Thesis for the master degree in molecular biology

"Induction of long-term potentiation in dorsal horn neurons and expression of immediate early genes Zif and Arc"

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Preface

This project leads to a master degree in molecular biology at the University of Oslo. The laboratory work was performed at the National Institute of Occupational Health (STAMI), Oslo, Norway.

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Abbreviations

ACC:	Anterior cingulated cortex
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
Arc:	Activity-regulated cytoskeleton-associated protein
BDNF :	Brain-derived neurotrophic factor
bp:	Base pairs
cAMP:	Cyclic adenosine mono phosphate
CaMK II:	Calcium calmodulin dependent protein kinase II
CNS:	Central nervous system
CRE:	Cyclic AMP-response element
CREB:	Cyclic AMP-response element binding protein
Ct:	Threshold cycle
DEPC:	Dietypyrocarbonate
EDTA:	Ethylen-diamine-tetra-acetous -acid
ERK:	Extracellular signal-regulated protein kinase
EPSP:	Excitatory post synaptic potential
GTP:	Guanosine 5'triphosphate
HFS:	High frequency stimulation
IEG:	Immediate early genes
LTP:	Long-term potentiation
LTD:	Long-term depression
NK1:	Neuro kinin 1
NMDA:	*N*-methyl-D-aspartate
MAPK:	Mitogen activated protein kinase
mGluR:	Metabotropic glutamate receptors
PCR:	Polymerase chain reaction
PKA:	cAMP-dependent protein kinase A
RT:	Reverse transcriptase
TE:	Tris/EDTA

Abstract

Increased pain response following injury is an important step in the process of protecting the injured site from further damage. However, pain can often be a more serious problem than the injury or disease process that initiated it. In postoperative-, neuropathic- and chronic inflammatory pain, the pain often causes long lasting suffering for the patients, without any clear adaptive function.

Increased sensitivity to stimuli may be a result of not only the activity in the primary afferent fibers, but also functional changes within the CNS, i.e. central sensitization. In central sensitization, responses to stimulation of sensory receptors are enhanced without any change in excitability of the primary afferent neurons. One form of central sensitization may be spinal long-term potentiation (LTP).

In this project, electrophysiological recordings of single cell activity was performed before and after LTP induction in dorsal horn neurons by high frequency stimuli (HFS) conditioning. Quantitative real-time RT-PCR was used to examine whether the induction of spinal LTP was associated with changes in expression of two immediate early genes (IEG), i.e. Zif and Arc. The LTP-associated Zif and Arc expression was measured at three time points, immediately after surgery, 30 minutes after HFS and 120 minutes after HFS.

HFS-induced LTP in dorsal horn neurons outlasted the duration of the experiments (2-6 hours). Zif showed an approximately twofold increase of expression 120 minutes after HFS, suggesting the involvement for this transcription factor in the transition from early- to late-phase LTP. The expression of Arc however, was not altered following the stimulation. It is concluded that Zif, but not Arc, is upregulated in spinal cord neurons in association with HFS-induced LTP, indicating a role for this gene in the transition from early- to late-phase LTP.

1. Introduction

1.1. Pain perception

The perception of pain is, like hearing, smelling, seeing and touching, a source of information about the environment of which the organism needs to behaviorally adapt to. The evolutionary importance of pain perception is evident in that even simple multicellular creatures can react to noxious stimuli by a withdrawal response. Higher organisms perceive noxious stimuli as pain, though the reaction to the stimulus is still withdrawal, or protection of the stimulated area. Being a subjective experience, pain is defined by the IASP (The International Association for the Study of Pain) as "*an unpleasant sensory and emotional experience associated with actual or possible tissue damage, or is described in terms of such damage*" (Ji et al. 2003; Merskey 1979; Walters 1994).

The sensation of pain has evolved to protect our body from damage internally and externally. Minor injuries like cuts and burns are frequent during every day life activities. The inflammatory process activated during the tissue damage increases pain sensation and so limits the activity of the injured part of the body. Activation of receptors in the peripheral nerve endings responding to noxious stimuli, i.e. the nociceptors, and the psychological experience of pain may however not coincide. It is possible to feel pain even though no nociceptors are activated as well as feeling no pain when nociceptors are in fact activated. These conditions may be explained by the ability of the central nervous system (CNS) to filter information before it reaches the consciousness (Brodal 2001a; Merskey 1979).

Although an increased pain response following injury is the organism's way of protecting the injured site from further damage, pain can often be a more serious problem than the injury or disease process that initiated it. Postoperative-, neuropathic- and chronic inflammatory pains are examples of such. In these cases the pain often causes long lasting suffering for the affected patients, without any clear adaptive function. By pre-treatment with presynaptic inhibitory drugs (such as μ-opiates) spinal cord sensitization can be attenuated and postoperative pain can be subdued (Terman et al. 2001). The requirement for more detailed understanding of the mechanisms of pain is obvious, in order to provide more efficient methods of treatment.

1.2. The somatosensory system

In order to sense its surroundings, an organism is equipped with various receptors, which transform external stimuli into nerve signals. The nociceptors responding to noxious stimuli are polymodal. They can react to diverse stimuli, like mechanical -, thermal - or chemical input. Each nociceptor has its own receptive field, defined as the area of the body from which a certain receptor intercepts signals.

Spinal nerves terminating in the spinal cord serves as the connection of the spinal cord with the rest of the body. A cross section of the spinal cord will reveal a characteristic "butterfly" pattern of gray and white, the gray and white matter. The gray matter consists mostly of cell bodies while the white color is due to high abundance of myelinized axons. There are three main types of nerve cells in the spinal cord. 1) Cells with axons leading to the peripheral organs, 2) cells with axons leading to higher levels of the CNS and 3) cells with axons leading to other parts of the spinal cord. The spinal cord is connected to the brain just above the first neck vertebra (Brodal 2001c).

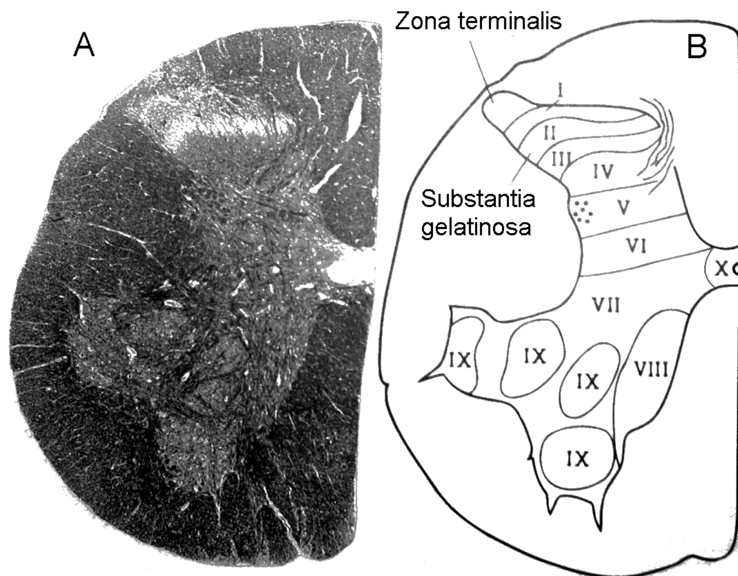

Fig. 1. Cross-section of the dorsal horn. A: Picture of cell and myelin staining. B: Diagram displaying the different laminae and their position in the dorsal horn. Lamina II, the substantia gelatinosa, pointed out in B, can be seen as a white zone on the picture in A. Adapted from (Brodal 2001c).

The spinal cord is made up by laminae, layers of cells that are different in anatomy and function (see Fig. 1). Ten different laminae can be identified in the spinal cord. Laminae I-VI constitute the dorsal horn, lamina VII is the transition between the dorsal and ventral horn, laminae VIII-IX comprise the ventral horn. Lamina II is also termed substantia gelatinosa and is involved in noxious stimuli input and regulation. Lamina X is the grey matter around the central canal, also found to be of some importance for the processing of noxious input. Cells with soma in one lamina may well have their dendritic tree ascending to higher laminae (Brodal 2001c; Brown 1981a).

The myelinated nerve fibers are called A-fibers, while the non-myelinated fibers are called C-fibers. Axons of larger diameter, the myelinated A-fibers, are further divided into Aα, Aβ and Aδ by decreasing diameter. The first two types react to stimuli like brush and touch, i.e. light innoxious stimuli from low threshold mechanoreceptors in muscles, joints and skin. Aα- and Aβ-fibers generally transmit their input to deeper layers of the spinal cord, lamina III-VI (Brodal 2001e; Brown 1981b).

Noxious stimuli are conducted through the Aδ- and C-fibers from the place of origin to the spinal cord. The sensory information from the lower body afferent fibers reaches the lumbar spinal cord, while the sensory information from the upper body (excluding the face) enters the thoracic and cervical spinal cord. The C-fibers give input to the dorsal horn to laminae I and II, but also V and X. Some neurons have dendritic trees linking laminae I and II to the deeper layers. Although C-fiber afferents terminate mostly in laminae I and II, the soma of their connecting second order neurons projecting to the brain, may be found in deeper laminae. The thin C-fibers split almost immediately into one short descending and one short ascending nerve branch continuing only one or two segments up or down. Branches from these fibers enter the grey substance in the dorsal horn ipsilaterally, forming synapses with other neurons. Because of the descending modulatory system, the dorsal horn strongly influence the regulation of information from nociceptors transmitted to higher order neurons in the central nervous system. Information from laminae II is transmitted to second-order projection neurons in laminae IV, V, and VI. These neurons receive direct innervations from the primary afferent fibers. The axons of these second order neurons ascend to the brainstem and thalamus through the spinothalamic tract, together with second-order neurons from laminae I, forming the anterolateral system. From thalamus the signals are lead to the primary somatic sensory cortex (Brodal 2001c; Brown 1981a; Purves 2001).

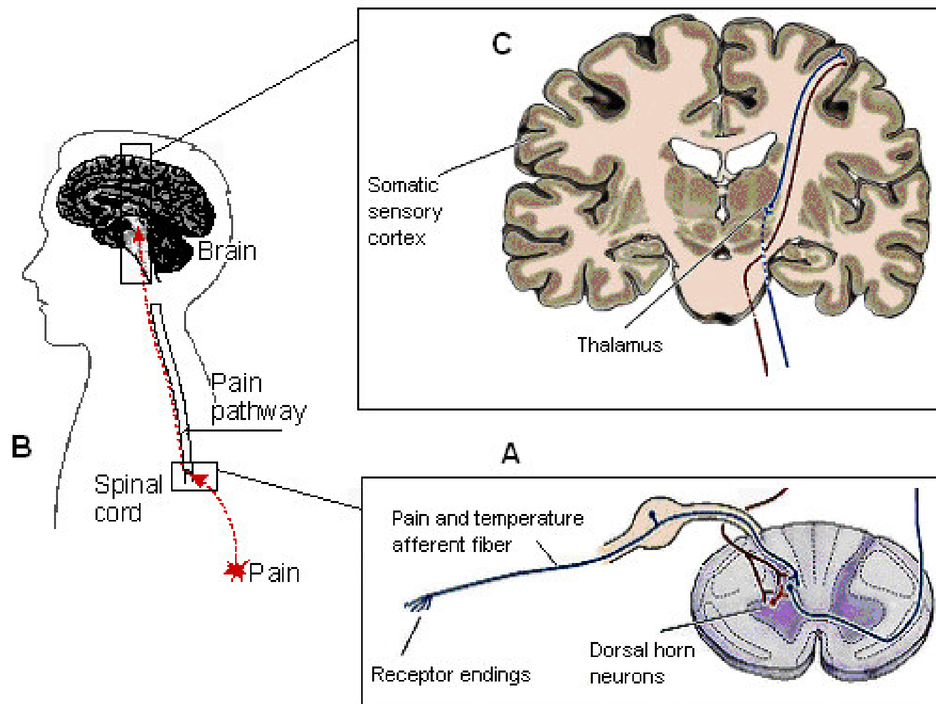

Fig. 2. Figure of the pain pathway form the periphery to the brain. A: The receptor endings transduce noxious stimuli to nerve signals. The stimulus is conducted through the afferent fiber (blue) into the gray matter of the dorsal horn where it connects to a second order projecting neuron and to the ascending fibers (blue). B: A schematic overview of the pain pathway. Painful input from the periphery reaches the spinal cord and is subsequently conducted to the brain. C: The ascending pathways reach the brain in the thalamus and connect with interneurons conducting the signal to the somatic sensory cortex. (Adapted from (Purves 2001).)

