracellular kinases, i.e. PKA, PKC and CaMKII. The increase in cytosolic Ca^{2+}-level may trigger activation of ERK, which in turn may activate transcription of genes encoding proteins important for synaptic transmission. Neurotransmitters released from neurons may also be a source of activation of glial cells. Upon activation, glia cells synthesize and release substances, i.e. cytokines, which may modulate synaptic transmission. These processes might be important for induction and maintenance of LTP. Adapted from (Gjerstad, 2007).

2. AIMS OF THE STUDY

The main purpose of this study was to investigate the cellular mechanisms of induction and maintenance of spinal LTP induced by HFS conditioning of the sciatic nerve. More specifically we aimed to:

I. Investigate the role of the NMDA receptors containing the NMDA receptor 2B (NR2B) subunit on the induction of LTP in single nociceptive dorsal horn neurons.

II. Examine whether the intracellular CaMKII in the spinal cord is necessary for induction of LTP in single nociceptive dorsal horn neurons.

III. Examine whether expression of the transcription factors Zif268 and c-fos are associated with maintenance of spinal LTP.

IV. Investigate whether maintenance of spinal LTP is associated with local spinal changes in the expression of genes encoding the proinflammatory substances IL-1β, GDNF, iNOS, p38 MAPK, COX2 and TNFα.

3. METHODS

The methods used are described in detail in paper I-IV. However, below is a brief description of the different methods used.

3.1. Animals

Female Sprague-Dawley (SD) rats delivered from Scandbur DK, Sollentuna, Sweden were used in all experiments. After arrival, the rats were housed in standard white plastic cages with four animals in each cage. The air temperature was kept at 20-22^{o}C and the relative humidity was kept at 50-55 %. The experiments were performed during the light period of an artificial 12h/12h dark/light cycle. The light was switched on at 06:00 am. All animals had free access to standard rat food and water. At least one week of acclimatization was allowed before the experiments. The rats used weighed 250-350 gram.

3.2. Anaesthesia and surgery

The rats were anaesthetized with urethane (1.3-2.1 g/kg body weight, intraperitoneally) and placed on a heating pad. Absence of foot withdrawal to pinch indicated adequate anaesthesia. The core temperature was kept constant at 36-37 ^{o}C by means of an electrical feedback control unit. In some experiments, a tube was inserted 10 mm into the trachea and fixed with a suture for artificial ventilation (Harvard Rodent ventilator, model 683). Laminectomy was performed at vertebrae Th13-L1, corresponding to the spinal cord segments where the sciatic nerve roots enter the cord.

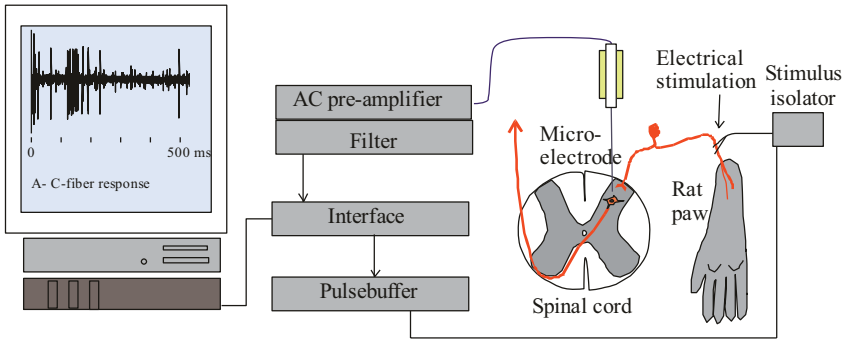
The sciatic nerve was dissected free at the mid-thigh level and isolated from the surrounding tissue by a plastic film. A bipolar silver hook electrode was placed proximal to the main branches of the sciatic nerve for electrical stimulation. To avoid muscle contractions in the experiments with single cell recordings, the rats received an intramuscular injection of 0.2 ml pancuronium bromide (2 mg/ml; Pavulon, Organon, the Netherlands). The animals were killed immediately after the experiments.

3.3. Single-cell recordings

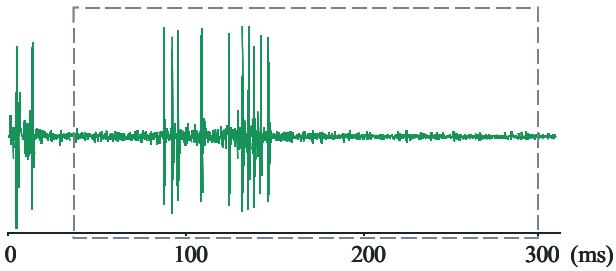
For single-cell recordings, parylene-coated tungsten microelectrodes with impedance 2-5 MΩ (Friedrick Haer & CO, Bowdoinham, USA) were lowered vertically into the dorsal horn of the spinal cord by an electronically controlled micromanipulator (Figure 4A). The recorded signals were amplified with an AC pre-amplifier, filtered with band-width 500-1250 Hz corresponding to the duration of the action potentials 0.8-2.0 ms, digitalized with the interface CED 1401μ and continuously captured on a PC with the software CED Spike 2 (Cambridge Electronic Design, Cambridge UK). The sampling frequency was 20000 Hz. Spikes 40-300 ms after stimulus were defined as C-fibre responses, i.e. responses in the neurons evoked by C-fibre activation (Figure 4 B). Single cell recordings were ensured on the basis of amplitude and shape of the action potentials (Figure 4 C).

After the microelectrode was lowered into the dorsal horn, the spinal cord segments where the sciatic nerve roots enter the cord were identified by the neuronal responses to finger tapping of the hind paw. Extracellular single cell activity was recorded from neurons at depths of 80 –1000 μm from the surface of the spinal cord. All single cells used in the experiments were identified by their ability to respond to natural stimuli: brush, fingernail pressure and surgical forceps pinch. The brush produced a feeling of touch, the fingernail pressure produced a feeling of firm pressure near pain threshold and the surgical forceps pinch produced pain well above the pain threshold when similar stimuli were applied to the human skin. Neurons responding to these modes in a graded manner were characterized as WDR neurons, whereas neurons responding only to surgical forceps pinch were characterized as NS.