The regions of the cortex involved in processing of nociceptive stimuli in man and animals are the anterior cingulated cortex (ACC) and insula. Particularly the ACC has been proposed to contribute to the perception of pain, the learning process of avoiding noxious stimuli as well as phantom limb pain. Patients with lesions in the ACC have reported a reduction in chronic pain (Wei and Zhuo 2001).

1.3. Synapses in the CNS

In general, the transmission of a signal from one nerve to another happens in the synapse. This connection between the presynaptic and the postsynaptic nerve serves as an important site for regulating signal transduction and enabling the plasticity of the nervous system. Neurotransmitters, like glutamate, are released in vesicles by the presynaptic neuron. The

neurotransmitter diffuses across the gap and binds to receptors on the postsynaptic neuron. Several receptors are present in the postsynaptic membrane, both slow acting, modulating metabotropic receptors and faster acting ionotropic receptors. Binding of neurotransmitters to postsynaptic receptors leads to opening of various channels, influx of Na^+, K^+ and Ca^{2+} and subsequent rise of membrane potential.

The most abundant groups of receptors in the CNS are the glutamate receptors. This group of receptors mediates permeability for different ions and is excitatory. The glutamate receptors are divided in three main groups; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors, metabotropic glutamate receptors (mGluR) and *N*-methyl-D-aspartate (NMDA) receptors (Brodal 2001b).

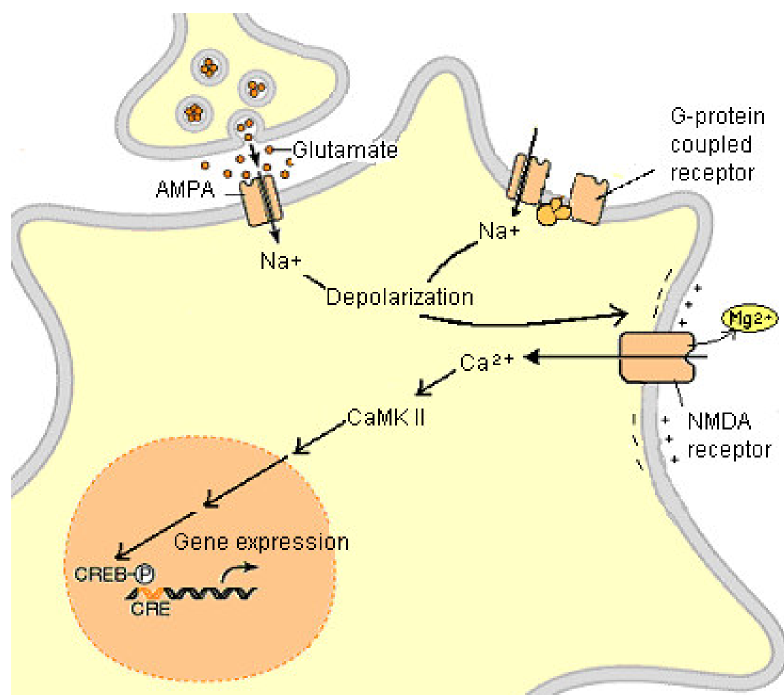

Fig. 3: Diagram of a synapse. The presynaptic neuron releases the neurotransmitter glutamate. This opens AMPA receptors that let Na^+ into the cell. Influx of Na^+ through both AMPA receptors and G-protein coupled receptors depolarizes the cell membrane, removing the Mg^{2+} block from the NMDA receptor and making it permeable to Ca^{2+}. Ca^{2+} in turn activates CaMK II eventually causing CREB phosphorylation and changes in gene expression.

Ionotropic receptors are ligand gated ion channels activated by binding of glutamate. Metabotropic receptors are coupled to G-proteins and activate signaling cascades by second messengers (Elliot 2001a). AMPA receptors are Na^+ permeable ion channels communicating fast and precise excitatory signals in the CNS. These receptors account for the majority of inward current for generating synaptic responses when the membrane potential of the cell is close to the resting potential (Malenka and Nicoll 1999). Metabotropic receptors like mGlu1-5 and NK1 are also found in many spinal excitatory synapses. NMDA receptors are ionotropic receptors, like AMPA receptors, but stand out because of some of their characteristics. First; the NMDA receptors are more permeable to Ca^{2+} than Na^+. Being an important intracellular signal, Ca^{2+} can initiate processes leading to long lasting changes in excitability in the postsynaptic neuron. Second; the NMDA receptors are voltage dependent. They do not open by binding of glutamate alone, unless the membrane is already depolarized. Mg^{2+} ions block the NMDA receptors in their resting state, however these ions dissociates from their binding sites by depolarization of the membrane. Third; an opened NMDA channel leads to a longer lasting Ca^{2+} influx than by normal EPSP. Activation of the AMPA-, mGlu-, NK1- and NMDA receptors may contribute to the generation of long-lasting changes in synaptic activity (Brodal 2001d; Liu and Sandkühler 1997; Malenka and Nicoll 1999; Platenik et al. 2000).

The strength of a synapse is not fixed, but varies both in response to changes in transmitter release from the presynaptic site and postsynaptic membrane receptor response to released transmitters (Ji et al. 2003). Increased postsynaptic ionic influx must be reversed in order to generate a new action potential. The Na, Na/K, Ca and H ATPases are important plasmatic membrane ion pumps maintaining the membrane potential at resting values. A noxious stimulus induces an increased discharge frequency in spinal dorsal horn neurons. Increased ATPase activity in the dorsal horn induced in a short time, is associated with and can be considered a marker of nociceptive neuronal activity (Czaplinski et al. 2005).

1.4. Peripheral - and central sensitization

Increased sensitivity and excitability of peripheral nociceptive nerve endings, by altered kinetics and reduced threshold, is known as peripheral sensitization. An example is the increased pain sensitivity after hot water burn, where water at 37 degrees feels burning hot.

Peripheral sensitization is also observed after injuries and inflammatory diseases. The peripheral increase of sensitivity is restricted to the inflammatory area (Ji et al. 2003; Ko et al. 2005). However, increased sensitivity may also be a result of functional changes within the CNS, i.e. central sensitization. In central sensitization, responses to stimulation of sensory receptors are enhanced without any change in excitability of the primary afferent neurons (Willis 2002). The term not only reflects synaptic strengthening, but also changes in the intrinsic membrane properties and/or neuronal networks (Ji et al. 2003). Woolf et al. described this phenomenon in spinal cord in the 1980s (Wall and Woolf 1984; Woolf 1983).

1.5. Long-term potentiation

Long-term potentiation (LTP) of synaptic strength in the hippocampus was discovered in 1966 and later described in detail by Bliss and Lømo in 1973 (Bliss and Lømo 1973). This long lasting increase in synaptic strength represents a cellular storage of sensory information.

Increased synaptic strength in the hippocampus following repetitive synaptic stimulation leading to LTP usually only occurs in the set of synapses being stimulated, rather than other synapses on the same cell. This specificity is an advantage, considering that it greatly increases the storage capacity of individual neurons. However, LTP can also be associative, which means that strong activation of one set of synapses can induce LTP in an independent, adjacent set of active synapses on the same cell. This associativity has been suggested as a cellular analogy of associative learning (Malenka and Nicoll 1999; Sandkühler 2000).

It has been suggested that LTP is involved in learning and memory in the hippocampus (Morris et al. 1986), fear conditioning in the amygdale (Rogan et al. 1997) and nociceptive processing in the spinal cord (Gjerstad et al. 2001). The increase in synaptic strength in dorsal horn neurons of the spinal cord may represent a somatosensory primitive form for "cellular learning and memory". Due to massive interest in the function of learning and memory, LTP has been studied extensively in the hippocampus. Understanding the molecular mechanisms underlying pain and sensitization is also an interesting and important field, requiring more studies of LTP in the dorsal horn. LTP of C-fiber evoked potentials in dorsal horn neurons may contribute to the phenomenon of hyperalgesia (Liu and Sandkühler 1997). Thus, it is

believed that LTP is part of the wider term central sensitization (Fang et al. 2002; Rygh 2002).

Somatic noxious stimulation of one part of the body can inhibit noxious spinal responses from other parts of the body in intact, but not in spinalized (reversible cold block of descending pathways) rats (Gjerstad et al. 1999). These data suggest that supra spinal and/or upper cervical cord mechanisms are important in mediating nociceptive inhibition following acupuncture or somatic noxious stimulation. Moreover, spinalized rats show a marked reduction of LTP intensity, compared to non-spinalized rats (Gjerstad et al. 2001). The level of attention and stress influences the activity in the descending pathways, thus emphasizing the importance of psychological factors in chronic pain syndromes (Rygh 2002).

The mechanisms behind LTP in different tissues are probably not identical from one tissue to another. Low frequency Aδ-fiber stimulation has reversed LTP in hippocampus and depressed LTP in spinal cord, although the time delay after which such stimulation can alter LTP differs. NK1 receptors are involved in spinal LTP but not in hippocampal LTP. These observations indicate that the mechanisms underlying reversal of LTP in hippocampus and LTP depression in spinal cord may be different (Liu and Sandkühler 1997; Zhang et al. 2001).

1.6. Plastic properties of the central nervous system

The nerves may store information for longer periods of time by changing their synaptic plasticity (Rygh 2002). Different cellular and molecular mechanisms underlie the distinct temporal phases of LTP. It has been suggested that while induction of LTP requires postsynaptic influx of calcium and activation of protein kinases, later phases leading to long lasting change depend on gene transcription and protein synthesis (Jones et al. 2001). Different stages of plasticity include diverse mechanisms. Activation of ion channels, both receptor-mediated and voltage controlled, leads to influx of positive ions, phosphorylation of intracellular signal proteins and eventually possible induction of gene expression (Platenik et al. 2000).

In the spinal cord both glutamate and substance P are released from C-fibers following noxious stimulation. Activation of the NK1-, NMDA-receptor currents leads to a marked rise

in Ca^{2+} levels, a trigger for LTP induction. The calcium ion is a universal and versatile second messenger. The concentration of Ca^{2+} is able to increase highly localized or throughout the whole cell (Ikeda et al. 2003; Platenik et al. 2000). As a local source, the increase in Ca^{2+} within the dendritic spine accounts for the specificity of LTP (Malenka and Nicoll 1999). Calcium calmodulin dependent protein kinase II (CaMK II) is found throughout the CNS and is believed to be a key component in induction of spinal LTP (Pedersen et al. 2005). This protein is activated by the influx of Ca^{2+} and regulates the calcium signaling in synapses by phosphorylating various proteins such as membrane receptors. An important property of CaMK II is that its activity no longer depends on elevated Ca^{2+} levels after it is autophosphorylated and thus in its active state (Elgersma and Silva 1999; Malenka and Nicoll 1999). Phosphorylating such molecules may lead to further activation of the synapse, or make the synapse more sensitive for further stimuli.

In hippocampus, blocking the NMDA receptor inhibits LTP and leads to marked reduction in gene expression (Cole et al. 1989; Link et al. 1995). The Ca^{2+} dependent pathways also seem to be important for the induction of certain forms of long-term changes in synaptic function in the dorsal horn (Malenka and Nicoll 1999). Disruption of receptors contributing to the mechanisms underlying synaptic plasticity, i.e. the NMDA receptor, impairs learning in the hippocampus.(Elgersma and Silva 1999)

1.7. Immediate early genes

When mammalian cells, including neuronal cells, react to external stimulation by alteration of gene transcription, two sets of genes can be distinguished. A number of genes are activated rapidly, yet transiently, thereby termed immediate early genes. Others show delayed induction and are termed late-response genes or target genes (Platenik et al. 2000). The term 'immediate early genes' is derived from lifecycle analysis of animal viruses, originally described as oncogenes required for replication and development of tumors. It was later shown that these genes responded to mitogenes or growth factors and was present in the DNA of all vertebrates (Davis et al. 2003; Lanahan and Worley 1998).

The IEGs are expressed within minutes after stimulation and can activate other downstream targets. IEGs are described as either 'regulatory' or 'effector' IEGs. The regulatory IEGs

encode transcription factors that can bind to DNA and may increase or decrease downstream gene expression. Effector IEGs encode proteins with a more direct functional role at the synapse. An estimated 30-40 genes (Lanahan and Worley 1998) are neuronal IEG. Of these 10-15 are regulatory genes, the rest are effector genes.

Eukaryotic gene expression is controlled at different levels, however the rate of initiation of new mRNA transcripts and the processing of precursor mRNA transcripts to mature mRNAs are the most sensitive to regulation. Molecules involved in this regulation are small DNA elements controlling cell specific transcription and transcription factors that binds to these elements. Transcription factors are in general proteins that bind to a specific DNA sequence in a promoter or enhancer region of a gene, activating or suppressing the transcription of this gene. The different levels of regulational control give rise to the great molecular and cellular diversity (Davis et al. 2003; Hall 1992; Lanahan and Worley 1998).

In the brain, many of the hormones and neuromodulators that regulate neuronal synaptic activity binds to G-coupled cell surface receptors linked to adenylate cyclase, an enzyme synthesizing cyclic AMP (cAMP). For these neuromodulators, cAMP is the intracellular second messenger signaling their presence. Through a signaling cascade of cAMP-dependent protein kinase A (PKA) and PKA-C binding, the cAMP response element binding protein (CREB) can be phosphorylated. Phosphorylated CREB can then bind to the regulatory region (the promoter) of a target gene and lead to transcription (see Fig. 4) (Hall 1992).

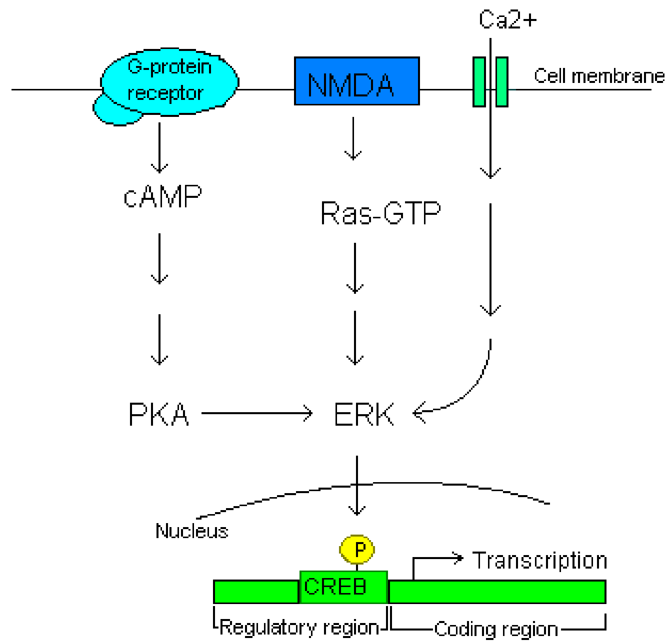

Fig. 4. Diagram of signaling transduction resulting in increased gene transcription. G-protein coupled receptors can activate intracellular messenger cAMP, leading to activation of PKA and subsequent ERK activation. The NMDA receptor may activate the Ras/MAPK pathway, leading to ERK activation resulting in CREB phosphorylation and gene transcription. Ca^{2+} may also contribute to the activation of ERK. (Adapted from (Elliot 2001b).)

In rodents, activation of ionotropic and metabotropic receptors by C-fiber nociceptor afferents activates various signal transduction pathways in dorsal horn neurons. NMDA receptors are involved in activating the Ras/MAPK pathway for gene expression by contributing to the GTP bound active state of the small GTPase Ras, which transmits the signal to a cytosolic cascade of protein kinases. A rise in intracellular Ca^{2+} concentrations, by influx of extracellular Ca^{2+} may also be required for induction of the Ras/MAPK pathway (Lever et al. 2003). The final effector kinase, the extracellular signal-regulated protein kinase (ERK), which is a member of the MAP kinase superfamily, then leads to CREB-mediated transcription regulation (see Fig. 4) (Platenik et al. 2000).

In hippocampus ERK may also be activated by brain derived neurotropic factor (BDNF) (Ying et al. 2002). This may possibly lead to both short-term hypersensitivity (Ji et al. 1999) and long-term sensory changes in synaptic plasticity, thus contributing to central sensitization in dorsal horn neurons. The observed long lasting changes in sensory processing may be due

to regulation of CREB-mediated transcription (Ji et al. 1999; Kawasaki et al. 2004). CRE sites are found on the promoter regions of many genes, among them the IEGs. The binding of phosphorylated CREB of the CRE-region leads to transcription of the genes following the promoter. In addition to a short-lived (<1h) contribution to central sensitization, ERK is involved in induction and maintenance of inflammatory pain, hence ERK is an important intracellular controller of activity dependent synaptic plasticity in the spinal cord (Ji et al. 2002; Kawasaki et al. 2004).

1.8. IEGs and LTP

Various conditioning stimuli have been used to initiate LTP and study the expression of IEG in the CNS. Noxious heat-, mechanical- or inflammatory stimuli in peripheral tissues activate IEGs in spinal dorsal horn neurons (Rahman et al. 2002). DNA microarrays have been used to investigate expression of thousands of genes following spinal cord injury. Early stages after injury include upregulation of genes involved in transcription and inflammation. An upregulation in IEG expression is observed in the acute phase of injury (minutes to days after injury) and continue in the subsequent days (Bareyre and Schwab 2003).

The maintenance of LTP may have different mechanisms according to its origination pathway (Roberts et al. 1996). The expression of the IEG c-Fos in the different laminae of the dorsal horn was influenced by the type of stimulus model used (inflammatory pain, arthritis induction or neuropathic pain) (Munglani and Hunt 1995). Moreover, different combinations of genes can be induced depending on the site of stimulation and the synaptic pharmacology (Wisden et al. 1990).

More than 100 molecules have been suggested as mediators for, or modulators of, hippocampal LTP. Many of those molecules may also be involved in spinal central sensitization (Sanes and Lichtman 1999). LTP in hippocampal slices leads to activation of CREB both in early stages as well as late stages of LTP (Leutgeb et al. 2005). Being an immediate early gene inducer, CREB activation may lead to upregulation of many IEGs. Estimates by Guzowski et al., suggest that perhaps 30 IEGs are induced in hippocampal neurons in association with LTP (Guzowski et al. 2000). Upregulation of IEG proteins

following conditioning stimuli have been shown in numerous studies. However, whether the increased level of mRNA is due to transcriptional activation or modulation of mRNA stability is not clear (Link et al. 1995; Wisden et al. 1990).

In the field of plasticity, the genes Zif and Arc are the most extensively studied, both considered to play an important role in memory (Guzowski et al. 2000; Jones et al. 2001). No previous studies have concentrated on the expression of these genes during induction and maintenance of LTP in the spinal cord.

1.9. Zif

The gene Zif (also referred to as Zif/268, NGFI-A, Krox 24, TIS8, Egr-1 or Zenk) was discovered due to its responsiveness to growth factor and serum treatment. The gene is regulatory, encodes a zinc finger transcription factor and is a member of the Egr family. Depending on the initiation sequence, the encoded Zif protein is 82 or 88 kDa in size.

In the rat brain Zif is constitutively expressed in the neocortex, primary olfactory and entorhinal cortices, amygdaloid nuclei, nucleus accumbens, striatum, cerebellar cortex and the hippocampus. Seizure activity, electroconvulsive shock, brain injury, nerve transaction, neurodegeneration, physiological stress and noxious stimuli, among others, have been shown to participate in regulation of Zif (Davis et al. 2003). Zif has been identified in monkey brain to participate in long-term memory (Miyashita et al. 1998). It is likely that the same gene is involved in long-term memory in humans, as the two species are so closely related.

Several different regulatory regions, including CRE sites, are found close to the promoter region of Zif, suggesting several different signaling pathways and kinases contributing to its expression. Various receptors may activate these pathways, including glutamatergic receptors (Davis et al. 2003).

It has been suggested that the Zif gene is essential in stabilizing synaptic plasticity in the hippocampus and for the expression of hippocampal and non-hippocampal forms of long-term memory. Zif has been reported to show upregulation from 10 minutes to 3 hours after conditioning of CNS tissue. In mice with a targeted inactivation of Zif, LTP in hippocampus

could not be maintained, probably due to a failure in the synthesis of downstream effector proteins encoded by genes for which Zif is an obligatory transcription factor (Jones et al. 2001; Roberts et al. 1996). The increased expression of Zif is induced more frequently with increased stimulus intensity. The minimum stimulus intensity required for induction of LTP is similar to that required to produce an increase of Zif mRNA (Cole et al. 1989). Zif upregulation has been found to correlate with the persistence of LTP rather than the magnitude of induction. However, induction of Zif is probably not necessary for all types of synaptic plasticity in all regions of the CNS (Davis et al. 2003).

Zif knock out mice has been used to show a direct connection between Zif expression and behavioral responses following persistent inflammatory pain, but not to acute noxious stimuli (Ko et al. 2005). Endomorphines has been known to reduce the expression of Zif by binding to mu-opiois receptors and suppressing the processing of nociceptive information in the CNS (Tateyama et al. 2002). Activation of ERK proteins has been shown to be spatially coincident with onset of Zif induction, (Sgambato et al. 1998) supporting a hypothesis of NMDA dependent ERK activation leading to CREB mediated Zif induction (Otahara et al. 2003). The activation of Zif may be NMDA dependent as NMDA has been suggested to induce ERK dependent transcription (Platenik et al. 2000).

Some characteristics are similar between Zif and Arc although others are radically different. Both genes share similarities of the promoter binding sites and both may be activated by the MAP kinase pathway. A difference however, is that Zif is a strictly nuclear mRNA, while Arc is located in the nucleus as well as the dendrites (Davis et al. 2003).

1.10. Arc

Lyford et al. and Link et al. first reported Arc (also termed Arg3.1.) in 1995. Lyford and colleagues termed the IEG activity-regulated cytoskeleton-associated protein (Arc) due to its subplasmalemmal cortex enrichment in cell bodies and dendrites, and subsequent co-localization with the actin cytoskeletal matrix (Lyford et al. 1995). The nuclear Arc mRNA is transported to the dendrites, where local synthesis of the protein occurs. There is some evidence that mRNA may be stored in storage granules in a translationally dormant state, awaiting a cellular triggering signal for its release (Håvik et al. 2003). After induction, Arc

mRNA is rapidly distributed throughout the dendritic tree and localizes to discrete regions that have received direct synaptic stimulation. This makes Arc unique among known IEGs (Guzowski et al. 2000; Steward et al. 1998).

In contrast to Zif, Arc is an effector IEG believed to act more directly on cellular function at the synapse. The differences between Zif and Arc became clear in experiments using BDNF-induced plasticity in the dentate gyrus (Ying et al. 2002), which induced Arc but not Zif expression, via the MAP kinase pathway (Davis et al. 2003). Local translation in the dendrites may be induced by BDNF and raises the possibility that the neurotrophin can induce activity-dependent increases in mRNA levels, which may affect synaptic efficiency. Support for the theory about local translation is found in that LTP induced by BDNF can be obtained in synapses severed from their cell bodies (Yin et al. 2002).

High frequency stimulation (HFS) of brain perforant path synapses both induces expression of Arc and causes newly synthesized Arc mRNA to localize selectively to the synaptically activated lamina as it migrates into the dendrites. The exact Arc mRNA localization pattern differs depending on the synapses activated and is not inhibited by protein synthesis inhibitors suggesting a localization signal within the mRNA sequence itself. Neurons possess mechanisms for both routing and localization, the latter mediated by signals generated by synaptic activation (Steward et al. 1998).

Arc is conserved across species (investigated by (Lyford et al. 1995) in rat, mouse and human), indicating an essential role for its gene product. The human ARC gene is located to chromosome 8 (Nagase et al. 1996). The fact tat this gene is present in both humans and rats makes it a good target for rat-model studies.

1.11. Project objective

In order to provide better treatment, there is an obvious need for a better understanding of the molecular mechanisms underlying pain. LTP in the CNS has been associated with central sensitization and has been extensively studied in various brain structures as well as the spinal cord. Central sensitization is linked to various pain states such as postoperative-, neuropathic-

and chronic inflammatory pains. Understanding the molecular mechanisms could provide more specialized pharmaca, with less pronounced side effects and a better quality of life for the patient.

LTP has been most extensively studied in the hippocampus and other brain regions due to its contribution to memory. Central sensitization or LTP in the spinal cord may be induced by a variety of stimuli. Here a model of HFS induced LTP is used to study spinal dorsal horn plasticity.

Although many studies have shown the importance of IEGs in the hippocampus, these genes have not been excessively studied in the dorsal horn. There is however a general consensus that immediate early genes are in fact participating in the maintenance of long-term plastic changes in the brain. The present work focuses on spinal expression of two IEGs, Zif and Arc, known to be upregulated in other parts of the CNS following LTP.

The thesis is divided in two main parts, initially addressing the physiological phenomenon LTP in the dorsal horn, and further if this is associated with altered expression of immediate early genes. The functional change in neuronal activity in the dorsal horn was studied by electrophysiological extracellular single unit recordings. Zif and Arc gene expression in the spinal cord was analyzed by quantitative real-time RT-PCR. The main question for this project is; is LTP in the spinal cord associated with upregulation of the immediate early genes Zif and Arc?