(A)



(B)



(C)

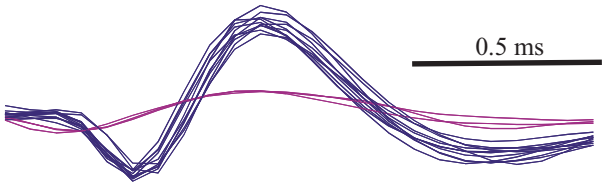


Figure 4. Extracelluar recordings in the spinal dorsal horn. The signals were captured by the software CED Spike 2 (Cambridge Electronic Design, Cambridge, UK). (A) Experimental apparatus set up for extracellular single cell recordings. (B) Neuronal activity evoked by a single test pulse applied to the sciatic nerve. Spikes 40-300 ms after stimulus were defined as C-fiber response. (C) Comparison of shape and amplitude of the action potentials from two cells.

3.4. Electrical stimulation

All electrical stimuli were applied to the sciatic nerve by the hook electrode. In the experiments for single-cell recordings, a test stimulus was delivered every 4th minute (2 ms rectangular pulses, 1.5 x C-fiber threshold). Three or six stable C-fiber control responses served as a baseline for the subsequent experiment with the cell. Induction of LTP was obtained by HFS conditioning (1 ms rectangular pulses, 3 x C-fiber threshold, five trains of 1 s duration, 100 Hz, 10 s intervals between the trains). The neuronal activity was monitored for 120 (paper I, II), 180 (paper III) or 360 (paper IV) minutes following application of HFS. Only one experiment was performed in each animal.

3.5. Drug administration

In paper I, we used the NMDA-2B receptor antagonist Ro 25-6981 hydrochloride, ((αR,βS)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol-hydrochloride), ($C_{22}H_{29}NO_2 \cdot HCl$) obtained from Sigma-Aldrich CO, St. Louis, Mo, USA. In paper II, we used the CaMKII inhibitor AIP ([Ala^9]-Autocamide 2-related inhibitory peptide trifluoroacetate salt, Myr-Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Ala-Val-Asp-Ala-Leu-OH) obtained from Sigma Aldrich, St. Louis, USA. Both drugs were applied directly onto the spinal cord.

3.6. Investigation of gene expression

For the investigation of changes in gene expression, the spinal cord segments L3-S1 were identified anatomically relative to Th13. Gene expression was investigated by tissue harvesting at five specific time points; approximately 15 minutes after laminectomy (native) (papers III-IV), 60 (paper III), 120 (paper III), 180 (papers III, IV) and 360 (paper IV) minutes after HFS conditioning of the sciatic nerve. In paper III, a section of the whole spinal cord approximately 5 mm in length was harvested, whereas in paper IV, the ipsilateral dorsal horn, approximately 10 mm in length was harvested. The intensity of the HFS conditioning used was 4.5 mA, which corresponded to the mean value of the HFS intensity applied in the experiments with single-cell recordings. To avoid long-lasting effects of the laminectomy on gene expression in paper IV, the surgical procedure associated with laminectomy was performed only 45-15 minutes prior to tissue harvesting.

3.7. Quantitative real-time RT-PCR

Total RNA was isolated from frozen spinal cord tissue with TRIzol reagent following the supplier's protocol (Life technologies, Inc., Rockville, Maryland, USA). RNA was reversibly transcribed by aid of the first-strand cDNA Synthesis Kit for reverse-transcriptase polymerase chain reaction (RT-PCR) (Roche Diagnostic, Mannheim, Germany). To avoid amplification from traces of possible DNA contamination in the RNA isolation, PCR primers were designed to span introns. All primers were checked for specificity by BLAST search.

Quantitative analysis of specific genes was performed on an ABI 7900 (Applied Biosystems, Foster City, California, USA), with the use of the Power SYBR green PCR mastermix (Applied Biosystems, Foster City, California, USA). The amount of template used in the PCR reaction was cDNA corresponding to i) 200 ng reverse-transcribed total RNA for iNOS, ii) 100 ng reverse-transcribed total RNA for Zif268, IL-1β, GDNF, p38 MAPK and TNFα, iii) 25 ng reverse-transcribed total RNA for COX2 and c-fos and iv) 5 ng reverse transcribed total RNA for β-actin. A final melting curve of fluorescence versus temperature was generated to screen for co-amplification products.

The quantity of the target cDNA template in each sample was presented with an amplification plot indicating the intensity of the fluorescence emitted by the SYBR-green-bound PCR product, as a function of number of cycles in the reaction (Figure 5A). Based on the computer-defined threshold, which is dependent on the background fluorescence, the threshold cycle (Ct) value for each sample was estimated with the software SDS, ABI (Applied biosystems, Foster City, California, USA). The amount of target cDNA in each sample was then calculated using the Ct value and a standard curve prepared for each gene (Figure 5B). The gene expression of the target genes Zif268, c-fos, IL-1β, GDNF, iNOS, p38 MAPK, COX2 and TNFα was normalized to the expression of the internal standard β-actin.

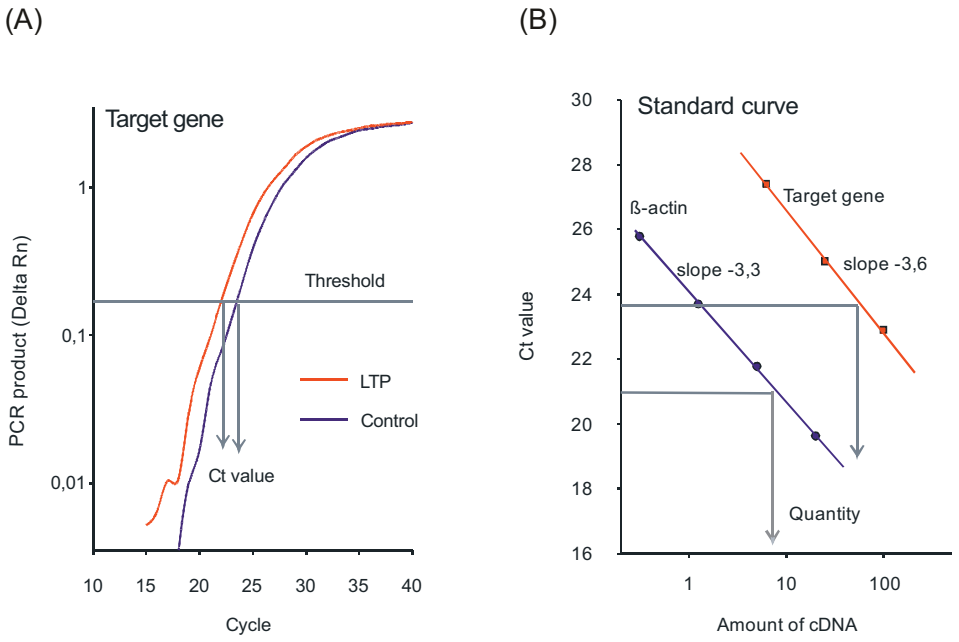


Figure 5. Detection of gene-expression. The data were analysed using the Applied biosystems' software SDS. (A) Amplification plots of the target gene in the control and the LTP group. Delta Rn; intensity of the fluorescence emitted by the SYBR-green-bound PCR product. Ct (threshold cycle); the number of amplification cycles required to obtain an amount of the product reaching a particular computer-defined threshold. (B) Standard curve for quantification of the internal standard and the target gene. The Ct value for each sample corresponds to a specific amount of cDNA.

3.8. Data analysis and statistics

The data are shown as representative examples, mean ± SEM and scatter diagrams. In paper I, III and IV, group means were compared using either paired or unpaired two-tailed Student's t-test. In paper II, two-tailed Mann-Whitney U test was used. Regarding the gene expression, fold change values at specific time points were defined by the expression of the target gene normalized to the expression of the internal standard β-actin and the native expression level. In paper I, III and IV, statistical analyses were performed on log-

transformed data to compensate for non-normal distributions. A p-value less than 0.05 was chosen as the level of statistical significance.

4. RESULTS

4.1. Spinal LTP is induced by HFS conditioning applied to the sciatic nerve

The mean C-fiber response, i.e. the mean value of the responses in the dorsal horn neurons evoked by C-fiber stimulation, increased significantly following HFS conditioning applied to the sciatic nerve. In most of the cells tested, HFS conditioning applied to the sciatic nerve induced an increase in the neuronal activity. The response reached a plateau after 60-120 minutes in the neurons affected by the conditioning stimulation. After this the neuronal response appeared to be stable and the increased response outlasted the experimental period of 120 (paper I and II), 180 (paper III) and 360 (paper IV) minutes.

The expression of LTP following HFS conditioning was inhibited by spinal application of the NMDA-2B receptor antagonist Ro-25-6981 (paper I) and the CaMKII inhibitor AIP (paper II). This may indicate that both the NMDA-2B receptor and the CaMKII play an important role in the induction of spinal LTP. Moreover a transient increase in the gene expression of the transcription factor Zif268 120 minutes (paper III) and an increase in the gene expression of IL-1β, GDNF and iNOS 360 minutes (paper IV) following HFS conditioning of the sciatic nerve were observed. Given that spinal LTP is induced by HFS conditioning, our results suggest that increased expression of these genes following HFS conditioning might be associated with the maintenance of spinal LTP.

4.2. Paper I

4.2.1. Spinal LTP is attenuated by the NMDA-2B receptor antagonist Ro 25-6981

The NMDA-2B receptor antagonist Ro 25-6981 was applied directly onto the exposed spinal cord in doses of 2.0, 4.0 and 8.0 mM. The C-fiber responses were inhibited in a dose-dependent manner with a significant reduction in the C-fiber response following application of 4.0 and 8.0 mM of the antagonist. Thus, the NMDA-2B receptor antagonist Ro 25-6981 showed a clear antinociceptive effect on spinal dorsal horn neuronal activity. Moreover, the effect of HFS was less pronounced following pre-application of 4.0 mM Ro 25-6981 and almost blocked following application of 8.0 mM Ro 25-6981. Administration of this

antagonist clearly attenuated the magnitude of the spinal LTP. It seems likely that expression of full LTP in dorsal horn neurons may be dependent on the NMDA receptors containing the NR2B subunit (paper I).

4.3. Paper II

4.3.1. Induction of spinal LTP is blocked by the CaMKII inhibitor AIP

The CaMKII inhibitor AIP was applied directly onto the exposed spinal cord in doses of 0.2 and 2.0 mM. The C-fiber mediated responses were not affected by 0.2 and 2.0 mM of the CaMKII inhibitor AIP alone or by vehicle. However, induction of LTP in single nociceptive dorsal horn neurons, induced by HFS conditioning of the sciatic nerve, was inhibited in a dose-dependent manner following pre-administration of the CaMKII inhibitor AIP. This indicates that CaMKII might be important for the induction of LTP in single nociceptive dorsal horn neurons in the spinal cord (paper II).

4.4. Paper III

4.4.1. Spinal LTP is associated with increased gene expression of Zif268

A transient increase in the gene expression of Zif268 was observed 120 minutes following HFS conditioning applied to the sciatic nerve. The observed increase in the gene expression of Zif268 was about twofold of the expression in the corresponding control. The expression in the control group was at about the same level as the expression in the native group. The increased gene expression of Zif268 120 minutes after conditioning was significantly higher than in the corresponding control. Regarding the gene expression of c-fos and COX2 a different pattern was observed. The expression of c-fos in the control group was significantly decreased at 120 minutes compared to the native group. No significant differences in the gene expression of c-fos or COX2 were observed between the HFS group and the corresponding control- or native group. The results indicate that changes in the gene expression of Zif268 might be associated with maintenance of spinal LTP after HFS conditioning applied to the sciatic nerve (paper III).