2. Materials and methods

All animal experiments were approved by The Norwegian Committee for Animal Research and were in accordance with European Community Council Directives.

2.1. Animals

Adult female Sprague-Dawley rats from Scanbur BK AB, Sweden (Scan: SPRD), weighing between 240 and 360 g were used in all experiments. After arrival the rats were housed in standard cages 25 x 41 x 15 cm (four rats in one cage). Rats of both sexes were kept in the same room. Food (Beekay feeds, B & K Universal, Nittedal, Norway) and water (tap water, Oslo) was freely accessible. One week of acclimatization was allowed before experiments. The air temperature and the relative humidity at were kept at 20-22 ºC and 50-55 %, respectively. The air ventilation rate was 15x the room volume pr hour. The rats were kept under an artificial light/dark cycle of 12 h /12 h (6:00pm-6:00am). All experiments were performed during the light period. A total of 54 rats were used in this project.

2.2. Surgery

The rats were anesthetized by intraperitoneal injections (i.p) of urethane (250 mg/ml, 1.6-1.8 g/kg bodyweight). To avoid overdose the anesthesia was administered by 4-8 injections; initial dose 1ml, subsequent doses 0.2 ml. Adequate anesthesia was indicated by absence of hind paw withdrawal to pinch (flexor reflex). The core temperature was controlled by means of an electrical feedback control unit (Harvard homoeothermic blanket control unit, model 50-7137), keeping the body temperature at a constant level of 36-37 ºC.

A section of 8-10 mm of the left sciatic nerve was dissected free and isolated from surrounding muscles and connective tissue by a plastic film. A bipolar silver hook electrode was placed under the sciatic nerve proximal to the main branches. A plastic tube for artificial ventilation was inserted 7-10 mm down the trachea and fixed with suture. Simplex, i.e. 80 % Vaseline and 20 % paraffin, was used to anoint the eyes to prevent them from drying. The rats

were mounted in a rigid frame by two ear bars. A laminectomy was preformed on vertebrae Th13-L1, corresponding to spinal cord segments L2-S2, where the sciatic nerve enters the spinal cord. Clamps, one rostral and one caudal to the exposed segments, fixed the spinal cord. The meninges were carefully punctured by a cannula and pulled apart with two forceps to make access for a microelectrode. To avoid muscle contractions, the rats received an intramuscular injection of a muscle relaxant (pancuronium bromide, 0.2-0.3 ml, 2mg/ml, Pavulon, Organon, the Netherlands) in the right thigh. After injection of muscle relaxant the animals were ventilated (Harvard rodent ventilator, model 683) with a breathing frequency of 83 breaths per minute. A microscope and a fiber optic light source were used for better precision during surgery.

2.3. Extracellular recordings

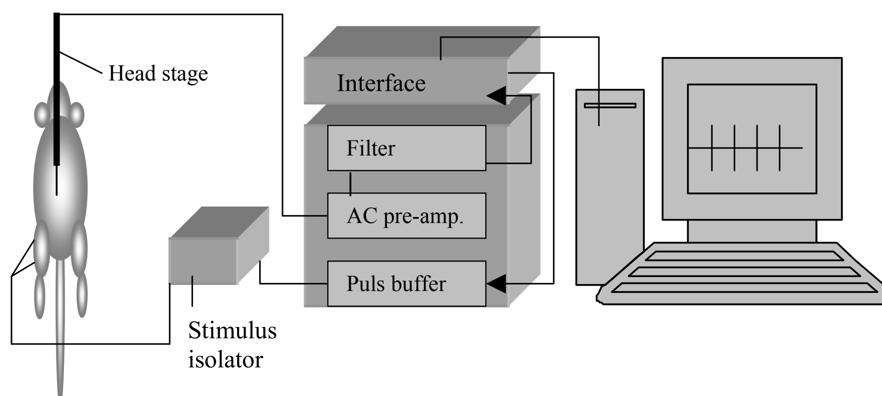

Fig. 5: Experimental apparatus set up for electrophysiological recordings. A bipolar silver hook electrode was used to stimulate the sciatic nerve in the left thigh. The signals from the recording electrode placed in the dorsal horn were digitized, filtered and amplified by the Neurolog and sampled on a computer using the Spike 2 computer program.

Single unit recordings were made with a parylene-coated tungsten microelectrode (2-4 MΩ impedance, Frederick Haer & Co, USA) lowered vertically into the dorsal horn using an electrically controlled micromanipulator (npi, model MW MS314, Germany). The reference-electrode was placed subcutaneously. The distance from the top of the dorsal horn to the tip of the electrode was noted. The recorded signals were amplified with an AC pre-amplifier, filtered (Digitimer) with bandwidth 500-1250 Hz (corresponding to the action potentials

duration of 0.8-2 ms), digitized with the interface CED 1401 µ and continuously captured on a PC with the software CED Spike 2 (Cambridge Electronic Design, Cambridge UK). The sampling rate was 20 kHz. (See Fig. 5)

2.4. Electrophysiological procedures

Touch and pinch to the hind paw were used as search stimuli. The neuronal activity in the spinal cord was presented graphically on the computer screen as well as acoustically through a loudspeaker to assist the search for relevant neuronal activity. A pulse buffer connected to a stimulus isolation unit (Digitimer) was used to control the intensity of the electrical stimuli given to the sciatic nerve. Extracellular single unit activity was recorded from neurons at depths of 250–1000 µm from the surface of the spinal cord. All electrical stimuli were given to the sciatic nerve through the bipolar silver hook electrode (1.5 mm distance between the hooks). The C-fiber threshold was defined as the stimulus intensity at which the cell generated only one C-fiber action potential.

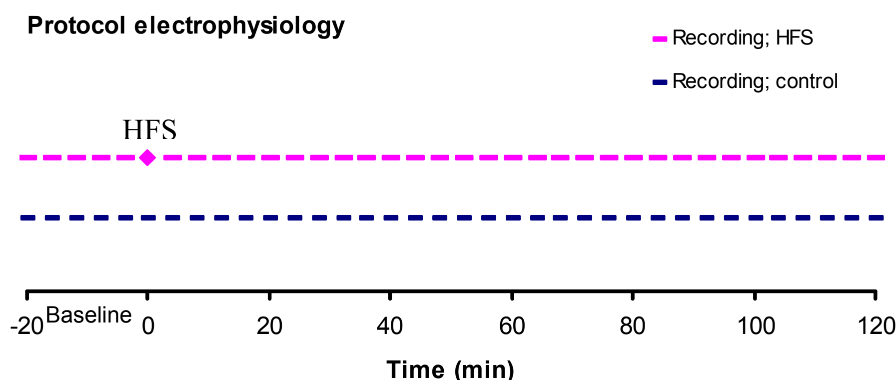

Fig. 6: Protocol for the electrophysiology recordings. Pink color represents HFS recordings. HFS was given after a baseline of 6 recordings, as indicated by diamond. Blue color represents control recordings where the animal received no HFS.

A rectangular test stimulus with pulse duration of 2 ms was delivered every 4th minute (see Fig. 6). The pulse current of the test stimuli was 1.5 times the C-fiber threshold. After an initial series of six test-stimuli, used to calculate the baseline response, the animal was given HFS. Each HFS consisted of 5 trains of 1 ms rectangular pulses at 100 Hz. Each train lasted 1

s, and the interval between trains was 10 s. The pulse current of the HFS conditioning was 3 times the C-fiber threshold.

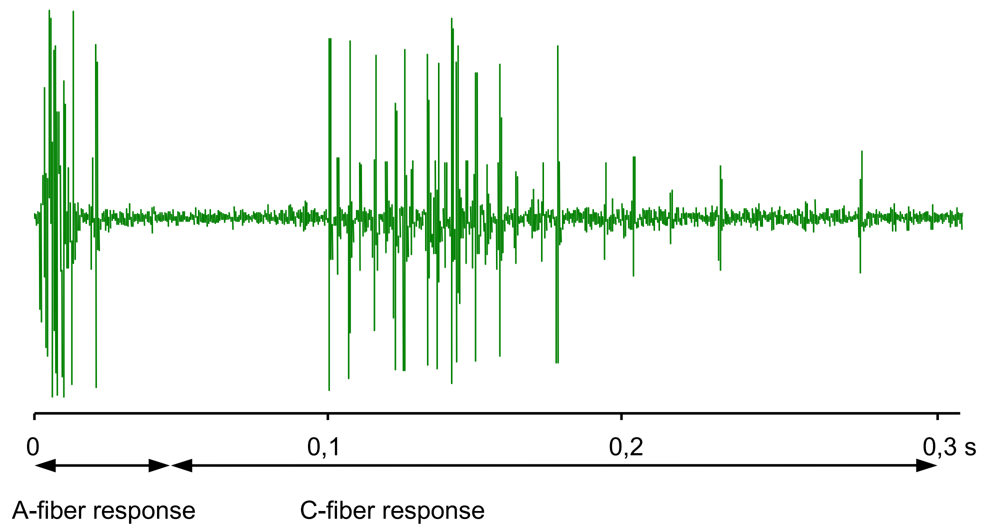

Fig. 7: Neuronal activity evoked by a single test pulse applied to the sciatic nerve, recorded on the computer.

Because A-fibers are faster conducting than C-fibers, the A-fiber and C-fiber signals were distinguished by latency. Action potentials recorded 0-40 ms after stimuli were defined as A-fiber responses, while action potentials with latencies between 40 and 300 ms were defined as C-fiber responses (see Fig. 7). The different action potentials were compared by shape and amplitude to determine if they originated from the same cell or not (see Fig. 8-10).

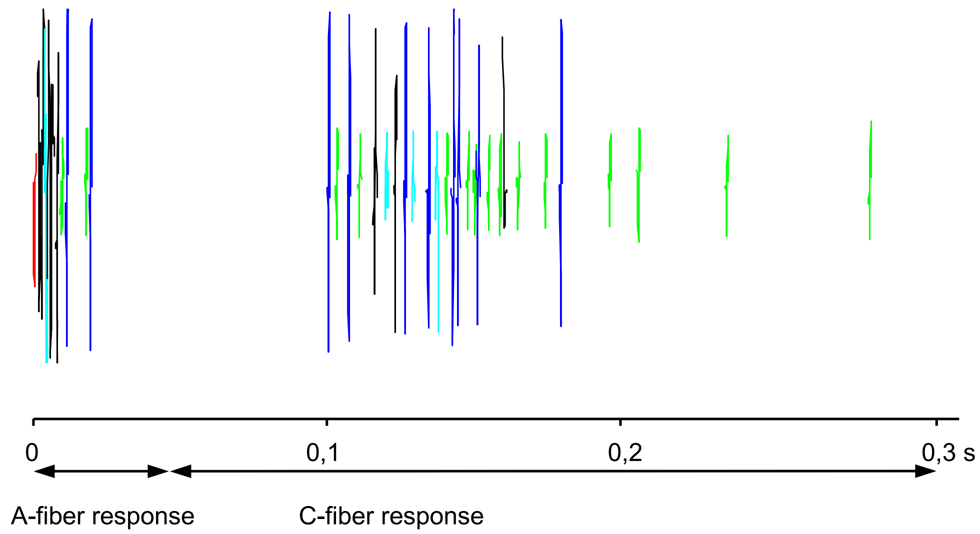

Fig. 8: Action potentials distinguished from each other (seen as different colors) based on amplitude and shape. The recorded neuronal activity in Fig. 7 was analyzed to identify action potentials from individual units.

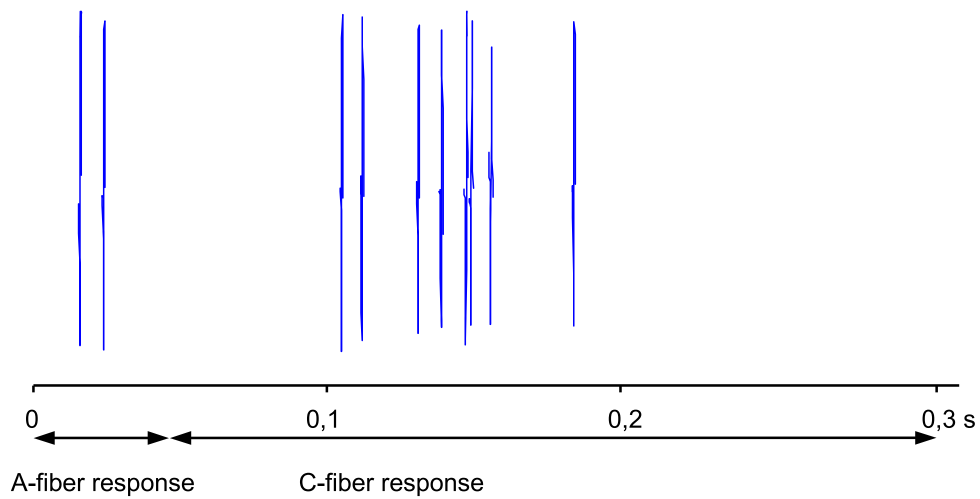

Fig. 9: Action potentials from a single cell. The identification of action potentials from individual cells (Fig. 8) made it possible to display action potentials generated by a particular cell.