4.5. Paper IV

4.5.1. Spinal LTP is associated with increased gene expression of IL-1β, GDNF and iNOS

A significant increase in the gene expression for IL-1β, GDNF and iNOS was observed 360 minutes following HFS conditioning applied to the sciatic nerve. The observed gene expression after HFS were for IL-1β 1.8 fold, for GDNF 1.3 fold and for iNOS 1.6 fold the gene expression in the corresponding control group. For both IL-1β and GDNF, the expression in the corresponding control was on the same level as the expression in the corresponding native group. In contrast, the expression of iNOS in the control group was higher than the expression of iNOS in the native group. However, there were no significant changes in gene expression for p38 MAPK, COX2 or TNFα 360 minutes after HFS conditioning of the sciatic nerve. The observed increase in the expression of the genes for IL-1β, GDNF and iNOS following HFS conditioning indicates that the neuronal events underlying the development of spinal LTP might be associated with an increase in the expression of these genes (paper IV).

5. DISCUSSION OF METHODS

The methods used to investigate the underlying mechanisms for induction and maintenance of LTP include electrophysiological extracellular single cell recordings in intact anaesthetized rats and real-time RT-PCR on spinal cord tissue following HFS conditioning of the sciatic nerve.

5.1. Animals and anaesthesia

All rats were anaesthetized with urethane, which is a commonly used anaesthetic for animals in non-recovery experiments. It is carcinogenic, which precludes its use as a human anaesthetic (Hara and Harris, 2002). The advantages of urethane in animal anaesthesia are that it produces a long-lasting steady level of surgical anaesthesia and, compared to other anaesthetics, has minimal effects on autonomic and cardiovascular systems and spinal reflexes, for review see (Soma, 1983; Maggi and Meli, 1986).

However, in an in vitro model using Xenopus laevis oocytes, it has been shown that urethane potentiates the function of neuronal $\alpha_4\beta_2$ nicotinic acetylcholine ($\alpha_4\beta_2$ neuronal nAch)-, $\alpha_1\beta_2\gamma_{2s}$ γ-aminobutyric $acid_A$ ($\alpha_1\beta_2\gamma_{2s}$ $GABA_A$)- and α_1 glycine receptors. In addition, it inhibits the NR1A/NR2A NMDA- and GluR1/GluR2 AMPA receptors (Hara and Harris, 2002). Thus, urethane seems to affect both inhibitory and excitatory systems, but the magnitude of the effect is less for urethane than the effect observed with other more selective anaesthetics (Hara and Harris, 2002).

Importantly, deep level of urethane anaesthesia has been shown in several studies insufficient to pre-emt LTP induction of C-fiber evoked potentials (Svendsen et al., 1997; Benrath et al., 2004). Thus, we assume that the background urethane anaesthesia does not interfere much with the measurement of electrophysiological responses. However, one cannot completely rule out that the dorsal horn single cell responses produced by nociceptive stimuli or pharmacological manipulation in the urethane anaesthetized animals are the same as those that would have been observed in awake animals (Hara and Harris, 2002).

5.2. Electrophysiological extracellular single cell recordings

Induction of LTP in the dorsal horn of the spinal cord has been shown both in vitro and in vivo using different techniques. Spinal LTP was first demonstrated in vitro in spinal cord slices (Pockett and Figurov, 1993). Today, the patch clamp technique, measuring changes in potentials or currents across the membrane, is the most used in vitro technique (Ikeda et al., 2003).

However, some aspects can only be studied in the entire animal with both the primary afferent nerve fibers and descending pathways intact. Extracellular single cell- or field potential recordings combined with HFS conditioning applied to the sciatic nerve has become a well established method to study LTP in the spinal dorsal horn in intact anaesthetized rats (Liu and Sandkuhler, 1995; Svendsen et al., 1997; 1998; Gjerstad et al., 2001; Afrah et al., 2002).

LTP is defined as long-term increase in synaptic strength. The LTP phenomenon may be demonstrated by measuring the sum of post-synaptic potentials from several cells, i.e. field potentials (Liu and Sandkuhler, 1997) or by the number of action potentials from single cells (Svendsen et al., 1997) in the spinal dorsal horn in response to peripheral stimuli. In our study, extracellular single cell recordings have been utilized because specific cells can be studied and a distinction between laminae can be made.

Single cell recordings were ensured on the basis of amplitude and shape of the action potentials. A successful extracellular single unit recording requires the action potentials from one cell to be clearly separated from the signals from the nearby cells. However, one cannot be absolutely certain that signals from one cell only are studied throughout the whole recording period. Moreover, neurons generating weaker electrical fields will more easily be overlooked.

Single cells used in the experiments were identified by their ability to respond to natural stimuli; brush, fingernail pressure and surgical forceps pinch. As little pinch as possible were used in the searching process to avoid sensitization of the neurons. However, when searching for superficial NS cells, moderate pinching was applied periodically as a search stimulus.

5.3. Investigation of gene expression

In our study, the level of mRNA expression is quantified to investigate the pattern of gene expression following HFS conditioning of the sciatic nerve. Reverse transcription of extracted RNA from the tissue sample followed by real-time RT-PCR is a very sensitive and flexible method for gene expression and quantification analysis, for review see (Bustin, 2000). The choice of candidate genes studied was based on the knowledge from earlier studies on the function of proteins and previous studies on gene- and protein expression observed after induction of LTP in hippocampus.

Spinal cord tissue was harvested by rapid dissection and freezing in liquid nitrogen to prevent degradation of RNA. Gene expression analysis was performed on RNA isolated from a 5 mm piece of the whole lumbar spinal cord in paper III and as a better approach 10 mm piece of the ipsilateral quadrant of the spinal dorsal horn in paper IV. Isolation of the quadrant instead of the whole spinal cord gave an increased specificity in relation to the dorsal horn neurons that is influenced by sciatic nerve conditioning. However, it may be argued that the results would have been even more conclusive if the gene expression was studied in specific laminae.

RNA quality is a critical factor for successful, reproducible and biological relevant gene expression analysis. The RNA molecule, once removed from its cellular environment, is extremely delicate and exposed to degradation by RNAse molecules and contamination under tissue- and RNA isolation. Another factor affecting the accuracy of gene expression analysis is DNA contamination (Zhang et al., 2005a), for review see (Bustin and Nolan, 2004). An important step to secure reliable results is to test the quality of the isolated RNA. Therefore, the RNA quality, in accordance with earlier studies (Zhang et al., 2005a), was evaluated by on chip gel electrophoresis with the Agilent Bioanalyzer. The technique detects the quality of ribosomal RNA which is supposed to be a good measurement for the quality of total RNA. The analyses showed satisfactory RNA quality in all the samples.

The primers used for real-time PCR were designed to span introns to avoid false positive results from amplification of possible genomic DNA contamination. Primer pairs were designed to be complementary to a sequence in the exons on each side of an intron, except for one set of primers, where the forward primer was complementary to the sequence of the

end of one exon and the beginning of the next. The optimal length for single stranded primers is about 18-24 bases with a GC (guanine/cytosine) content of between 40-60%. The melting temperature (Tm) of the primer pair should be between 58-60 ^{o}C and not differ more than 1-2 ^{o}C. The primers used in this project were within these parameters, except the primers used for iNOS. The possibility of hairpin formation, caused by primers being self-complementary, or dimerization, caused by primer pairs being complementary, was minimized under the design. High GC content in the primer pair, especially at the 3' end was avoided as this can lead to false priming. The specificity of the primers was evaluated by BLAST search.

The primer concentration used in the PCR reaction should be kept at an optimal level. Too high primer concentration may promote mispriming and accumulation of non-specific products. Too low primer concentration is on the contrary a smaller problem at real time analysis, as target copy number is calculated at a time point (exponential phase) well before the primer supply is exhausted, for review see (Bustin, 2000). However, even if the specificity of the primers and the parameters for the reaction were optimized, unspecific priming might still have occured. The final PCR product was therefore investigated for formation of unspecific products. A melting curve with fluorescence as a function of temperature was constructed at the end of all PCR reactions to visualize eventually bi-products. Theoretically, some bi-products might have melting points close to the main product and ideally to exclude this, gel electrophoresis may be performed to analyze the amplified PCR product (Zhang et al., 2005a).

To correct for sample to sample variation, i.e. differences in tissue weight and differences in the efficiency of the cDNA synthesis, an internal standard was co-amplified with the target gene. The expression of the target gene was normalized to the expression of the internal standard. Suitable internal standards have a constant expression independent of experimental intervention (Zhang et al., 2005a). The gene for β-actin encodes a ubiquitous cytoskeleton protein and is most likely independent of the conditioning. As expected, pilot studies showed no indications of changes in the expression of β-actin. Hence, β-actin was used as an internal standard.

6. DISCUSSION OF RESULTS

6.1. Induction and maintenance of spinal LTP

Spinal LTP has been studied since 1993 (Pockett and Figurov, 1993; Randic et al., 1993) and is now a well accepted phenomenon that fall into the term central sensitization or vice versa, for review see (Sandkuhler, 2000; Willis, 2002). In accordance with previous studies, we demonstrated that LTP in the spinal dorsal horn in anaesthetized animals can be induced by HFS conditioning of the sciatic nerve (paper I-IV) (Svendsen et al., 1997; Sandkuhler and Liu, 1998; Gjerstad et al., 2001; Afrah et al., 2002). In this work, we used this established method to investigate some of the mechanisms involved in spinal LTP.

Previous studies show that induction of LTP involves activation and modification of receptors and intracellular proteins (Liu and Sandkuhler, 1995; Svendsen et al., 1998; Ikeda et al., 2003; Yang et al., 2004; Xin et al., 2006), which may increase the synaptic strength. The maintenance of LTP, on the other hand, may involve more long-lasting alterations like changes in expression of genes and de novo protein synthesis (Hu et al., 2003). Based on this, we wanted to investigate whether the NMDA receptors containing the NR2B subunit (paper I) and the intracellular CaMKII (paper II) were involved in induction of spinal LTP. In addition, to study the mechanisms for the maintenance of HFS-induced spinal LTP, changes in gene expression of the Zif268, c-fos and COX2 (paper III) and changes in the gene expression of IL-1β, GDNF, iNOS, p38 MAPK, COX2 and TNFα (paper IV) were investigated.

6.2. Induction of spinal LTP and activation of the NMDA-2B receptor

Earlier observations have suggested that spinal LTP involves activation of the NMDA receptors (Randic et al., 1993; Liu and Sandkuhler, 1995; Svendsen et al., 1998; Ikeda et al., 2003). Functional NMDA receptors exist as heteromeric complexes including the essential and ubiquitous NR1 subunit in combination with one or more of the NR2A-NR2D subunits. In addition, some NMDA receptors contain the subunits NR3A and NR3B (Al-Hallaq et al., 2002; Matsuda et al., 2002). Compared to the other subunits, it has been shown that the NR2B subunit has a relatively restricted distribution in nociceptive transmission and pain

regulatory pathways such as in the forebrain (Laurie et al., 1997) and in the superficial dorsal horn of the spinal cord (Yung, 1998; Boyce et al., 1999; Nagy et al., 2004), suggesting that NMDA-2B receptors may play a critical role in nociceptive transmission.

In our study, we applied the selective NMDA-2B antagonist Ro 25-6981 directly onto the spinal cord to investigate the role of the receptor on the induction of LTP in the dorsal horn. Spinal administration of the NMDA-2B receptor antagonist showed a clear antinociceptive effect on spinal dorsal horn neurons and in addition attenuated the magnitude of spinal LTP. These results indicate that activation of the NMDA receptors containing the NR2B subunit may be involved in spinal nociceptive synaptic transmission and important for the induction of spinal LTP.

Supporting our findings, a recent study showed that spinal application of the NMDA-2B antagonist Ro 25-6981 caused a clear antinociceptive effect and a significant inhibition of dorsal horn LTP induced by HFS conditioning applied to the sciatic nerve (Qu et al., 2009). Moreover, previous behavioural findings have indicated that different selective NMDA-2B receptor antagonists possess antinociceptive activity in both acute and chronic pain models (Bernardi et al., 1996; Taniguchi et al., 1997; Sakurada et al., 1998; Boyce et al., 1999; Chizh et al., 2001).

Patch clamp studies of single channels in substantia gelatinosa neurons of spinal cord slices show that NMDA receptors containing the NR2B subunit seem to have higher Ca^{2+}-conductance than other NMDA receptors (Momiyama, 2000). Ca^{2+} is a critical intracellular signaling molecule for triggering plastic changes (Ikeda et al., 2003). The increase in intracellular Ca^{2+}-concentration may lead to activation of Ca^{2+}-dependent enzymes in signal transduction pathways, including CaMKII, important for long-lasting modifications and sensitization.