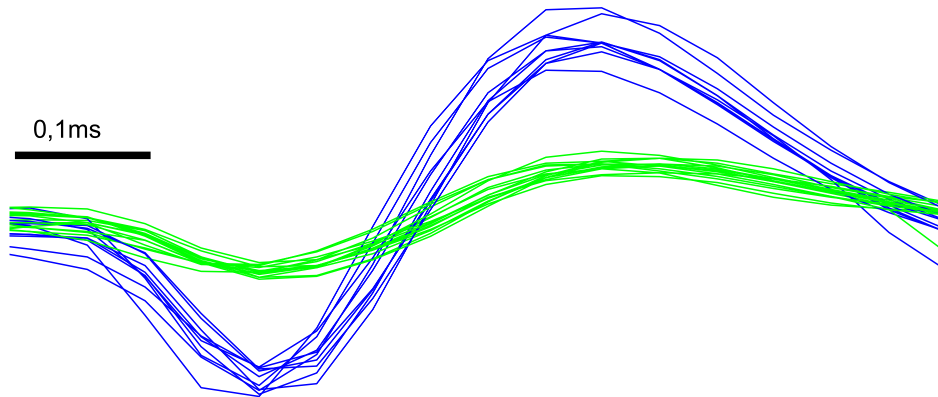

Fig. 10: Comparison of the shape of action potentials. Action potentials from two different cells in Fig. 8 (green and blue) were superimposed, in order to show differences in both shape and amplitude.

The neuronal activity in animals receiving HFS was compared to the neuronal activity in animals receiving no HFS, i.e. the controls. Neuronal activity was measured as number of spikes from one unit, recorded within the period defined as C-fiber response, i.e. 40-300 ms after stimulating. Only one unit was studied in each animal.

2.5. Tissue harvesting for PCR

At the end of the experiments, the animals were killed by removing the ventilation tube and crushing the spine at neck level. A 50 mm section of the spinal cord tissue corresponding to the spinal cord segments L3-S2 was collected, rapidly frozen in liquid nitrogen and stored at -80 ºC for further analysis. Sampling of neuronal recordings was not performed in the experiments for native - or 30 min tissue. Gene expression was investigated from tissue harvested at three time points (see Fig. 11):

1) Immediately after surgery (native)
2) 30 min after the initial stimuli (HFS and control)
3) 120 min after the initial stimuli (HFS and control)

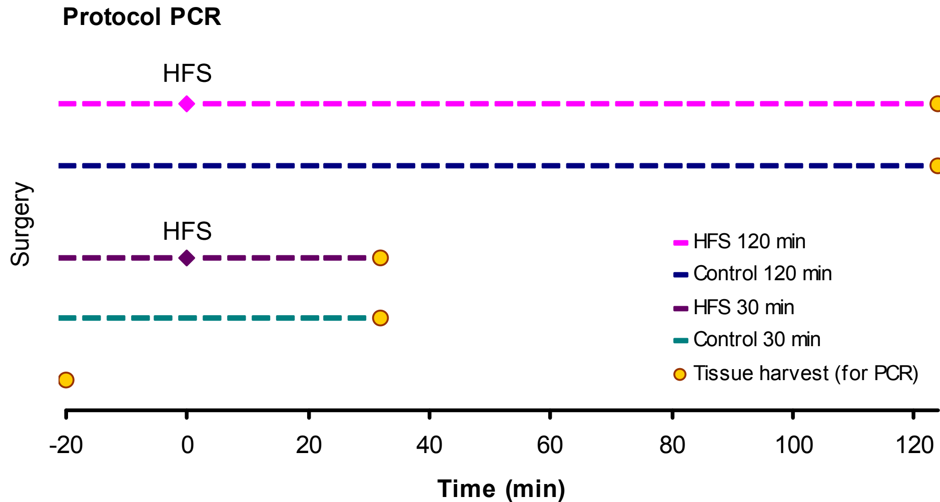

Fig. 11: Protocol for the PCR experiments. HFS (administered at time 0, indicated by diamond) and control (no HFS) tissues were harvested at 30 and 120 min. Native tissue was harvested without prior electrophysiological recordings.

2.6. Quantitative real-time RT-PCR

To isolate total RNA, samples of spinal cord were thawed, added TRIzol (Life Technologies, Inc., Rockville, MD) and homogenized by a Polytron for 30 s to disrupt the cells and denature proteins. Non-solubilized cell material was removed by centrifugation. Chloroform was added to separate the sample in an organic phase, an interphase, and an aqueous phase containing the RNA. Isopropanol was then added to the aqueous phase to precipitate the RNA. The pellet was washed with 75 % ethanol, dried and redissolved in DEPC-water. The amount of RNA was quantified by optical densitometry and diluted to a concentration of 1 µg/µl by adding DEPC-water. (For further details see appendix I.)

The 1st strand cDNA Synthesis Kit for RT-PCR (cat no. 1 483 188, Roche Diagnostics, Mannheim, Germany) was used to synthesize cDNA, as presented in detail in Appendix II. In short, a mix of 1.5 µg total RNA, deoxynucleotides and random primer was incubated at 65 ºC. AMW reverse transcriptase was then added, and the reverse transcription was run at the following schedule [42 ºC 60 min, 99 ºC 5 min and 4 ºC 5 min] (PerkinElmerCetus DNA Thermal Cycler 480). The cDNA product was diluted in TE-buffer and stored at –80 ºC.

Two different immediate early genes were investigated, Zif and Arc. The expression of the target genes Zif and Arc was normalized to the expression of a reference gene, i.e. β-actin. All primer pairs were designed to span introns, to avoid amplification from traces of possible DNA contamination in the RNA isolation, and gave PCR products of 245 bp for Zif, 265 for Arc and 87 bp for β-actin (see table 1). All primers were checked for specificity by Blast search. Quantitative analysis of the genes were performed on an ABI 7900 (AppliedBiosystems) with Sybr Green 1(qPCR Core Kit Sybr Green 1, Eurogentec) at the following schedule: 50 ºC 2 min, 95 ºC 10 min and 40 cycles of [95 ºC 15 s and 60 ºC 1 min]. (For further details see appendix III.) A final melting curve of fluorescence versus temperature was generated to screen for co-amplification products. The software SDS 2.2 (AppliedBiosystems) was used for data analysis and melting curve generation.

Table 1: Primers used for real time PCR

Primer	Sequence (written 5'- 3')	bp	%GC	Tm ºC
β-actin forward	CTA AGG CCA ACC GTG AAA AGA	21	47.6	58.0
β-actin reverse	ACA ACA CAG CCT GGA TGG CTA	21	52.4	59.2
Zif forward	TAC CCC AAA CTG GAG GAG ATG A	22	50.0	59.4
Zif reverse	TCG TTA TTC AGA GCG ATG TCA GA	23	43.5	58.6
Arc forward	AGC AGA CTT CGG CTC CAT GA	20	55.0	59.5
Arc reverse	GCA CCT CCT CTT TGT AAT CCT ATT TTC	27	40.7	59.8

The amount of template corresponded to 100 ng reverse transcribed total RNA for Zif/Arc and 5 ng reverse transcribed total RNA for β-actin, 20 µl in each well. In addition a dilution series was made to make a standard plot for each PCR run. A final melting curve of fluorescence versus temperature was generated to screen for co-amplification products.

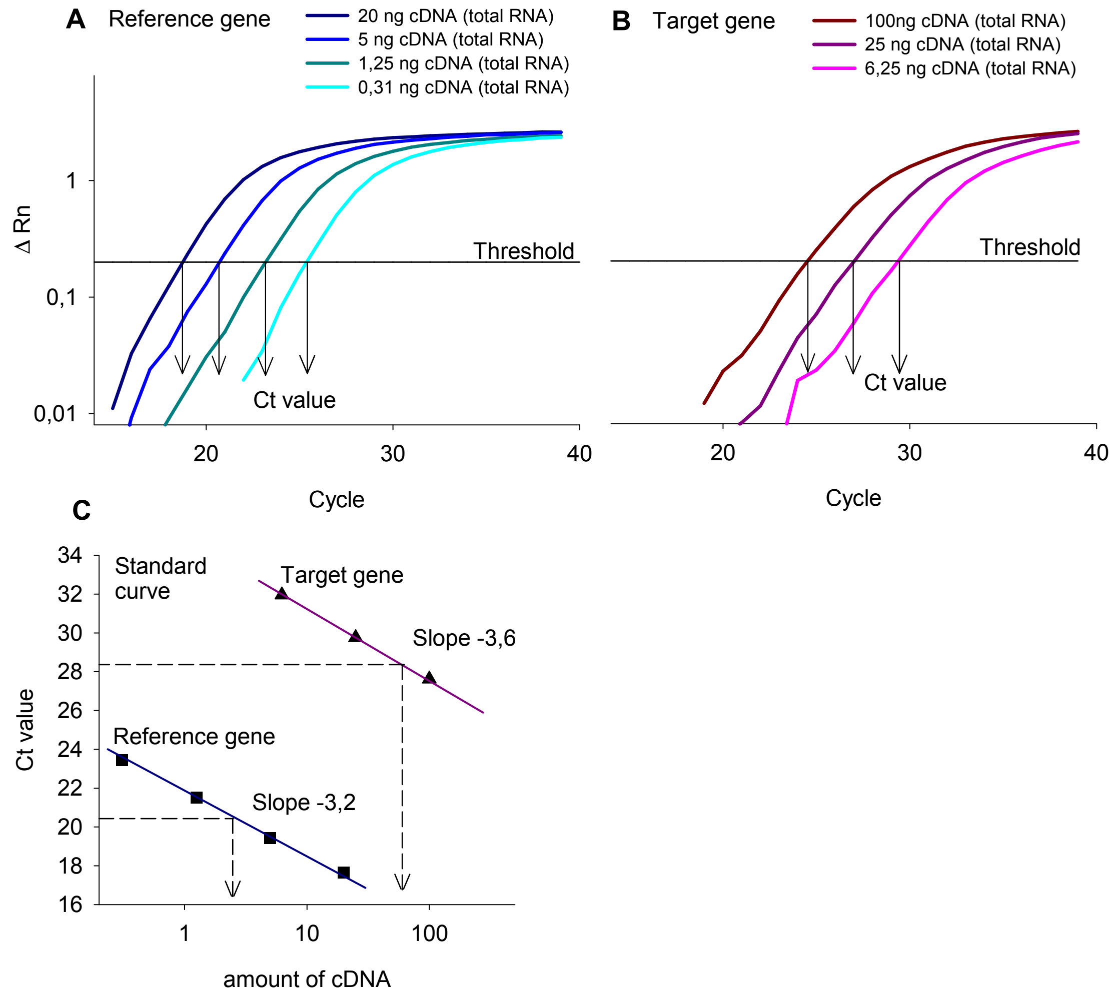

Fig. 12: Amplification plot of dilution series for the reference gene (A) and target gene (B) presented as delta Rn as a function of number of cycles in the PCR reaction. (Data taken from Zif raw data). Ct-values are read from the x-axis from a computer estimate of the steepest point in the exponential phase (Threshold, horizontal line at delta Rn=0,2). C; Standard curve for quantification of data from the reference gene and target gene. The Ct value is plotted on the y-axis while the amount of cDNA is plotted on the x-axis. (Data taken from Arc raw data).

Analysis of the quantity of the added cDNA template is based on the exponential phase of the reaction. Based on the background fluorescence, the computer defined the threshold (Fig. 12 A and B). The data are presented as delta Rn, a measure of the intensity of the fluorescence the SybrGreen-bound PCR product emits, as a function of number of cycles in the reaction. Based on the computer-defined value of delta Rn on the y-axis, the threshold cycle (Ct) value for each sample was estimated (Fig. 12 A and B). The amount of cDNA was then calculated using the standard curve; a specific Ct value for one sample corresponded to a specific amount of cDNA (Fig. 12 C).