6.3. Induction of spinal LTP and activation of the intracellular CaMKII

Several lines of evidence demonstrate that induction of LTP in the hippocampus might be dependent on the molecule CaMKII (Ito et al., 1991; Silva et al., 1992; Otmakhov et al., 1997; Giese et al., 1998). Thus, CaMKII, whose activation is dependent on intracellular

Ca^{2+}, has long been considered to be important for LTP in hippocampus. However, since CaMKII is abundantly expressed in the superficial layers of the spinal cord (Bruggemann et al., 2000), it is likely that this molecule plays a role not only in the brain, but also in the superficial laminae of the spinal cord receiving nociceptive input from primary afferents.

In our study, we applied the specific CaMKII inhibitor AIP directly onto the spinal cord to investigate whether the enzyme is involved in the induction of spinal LTP. We observed that LTP in single nociceptive neurons was attenuated in a dose-dependent manner following pre-treatment of AIP. In experiments with no conditioning, no effect of AIP was observed. Our results indicate that CaMKII may be important for the induction of LTP in single nociceptive dorsal horn neurons.

Recent data demonstrating that CaMKII contributes to induction of spinal LTP of field potentials, when the inhibitor is applied before conditioning (Yang et al., 2004), support our results. Moreover, the observation that capsaicin-induced central sensitization is prevented by the CaMKII inhibitor KN-93 further supports the importance of this kinase at the spinal level (Fang et al., 2002).

In some cases CaMKII can be activated and then locked in an active formation. This process may either arise from binding of Ca^{2+}/calmodulin (Hanson et al., 1994) or by binding to the NMDA receptor subunit NR2B (Bayer et al., 2001). In this situation the CaMKII stays active independent of Ca^{2+}/calmodulin binding (Thiel et al., 1988; Bayer et al., 2001), allowing its enzymatic activity to continue long after the Ca^{2+} signal has returned to baseline.

Activation of CaMKII mediates a variety of different cellular responses to calcium, which may affect synaptic plasticity. These include phosphorylation of various receptors and transcription factors (Barria et al., 1997; Fang et al., 2002; Fang et al., 2005a). For example, in the spinal cord, it has been shown that CaMKII activity enhances both the phosphorylation of AMPA receptor subunit GluR1 and the transcription factor CREB during capsaicin-induced central sensitization (Fang et al., 2002; Fang et al., 2005a). Moreover, in hippocampus, CaMKII also promotes incorporation of new AMPA receptors in the membrane (Hayashi et al., 2000).

Taken together, these studies indicate that CaMKII activity might be important for synaptic plasticity, probably through several different cellular responses.

6.4. Spinal LTP and gene expression

Earlier data have shown that the late-phase spinal LTP may be inhibited by the protein synthesis inhibitor anisomycin and cycloheximide (Hu et al., 2003). It has therefore been suggested that maintenance of LTP in the spinal cord may involve altered gene expression and protein synthesis. Changed gene expression may involve at least two steps; i.e. i) changes in expression of genes encoding transcription factors and ii) changes in expression of LRGs encoding other proteins. There are few studies discussing possible changes in the expression of specific genes following HFS-induced spinal LTP. Thus, investigation of gene expression of the transcription factors Zif268 and c-fos and the LRGs encoding IL-1β, GDNF, iNOS, p38 MAPK, COX2 and TNFα following HFS conditioning applied to the sciatic nerve was performed.

6.4.1. Gene expression of Zif268, c-fos and COX2

Previous studies have shown that the expression of Zif268 and c-fos is increased after induction of LTP in hippocampus (Nikolaev et al., 1991; Jones et al., 2001). However, since long-term changes in spinal sensitivity are controlled by similar mechanisms as for hippocampal LTP, for review see (Ji et al., 2003), increased expression of Zif268 and c-fos may also occur at the spinal cord level. Moreover, evidence exists that peripheral inflammation increases the expression of Zif268, c-Fos and COX2 in the spinal cord (Herdegen et al., 1994; Samad et al., 2001).

In our work, we demonstrated a transient increase in the expression of the Zif268 gene 2 hours following HFS conditioning of the sciatic nerve. Thus, our results indicate that a change in the gene expression of Zif268 might be associated with the HFS conditioning-induced spinal LTP. Expression of the Zif268 gene is presumably regulated by different Ca^{2+}-dependent signaling pathways, as several different regulatory regions, including CRE sites, are found close to the promoter region. Previous studies have indicated that induction of the Zif268 gene is essential for stabilization of the late-response LTP in hippocampus

(Jones et al., 2001). The transient increase of the expression of Zif268 observed in our work is consistent with an involvement in the transition from the early- to the protein synthesis-dependent late-phase of spinal LTP. Interestingly, recent studies have shown that the immunoreactivity for Zif268 expressing neurons is up-regulated in the superficial spinal cord 3 hours following induction of LTP (Rygh et al., 2006; Haugan et al., 2008), i.e. approximately at the time point where the early- to late-phase transition is believed to occur (Jones et al., 2001; Hu et al., 2003).

The Zif268 gene encodes a zinc-finger transcription factor and activates the expression of different downstream target genes. Thus, induction of Zif268 may be an important element in altered gene- and protein expression and in this way participate in the signaling cascade required for maintenance of LTP. Moreover, recent data using antisense treatment towards this gene resulted in deficit long-term maintenance of inflammatory hyperalgesia (Rygh et al., 2006). This indicates a role for the Zif268 gene in the process leading to sensitization.

No clear changes were observed for c-fos or COX2 following conditioning. Compared to previous data on c-Fos and COX2 expression in inflammatory pain (Herdegen et al., 1994; Ji and Rupp, 1997; Samad et al., 2001), it was expected to observe an increase in the expression of these genes. Apparently, our data showed that the expression level of c-fos in the control at 2 hours was lower than the expression in the native group. Since the expression of c-Fos and COX2 are considered to be important for noxious stimuli in other models and c-Fos has been used as a marker of activation of nociceptive neurons, it was surprisingly that induction of spinal LTP did not increase the expression of c-fos or COX2. It is possible that this is related to the nature of the noxious stimuli. c-Fos expression may also be suppressed by anaesthesia (Buritova and Besson, 2001).

6.4.2. Gene expression of IL-1β, GDNF, iNOS, p38 MAPK, COX2 and TNFα

In our study, we demonstrated a significant increase in the gene expression of IL-1β, GDNF and iNOS in the ipsilateral dorsal horn compared to the corresponding controls 6 hours following HFS conditioning of the sciatic nerve. The increase in both the C-fiber response and the gene expression of IL-1β, GDNF and iNOS 6 hours following HFS conditioning

applied to the sciatic nerve indicates that spinal LTP might be associated with an increased expression of these genes.

The increased gene expression of the proinflammatory IL-1β observed in our work 6 hours following conditioning is in line with a previous animal study showing an up-regulation of IL-1β in the spinal cord following peripheral inflammation (Raghavendra et al., 2004). Supporting a role for spinal cord IL-1β in sensitization, earlier data have shown that intrathecal administration of IL-1β produces enhanced dorsal horn neuronal response, but also mechanical and thermal hyperalgesia in animal models (Reeve et al., 2000; Sung et al., 2004). Moreover intrathecal administration of IL-1 receptor antagonists reduced inflammatory pain in animals (Zhang et al., 2008).

Earlier studies indicate that IL-1β is mainly induced in glial cells after inflammation (Zhang et al., 2008). Thus, the up-regulation of IL-1β following HFS conditioning may be due to activation of glial cells and increased gene expression of IL-1β in these cells. Supporting this, it is suggested that induction of spinal LTP not only involves activation of neurons, but also activation of glial cells (Ma and Zhao, 2002).

Previous findings have suggested that IL-1β exerts its action through different mechanisms including increased excitatory synaptic transmission, enhanced NMDA-induced currents, decreased inhibitory synaptic transmission, suppressed GABA- and glycine-induced currents, increased CREB phorphorylation (Kawasaki et al., 2008) and increased expression and/or release of other substances like GDNF (Verity et al., 1998; Tanabe et al., 2009), iNOS and NO (Sung et al., 2004; Sung et al., 2005). Interestingly, all these events may influence on nociceptive transmission and contribute to increased synaptic strength. Thus, it has been suggested that IL-1β may be important for development of sensitization in the spinal cord.

GDNF was initially purified from conditioned medium of rat B-49 glial cells as a potent trophic factor for mesencephalic dopaminergic neurons (Lin et al., 1993). Later, it has been shown that GDNF also may be a neurotrophic factor for sensory neurons (Matheson et al., 1997). Interestingly, we observed an increase in the gene expression of GDNF in the spinal cord 6 hours after HFS conditioning of the sciatic nerve. Although the number of

experiments on gene expression was low at 3 hours after condtitioning, we saw a tendency for an increased expression of GDNF already at this time point. Despite different stimulation protocols, this is in line with the results showing an upregulation of the gene for GDNF already at 30 minutes, with a peak at 3 hours (Satake et al., 2000). Our findings suggest that upregulation of mRNA for GDNF 6 hours after conditioning might be associated with spinal LTP.

The GDNF protein has previously been shown to be present mainly in superficial layers, but is also observed in deeper laminae of the spinal dorsal horn (Holstege et al., 1998; Fang et al., 2003). Since this region of the dorsal horn receives many afferent inputs, it seems likely that GDNF are involved in spinal processing of sensory information. However, there are conflicting data about its role in nociceptive transmission. One report describes reduced hyperalgesia in complete Freund's adjuvant-induced inflammation following intrathecal administration of an antibody blocking the GDNF function (Fang et al., 2003). In contrast, intrathecal administration of GDNF showed potent analgesic effects in rat models of neuropathic pain (Boucher et al., 2000; Sakai et al., 2008). Different type of administrations, pain models and dosage may account for these seemingly contradictory observations.
It has been suggested that GDNF is present not only in glia, but also in primary afferent terminals in the dorsal horn (Holstege et al., 1998; Satake et al., 2000). Whether the increased expression of GDNF observed in our work takes place in neurons and/or in glial cells remains to be investigated. However, our data showing an increase in both C-fiber response and gene expression of GDNF following HFS conditioning suggest that increased GDNF gene expression might be associated with spinal LTP.

GDNF acts on neurons and glial cells that express the receptor components for GDNF signaling, i.e. the signal transducing elements RET or neuronal cell adhesion molecule (NCAM) and the ligand binding domain GDNF family receptor α1 (GFRα1) (Bennett et al., 1998; Honda et al., 1999; Paratcha et al., 2003; Jongen et al., 2007). Through activation of the receptor, GDNF is known to trigger multiple signaling pathways and is found to have influence on glial NO production (Chang et al., 2006).

The diffusible messenger NO is produced from L-arginin by three distinct isoforms of NOS, i.e. nNOS (neuronal), eNOS (endothelial) and iNOS. NO is a product of activated neurons

as well as glial cells and exert its effect on nearby cells. While nNOS is found in dorsal horn neurons in superficial laminae, iNOS expression is found to be upregulated in spinal glial cells following inflammation (Maihofner et al., 2000). Thus, the increased gene expression of iNOS observed in our work may result from amplified expression of iNOS in glial cells. Interestingly, it has been suggested that NOS and NO is involved in the LTP of C-fiber evoked field potentials in the spinal cord and thermal hyperalgesia induced by tetanic stimulation (Zhang et al., 2005b).

Our findings revealing an increased mRNA expression of iNOS in the ipsilateral dorsal horn 6 hours after HFS conditioning of the sciatic nerve, is in line with previous observations showing an increased expression of iNOS in the spinal cord with a maximum at 4 hours following hindpaw zymosan injection (Guhring et al., 2001). However, our data also showed that the expression of mRNA for iNOS in the control group at 6 hours was high compared to the native group.