2.7. Acryl amide gel electrophoresis

To confirm that the product measured in the real time PCR reaction was uncontaminated, an acryl-amid gel electrophoresis was preformed. A 6.7 % PAGE gel was used. The gel was loaded with 5 µl PCR product and 4 µl φ-χ standard ladder. 1xTAE buffer was used and the gel was run on 180V (approx. 27 V/cm) for 35 min. SYBER Green I (FMCBioProduct) diluted 10.000 times in 1xTAE buffer was used for detection. All primer pairs produced a single band corresponding to the predicted size.

2.8. Statistics

The baseline was defined as the average number of C-fiber spikes of the six first initial C-fiber recordings in the beginning of each experiment. The C-fiber response at the end of the experiments was calculated as the average of the six last recordings in each experiment, in percent of baseline. The calculated C-fiber responses were log transformed due to the dispersion in the group, to acquire normally distributed data. A one-tailed Students T-test was used to compare the results from the HFS group to the control at the end of the experiments.

Fold change values for each sample was defined by the gene expression of the target gene divided by the gene expression of the reference gene. The fold change values were log-transformed due to the dispersion in the group, to obtain normally distributed data. A one-tailed Students T-test was used to compare the results from the HFS group to the control group at 30 min and 120 min.

The software Microsoft Excel was employed for all statistic analyses. A *P* value of 0.05 was accepted as the level for statistical significance.

3. Results

3.1. Electrophysiology

The objective of this project was to examine whether dorsal horn LTP is associated with altered expression of immediate early genes Zif and Arc. Electrophysiological recordings of single cell activity were performed before and after LTP induction in dorsal horn neurons by high frequency stimuli conditioning.

All cells had their receptive field in the right hind paw (see example in Fig. 13.A). Measurement of C-fiber threshold and dorsal horn depth at the start of each experiment indicated a decrease in C-fiber threshold downwards in the dorsal horn laminas (Fig. 13B). The response of one unit was measured by the number of C-fiber action potentials in the period 40 - 300 ms after a stimulus. In most cells tested, HFS produced an increase in neuronal activity leading to more action potentials, with higher frequency, in response to the test stimuli after HFS than before. The increase in the A-fiber response was less prominent than the increase in C-fiber response. The effect of the HFS lasted for more than six hours (see example Fig. 13.C).

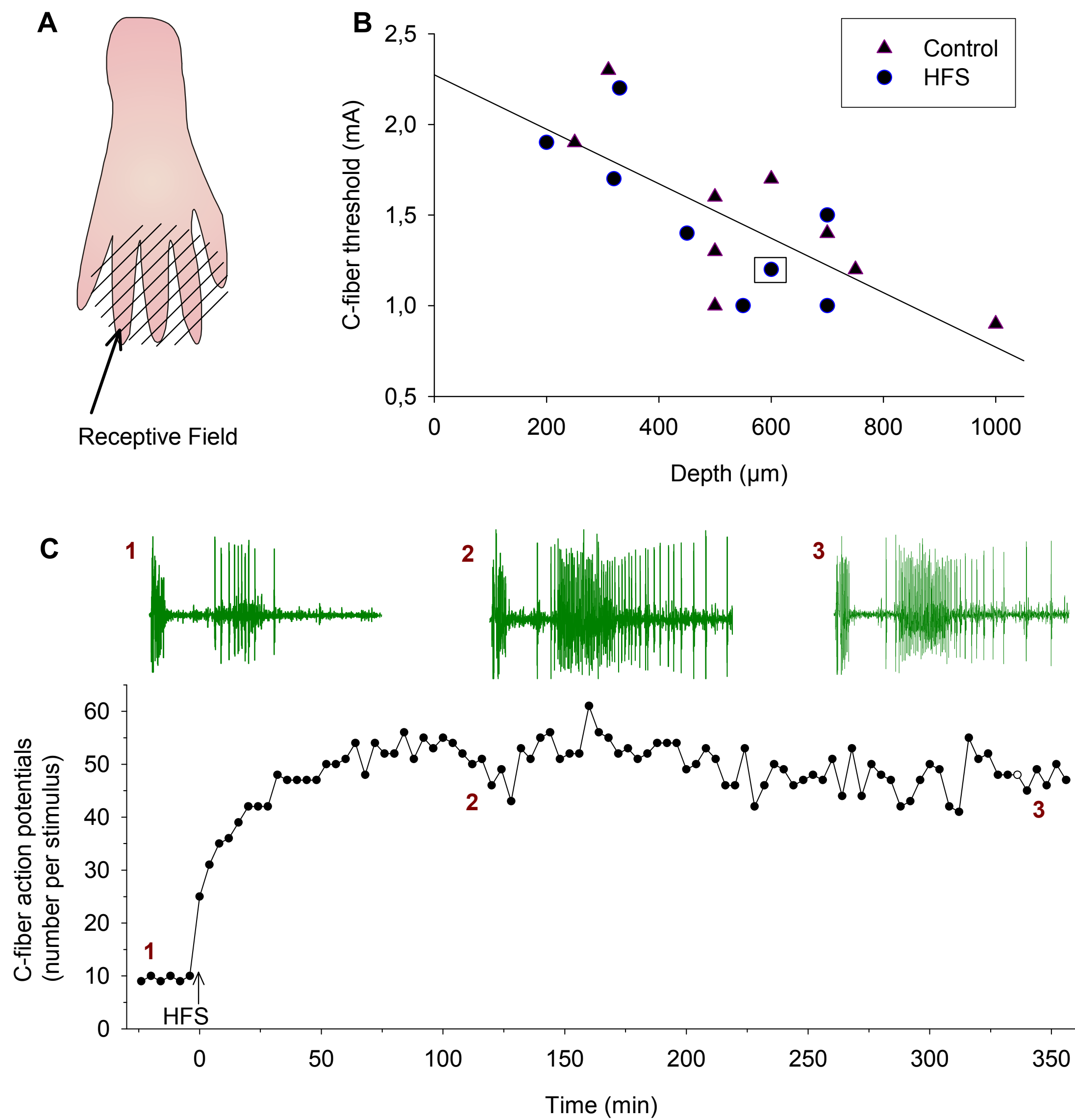

Fig. 13: A; Example of receptive field for one cell, measured at the start of the experiment. B; A plot of dorsal horn depth and C-fiber threshold of all cells (control and HFS) included in this study. A regression line was added to the plot to show the relationship between C-fiber threshold and dorsal horn cell depth. C; Top: Examples of raw data of the cell in A, marked with a square in B, at different time points before and after HFS. Bottom: Electrophysiological recordings of the same cell as above, receiving HFS and followed for 6 hours, making sure the LTP was stable and did not decrease with time.

Test and control cells (n=7 in both groups) were recorded before HFS (baseline neuronal response) and for 120 min after HFS. The time at which HFS was administered was termed 0 min, although control groups received no HFS.

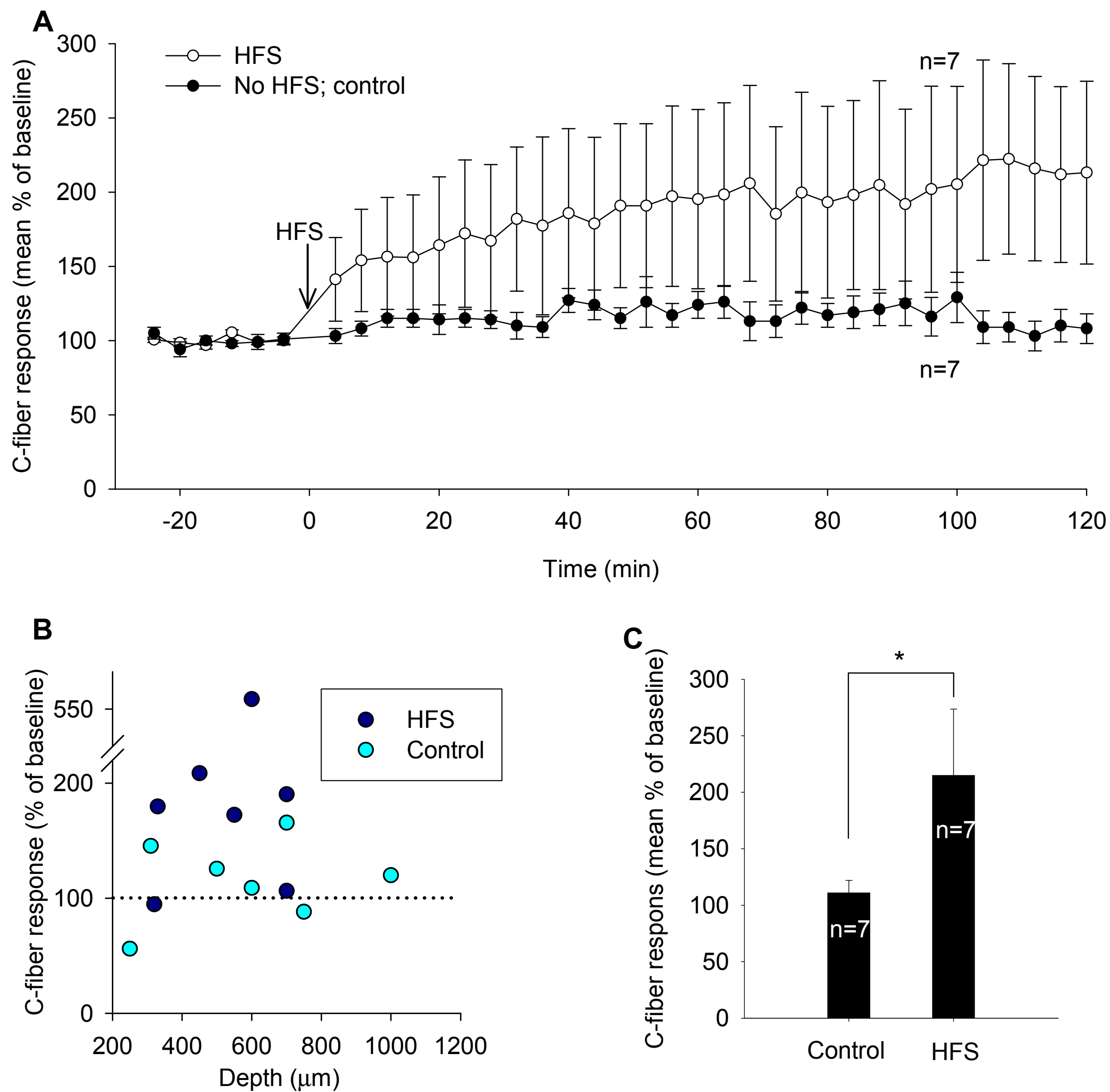

Fig. 14: A; Graph of electrophysiological data presented as mean % of baseline. Black circles represent the control, white circles represent the test-group. N=7 in both groups, HFS was administered at time 0 min. Error bars indicate variance. B; C-fiber responses (mean value of the last 6 recordings in both groups) of the cells from the electrophysiological recordings plotted against the depth in the dorsal horn. C; C-fiber response (mean value of the last 6 recordings in both groups) in control and HFS-group 120 minutes after test-group received HFS. Error bars indicate variance. The increase in C-fiber response after HFS was statistically significant as indicated by * (*P*=0.037).

The baseline neuronal response appeared to be stable for both the HFS and the control group. The HFS group showed a gradual increase in neuronal activity, whereas the control group remained unaltered (Fig. 14.A). No obvious correlation between the C-fiber responses and the depth of a cell was observed (Fig. 14.B). The mean C-fiber responses at the end of the experiment (mean value of the last six recordings) showed a significant increase in the HFS group compared to the control group (Fig. 14.C). Thus, the data showed an induced LTP of the cells, making them more sensitive to test stimuli.

3.2. Real-time PCR

The expression of target genes Zif and Arc was quantified at three different time points; immediately after surgery, 30 min after HFS and 120 min after HFS. To show that an alteration in expression of the target gene was due to an actual alteration, and not caused by an altered expression of the reference gene, the time course of the expression of β-actin was examined.

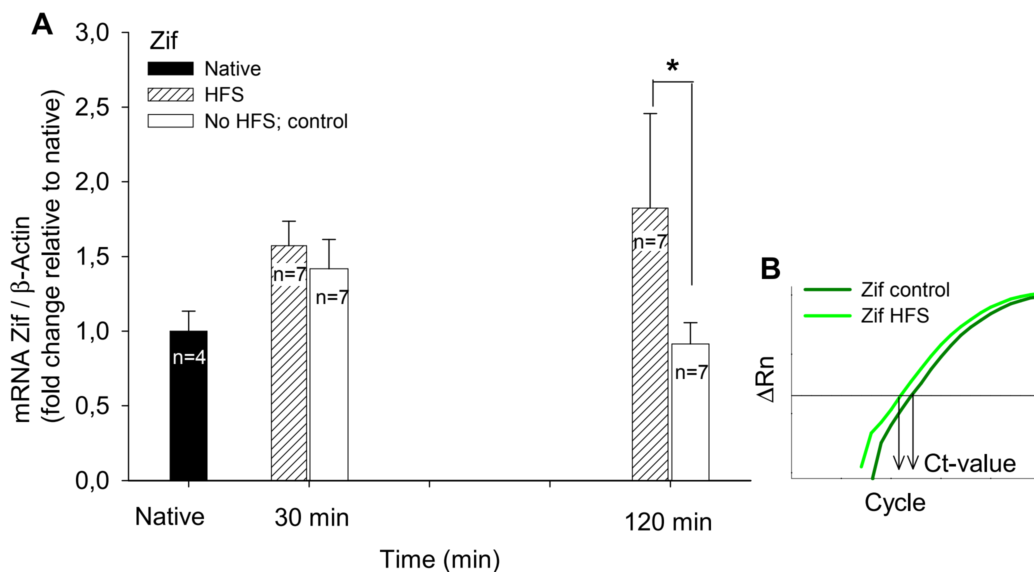

Fig. 15: A; Results of real time PCR on Zif immediately after surgery, native, after 30 min (HFS and control) and after 120 min (HFS and control) presented as fold change relative to native. The increase in expression in the HFS group after 120 min was statistically significant, indicated by * (*P*=0.047) Error bars indicate variance. B; Example of upregulation (left shift) of Zif, HFS compared to control.

Compared to the native expression, HFS produced no obvious alteration in the expression of Zif at 30 min, but an almost twofold increase in Zif expression at 120 min (Fig. 15). The variance was larger in the HFS group than in the control group. The expression of the reference gene β-actin in the Zif-run appeared to be stable (Fig. 16).

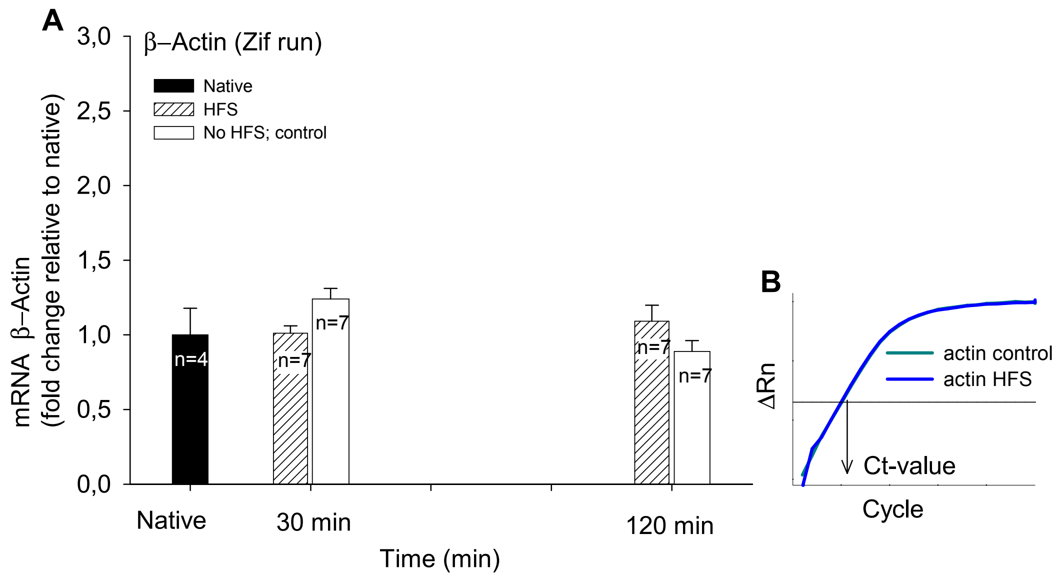

Fig. 16. A; Results of real time PCR on β-actin (Zif run) immediately after surgery, native, after 30 min (HFS and control) and after 120 min (HFS and control) presented as fold change relative to native. Error bars indicate variance. B; Example of PCR amplification plot for β-actin HFS and control.

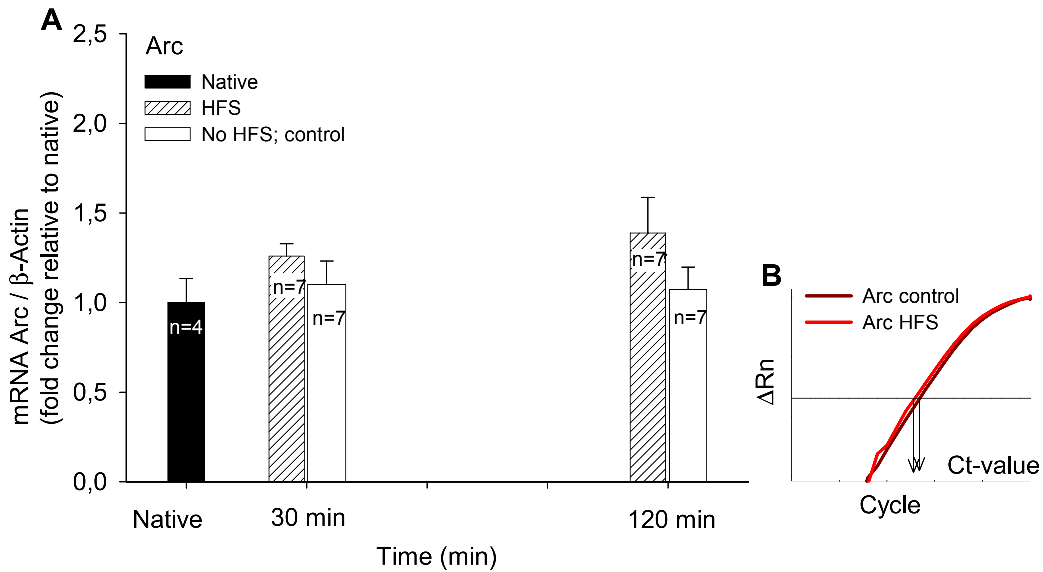

Fig. 17. A; Results of real time PCR on Arc immediately after surgery, native, after 30 min (HFS and control) and after 120 min (HFS and control) presented as fold change relative to native. Error bars indicate variance. B; Example of upregulation of Zif HFS compared to control.

HFS did not alter the expression of Arc. No obvious difference between the HFS group and the control at 30 and 120 was observed (Fig. 17). Notably, the expression of β-actin in the Arc-run was lower at 30 and 120 min than in the native group (Fig. 18).

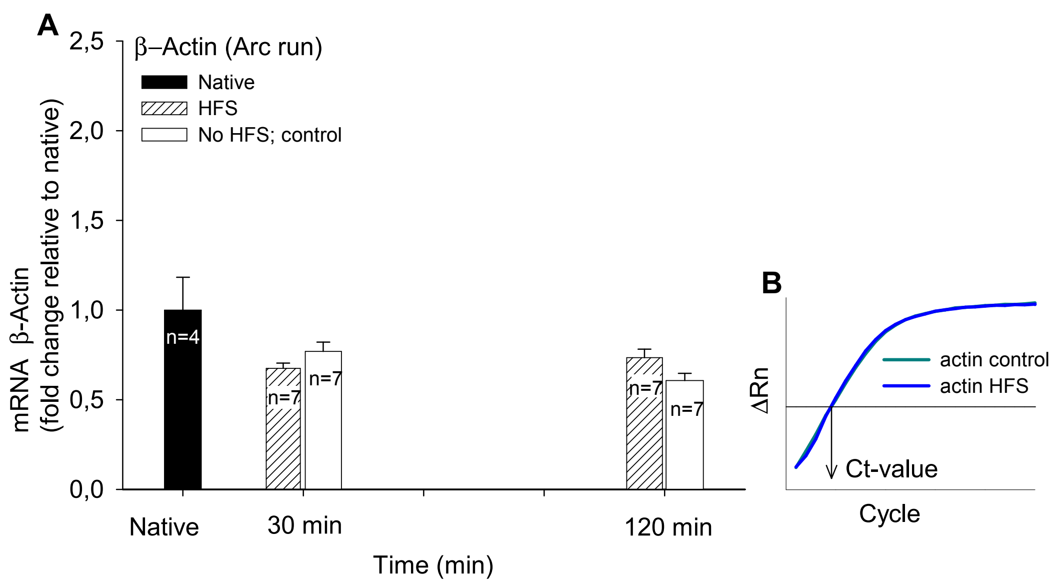

Fig. 18: A; Results of real time PCR on β-actin for Arc immediately after surgery, native, after 30 min (HFS and control) and after 120 min (HFS and control) presented as fold change relative to native. Error bars indicate variance. B; Example of PCR amplification plot for actin HFS and control.

4. Discussion

4.1. Discussion of materials and methods

In order to study pain, a model system is needed. It is obvious that *in vivo* human models cannot be used in terms of studying molecular mechanisms in the central nervous system (CNS). Human tissue might be available to some extent, however there will probably not be uniformity in the underlying material and no solid research can be based on it. SPD rats are readily used as model systems for pain research, both *in vivo* and *in vitro*. Female instead of male SPD rats were used since they exert a smaller risk for the personnel handling the animals and the researcher to develop allergies. Hormonal changes in the rats were not considered in this project. Effort was made to minimize the influence of transporting and handling prior to the experiments.

Electrophysiology

Spinal long-term potentiation (LTP) and injury-induced hyperalgesia share signal transduction pathways, time course and pharmacological profile. This makes use-dependent LTP at C-fiber synapses an attractive model of injury-induced central sensitization and hyperalgesia (Sandkühler 2000). The functional change in the spinal cord may be studied by single unit recordings or by field potentials. However, field potentials may measure potentials from afferents, which is not the case for single unit recordings (Hu et al. 2003). Thus, in accordance with previous studies (Gjerstad et al. 2001; Pedersen et al. 2005; Rygh 2002; Svendsen et al. 1999; Zhang et al. 2001) single unit recordings were used for this project.

There is a possibility that the observed changes in neuronal response are due to a switch in measuring one cell to measuring another. However, the waveform of the action potential indicates that this is unlikely. Another factor in consideration was the interspike intervals. In conformity with Miki et al. (2002), no spikes were within the range of the absolute refractory period of an action potential (approximately 0.5-1 ms) in these recordings, further suggesting that only one neuron was recorded. However, one cannot be absolutely certain that only one cell is studied throughout the whole recording period, as the form of the action potential from one cell may change over time and two cells can have nearly identical action potentials (Miki et al. 2002).

Gene expression

The induction of a particular transcription factor can be measured at the level of its mRNA or at its protein level. Upregulation of mRNA is not always consistent with upregulation of the protein. Assessment of the protein on the other hand can suffer from cross immunoreactivity (Platenik et al. 2000). Both methods are widely used for studying expression, but their weaknesses should be recognized.

mRNA quantification can be measured by northern blotting and in situ hybridization, RNAse protection assays, cDNA arrays and reverse transcription followed by real-time PCR. Northern blots provide information about mRNA size, alternative splicing and mRNA integrity, however suffers from comparatively low sensitivity. In situ hybridization is the most complex method, but allows localization of transcripts to specific cells within a tissue. The RNAse protection assay is most useful for mapping transcript initiation and termination sites, intron/exon boundaries, and for discriminating between similarly sized and related mRNAs. Low sensitivity is the main limitation of this method. Reverse transcription of extracted RNA from a tissue sample followed by reverse transcription and real-time PCR is a very sensitive and flexible quantification method (Bustin 2000). Being one of the most sensitive and flexible quantification methods for gene expression analysis, quantitative real-time RT-PCR was used for this project.

RNA quality and quantity are critical factors for successful, reproducible and biologically relevant gene expression analysis. Degraded and inaccurately quantified RNA would give misleading results. RNA, once removed from its cellular environment, is extremely delicate. This makes the purification step very important, though tricky. Another important factor affecting the accuracy of gene expression analysis is DNA contamination (Bustin and Nolan 2004; Zhang et al. 2005). To increase reproducibility in the purification steps of mRNA isolation and cDNA synthesis it is important to follow the protocol punctiliously (Lekanne Deprez et al. 2002). For this project cDNA synthesis was performed with primers resulting in nonspecific binding to the mRNA and subsequently synthesizing cDNA from all available mRNA.