Why the expression of mRNA for iNOS increased in the control experiments, remains to be investigated. The methodological difference between the native group and the control group was the isolation of the sciatic nerve and the time lag of 6 hours. One possible explanation for the increased gene expression for iNOS in the control group compared to the native group may be the result of long-lasting sensory input from the periphery following the surgery and the process of the sciatic nerve isolation. In addition, the expression of iNOS might be influenced by local spinal release of cytokines after splitting the meninges.

Based on previous data showing increased mRNA for COX2 (Beiche et al., 1998; Samad et al., 2001) and TNFα (Raghavendra et al., 2004) in the spinal cord following peripheral inflammation, it was expected to observe an increase in COX2 and TNFα in our LTP model. However, no clear changes in the gene expression of COX2 or TNFα, nor p38 MAPK were observed in our work 6 hours after HFS conditioning of the sciatic nerve, suggesting that spinal LTP may not be associated with clear changes in the expression of these genes.

6.5. Clinical implications of spinal LTP

It has been suggested that the neuronal events leading to spinal LTP may at least in part be important for the development of central sensitization and hyperalgesia, for review see (Sandkuhler, 2000). However, the relevance of LTP induced by HFS conditioning as an underlying mechanism for the development of central sensitization and hyperalgesia observed following inflammation, tissue- or nerve injury may be questioned. The most frequently used form of conditioning to induce LTP in spinal nociceptive superficial and deep dorsal horn is brief electrically HFS (about 100 Hz) of the sciatic nerve (Liu and Sandkuhler, 1995; Svendsen et al., 1997). This form of conditioning has lately been criticized to be supra-maximal of the physiological frequency range of primary afferent C-fibers. However, it has been shown that some C-fibers may reach discharge rates up to approximately 200 Hz (Fang et al., 2005b).

Earlier data demonstrate that in deep dorsal horn WDR neurons HFS (30 or 100 Hz) induces a robust LTP, whereas LFS (3 Hz) conditioning induces a short-lasting LTP (Haugan et al., 2008). However, supporting the relevance of LTP in central sensitization and hyperalgesia, it has recently been shown that LFS (2Hz) conditioning successfully may induce LTP in lamina I neurons projecting to the PAG (Ikeda et al., 2006). Moreover, strong natural stimulation such as inflammation and tissue- and nerve injury has also induced LTP in the spinal dorsal horn (Sandkuhler and Liu, 1998; Rygh et al., 1999; Ikeda et al., 2006).

Interestingly, it has been shown that HFS conditioning of sciatic nerve fibers, which induces LTP at synapses of C-fibers in the spinal cord, has behavioral consequences in rats and causes ipsilateral hind paw thermal hyperalgesia for 6 days (Zhang et al., 2005b) and bilateral mechanical allodynia for 4-6 days (Ying et al., 2006). This suggests that LTP at C-fiber synapses has an impact on nocifensive behavior. In addition, a perceptual correlate to LTP in the nociceptive pathways has been described in humans and further suggests clinincal implications of this phenomenon (Klein et al., 2004).

Finally, superficial NK1 expressing neurons important for the expression of spinal LTP (Ikeda et al., 2006), also seems to be involved in the expression of hyperalgesia in behaving animals (Mantyh et al., 1997; Nichols et al., 1999).

6.6. Future perspectives

Spinal LTP, central sensitization and hyperalgesia seems to involve the same essential elements, i.e. activation of primary afferent C-fibers, induction protocols and signal transduction pathways, for review see (Sandkuhler, 2007). Moreover, the ease with which LTP is induced during inflammation supports the proposal that the mechanisms of LTP are similar to those of central sensitization associated with peripheral inflammation (Vikman et al., 2003). Based on these observations, it is a good support for LTP having a clinical relevance. Furthermore, it has been suggested that induction of spinal LTP by noxious stimulation may be one mechanism whereby acute pain is turned into chronic pain (Klein et al., 2004).

Hence, it is important to further investigate the cellular mechanisms responsible for plasticity within the spinal nociceptive circuits. Moreover, further research may also reveal novel and maybe better therapy to prevent or reverse the development of central plastic changes leading to hyperalgesia.

Taken together, more knowledge about the mechanisms underlying the cellular events leading to hyperalgesia may be important for future treatment of long-lasting pain states.

7. CONCLUSIONS

I. Spinal administration of the NMDA-2B receptor antagonist Ro 25-6981 showed an antinociceptive effect on spinal dorsal horn neuronal activity and clearly attenuated the magnitude of the spinal LTP induced by HFS of the sciatic nerve. These observations indicate that activation of the dorsal horn NMDA-2B receptors may be involved in spinal nociceptive transmission. Furthermore, full expression of LTP in dorsal horn neurons seems to be dependent on activation of these receptors. Our results suggest that activation of the NMDA-2B receptors may be important for induction of LTP in single nociceptive dorsal horn neurons.

II. Induction of LTP in nociceptive dorsal horn neurons, induced by HFS conditioning of the sciatic nerve, was not observed following pre-treatment of the highest dose used of the CaMKII inhibitor AIP. The clear inhibition of LTP by spinal pre-administration of AIP indicates that CaMKII may play an important role in the induction of spinal LTP in single nociceptive dorsal horn neurons.

III. Our data demonstrated a transient increase in the expression of the Zif268 gene 120 minutes following HFS conditioning of the sciatic nerve. In contrast, no clear change was observed in the expression of c-fos and COX2 following HFS conditioning. These results indicate that the transient increase in the expression of Zif268 may be associated with spinal LTP. Since Zif268 is a transcription factor, which controls the expression of other genes, it seems likely that spinal LTP is associated with de novo synthesis of proteins.

IV. The expression of the genes for IL-1β, GDNF and iNOS significantly increased in the ipsilateral spinal dorsal horn 360 minutes following HFS conditioning of

the sciatic nerve. No clear changes were observed in the gene expression for p38 MAPK, COX2 and TNFα. The increase in both the C-fiber response and the gene expression of IL-1β, GDNF and iNOS following HFS conditioning indicate that induction of LTP might be associated with changes in the expression of these genes. The increase in the gene expression of IL-1β, GDNF and iNOS is consistent with the hypothesis that these genes might be associated with maintenance of spinal LTP. Whether or not there is a causal relationship between up-regulation of IL-1β, GDNF or iNOS and maintenance of LTP remains to be investigated.

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