Primers used for real-time PCR were designed to span introns to avoid false positive results arising from amplification of possible genomic DNA contamination. The optimal length for single stranded primers is about 18-24 bases, with a GC content of between 20 % and 70 %.

The T_m of the primer pair should not differ more than 1-2 ˚C. The primer concentration should be kept at an optimal level. Too high concentration may promote mispriming and accumulation of non-specific product. Too low concentration is considered less of a problem, as target copy number is calculated at a time point well before the primer supply is exhausted (Bustin 2000). The primers used for this project had GC % and T_m within these optimal parameters. All primers except the Arc reverse primer were within optimal parameters.

A method for minimizing errors and correcting for sample-to-sample variation is to amplify a reference gene, to which the measured RNA can be normalized, at the same time as the target gene. Reference genes, also referred to as 'housekeeping' genes, are genes expressed at a relatively constant level among different tissues and should not be affected by experimental methods. One such 'housekeeping' gene commonly used is β-actin. This gene encodes a ubiquitous cytoskeleton protein and was one of the first to be used as internal standard. The selection of a reference gene is may affect the results, due to the varying expression levels of the gene in different tissues at different times. However, β-actin has been found to be a good choice for reference gene selection, depending on the experimental model used (Bustin 2000; Zhang et al. 2005). Indeed, the level of β-actin in this project appeared to be stable.

SYBR green I dye was used for detection. This dye binds to the minor groove in double stranded DNA, increasing its fluorescence over 100-fold upon binding. This detection method is considered a good method although there is no discrimination between specific and nonspecific PCR fragments – both will be measured (Lekanne Deprez et al. 2002). To test for possible nonspecific PCR products, a melting curve analysis was performed, in all cases revealing only one product. In addition a gel analysis was performed, yielding only one band of the predicted size for each assay.

The Ct value is defined as the cycle when sample fluorescence exceeds a chosen threshold above calculated background fluorescence. The background fluorescence may vary from sample to sample or run to run, influenced by changing reaction conditions. The Ct value is used to determine copy numbers and is hence the most frequently quoted parameter when reporting quantitative real-time PCR (Bustin and Nolan 2004).

An ideal real time PCR reaction follows the equation;

$$y = k * 2^n$$

Where -y is the number of PCR products (quantity)
-k is the number of cDNA copies before the reaction starts
-n is the number of cycles

In an ideal PCR reaction based on a factor four dilution series, there is two cycles between each graph. However, since the PCR reaction was not ideal for the target genes a standard curve plot, instead of only Ct values, was used for quantification in this project.

The gene expression measurements were made with the whole spinal cord as substrate. It may be argued that the results could have been more conclusive if only half the spinal cord was examined and that investigating just the laminas in question would prove a better method. Li et al. used laser capture microdissection (LTM) to investigate gene expression in lamina I and II, on one side of the spinal cord only. After ipsilateral injection of formalin in one hindpaw, they achieved greater induction of gene expression in some of the LTM samples, than by samples of homogenates. The variance in the material was, however, too large for the groups to make any conclusions to whether the LTM method is better than homogenates (Li et al. 2004). The possibility remains that more accurate results may be achieved by studying gene expression exclusively in the laminas receiving C-fiber input i.e. I, II, III, V and X.

4.2. Discussion of results

Long-term potentiation

The cells studied in this project all had their receptive fields in the left hindpaw. All units reacted to noxious stimuli, indicating that the neurons that were recorded belonged to the pain pathway. In accordance with previous findings, the C-fiber threshold and depth of the cell seemed to correlate; the lower the threshold the deeper the cell (Liu and Sandkühler 1997; Seagrove et al. 2004). The effect of high frequency stimulation (HFS) lasted for more than six hours, indicating a long lasting increase in synaptic strength. In other studies on different parts of the CNS, the effect of HFS has lasted for several days (Håvik et al. 2003). This has not yet

been done on spinal cord, *in vivo* on anesthetized rats, probably due to methodological difficulties.

Spinal cord LTP can be induced by various noxious stimuli like formalin injection (Li et al. 2004), complete Freunds adjuvant injection (Miki et al. 2002), crushing of bones and soft tissue (Rygh et al. 1999) or electric stimulation to the sciatic nerve (Svendsen et al. 1999). In the spinal cord, inflammatory pain models may result in a long lasting (several weeks) sensitization with upregulation of the immediate early gene (IEG) c-Fos in laminae V and VI. In contrast, neuropathic pain models are associated with c-Fos upregulation in laminae III and IV (Munglani and Hunt 1995).

NMDA and AMPA receptors may be differentially involved in regulation of c-Fos and Zif expression in the spinal dorsal horn following noxious thermal, - formalin or - mechanical stimulation. Thus, the cellular mechanisms, such as the signal transduction pathway involved in regulating c-Fos and Zif are most likely different (Rahman et al. 2002). The onset and duration of spinal LTP critically depends on the type and intensity of conditioning stimulation and the activity of inhibitory controls. Full expression of LTP may be achieved after only a few minutes or it may take up to an hour, the effect may last for several hours. In awake animals hippocampal LTP may last for the whole lifespan of an animal (Sandkühler 2000).

LTP has been studied in numerous areas of the CNS, among them the hippocampus, amygdalae, brain stem and dorsal horn. The mechanisms of induction and maintenance of LTP may vary between different types of synapses (Sandkühler 2000). Blockage of spinal NMDA receptors and mGluR1 has been shown to lead to a significantly reduced C-fiber induced sensitization. This suggests that the ability of these receptors to increase intracellular levels of Ca^{2+} and subsequent ERK activation after glutamate release from C-fibers is important for the maintenance of long lasting functional changes in the spinal cord (Lever et al. 2003).

Peripheral inflammation has been known to upregulate NK1 in dorsal horn neurons. Ji et al. found that the protein kinase pathway involving MAPK and ERK co localizes with NK1 in superficial dorsal horn neurons (Ji et al. 2002). However, NK1 antagonists did not affect the number of neurons labeled with activated ERK in a study by Lever et al. (Lever et al. 2003). Interestingly NK1 antagonists have been shown to reduce inflammatory pain (Ji et al. 2002).

NK1 receptors are probably important for induction of C-fiber evoked LTP in the spinal cord, but most likely not necessary for the maintenance of LTP (Liu and Sandkühler 1997).
Both LTP and the opposite phenomenon long-term depression (LTD) can be induced by increased intracellular concentrations of Ca^{2+}. The magnitude of synaptic stimulation, the pattern of stimulation, the specific concentration of intracellular Ca^{2+} or the postsynaptic membrane potential (Kim et al. 2003; Randic et al. 1993) may be deciding factors in whether LTP or LTD is induced (Brodal 2001d). Depolarization of the postsynaptic membrane before conditioning favors induction of LTP while the opposite i.e. hyperpolarization before conditioning favors induction of LTD (Sandkühler 2000).

Blocking of de novo protein synthesis before titanic stimulation selectively inhibits the late-phase maintenance of LTP of C-fiber evoked field potentials without affecting the induction and early-phase maintenance of the spinal LTP (Hu et al. 2003). This indicates a need for expression of new genes in the late-phase of LTP, but not in the early-phase.

In accordance with previous studies, the electrophysiological recordings showed that LTP was induced in spinal dorsal horn neurons following a brief HFS. The cells became more sensitive to the test-stimuli after the induction of LTP. The LTP outlasted the experimental time course and the proposed early-phase of LTP.

Gene expression

The expression of Zif and Arc was normalized to the expression of the reference gene β–actin. To show if the increase in fold expression of Zif was due to an actual upregulation or if it was caused by a lower expression of the reference gene, the time course of the expression of β-actin was examined. Comparing the β–actin graph for the Zif run to the Zif graph, it is clear that the almost twofold expression of Zif after 120 min was not caused by a HFS induced low β–actin level. The present data indicated that Zif, but not Arc is associated with spinal LTP.

Wisden et al. (1990) did not find a link between Zif activation and sensitization in the spinal cord, but upregulation of Zif in hippocampus following LTP was observed (Wisden et al. 1990). Many previous studies of various brain tissues have confirmed a raise in expression of both Zif and Arc. In the brain, Link et al. found that mRNA transcripts of IEGs, Arc in particular, were localized in the soma and the dendrites of granule cells. This localization offers a potential for local protein synthesis at activated postsynaptic sites, possibly

underlying the synapse-specific modulations during long-term plastic events (Link et al. 1995).

The inconclusive results of the Arc study may be due to markedly lower or lacking expression in the spinal cord compared to the brain. Arc may in fact not be part of the system of sensitization in the spinal cord. Another factor may be the possible influence of the anesthetic Urethane on expression of this gene. In the hippocampus it has been shown that Urethane probably blocked the induction of Fos-like immunoreactivity, and attenuated Zif expression (Richardson et al. 1992). There is a possibility that Urethane may block or attenuate the expression of Arc in dorsal horn neurons.

Repeated LTP episodes may lead to opening of further channels in the synapse or addition of 'hyperslots'; AMPA receptors that are anchored to the postsynaptic membrane, further activating the synapse and possibly increasing its size (Lisman 2003). When one form of plastic alteration has occurred, the synaptic response to subsequent stimuli may have changed (Zhang et al. 2001). These changes observed in spinal cord tissue do not occur spontaneously. All cellular activity is regulated and controlled by the genome and its expression, so also in a synapse in a nerve cell. The question is what genes are expressed, at what levels and at what time.

As early as one hour after injury of spinal cord tissue, the mRNA of several ion channels, transporters and receptors are down regulated. This down regulation of receptors and transmitters is likely to contribute to both long-term neuronal damage and impaired synaptic transmission (Bareyre and Schwab 2003). Plasticity in the synapses includes structural changes (Toni et al. 1999). Local translation in the dendrites contributes to synaptic and behavioral plasticity (Miller et al. 2002). Upregulation of growth factors and of molecules involved in axonal targeting and neurite outgrowth is observed after nerve injury. Nerve cells thus demonstrate an effort to adapt to lesions, regeneration and re-growing of axons (Bareyre and Schwab 2003).

More research remains in order to fully understand all the mechanisms behind spinal cord LTP. Further studies could concentrate on late response genes, correlation between immediate early genes and late-response genes, the function of Zif or what genes it associates with. The

expression of IEGs in the spinal cord could also be investigated associated with various noxious LTP-inducing stimuli.

Clinical implications

Reduction of central sensitization is important for minimization of postoperative pain. Administration of pre-emptive analgesics, like μ-opioid receptor agonists, is currently used to diminish such pain. μ-opioid receptors play an important role in the processing of nociceptive information and may also inhibit depolarization of dorsal horn neurons needed for central sensitization. Indeed central sensitization and LTP may share similar mechanisms. Previous data show that the μ-opioid agonist morphine reverses LTP in spinal dorsal horn neurons (Rygh et al. 2000). Clinically used μ-opioid receptor agonists may effectively diminish central sensitization. Some opiates may possibly inhibit induction of immediate early genes involved in spinal sensitization. Activation of pre- or post-synaptic μ-opioid receptors reduces influx of Ca^{2+} and depolarization of the cell. Analgesics, like morphine, are widely used to diminish pain, however other drugs can be substitutes for morphine in this treatment. Endomorphins has been suggested as a substitute for morphine in treatment of pain (Tateyama et al. 2002). Moreover, the effect of volatile anesthetics, halothane and isoflurane, on hyperexcitability of spinal dorsal horn neurons has been investigated. The results showed suppression of excitation and spontaneous activity during the first 30 min after noxious stimuli, but not attenuation of development of long lasting hyperexcitability (Kawamata et al. 2005).

Long lasting analgesia can be achieved by other means, such as low frequency transcutaneous electrical nerve stimulation (Rong et al. 2005). The balance and variety between inhibitory and facilitating systems during counter-stimulation is not known. The components of the central neuropathic pain may have different sensitivities to analgesics or to the route of administration (Wang et al. 2005). Such diversity can explain the clinical observations that identical stimulation protocols may produce long lasting strong analgesia in some patients while proving ineffective or even initially aggravate pain in others (Sandkühler 2000).

Interestingly, the LTP model may also be transferred to humans. A recent study by Klein et al. claims to present the missing link between clinical observations in humans and basic neurobiological mechanisms that were proposed to turn acute pain into chronic pain. By electrically stimulating primary afferents in the skin on humans, they showed that neurogenic

hyperalgesia shares characteristics with both homosynaptic and heterosynaptic types of LTP (Klein et al. 2004).

Identifying Zif as an immediate early gene possibly involved in the transition from early to late phase LTP in the spinal cord is a small step on the way to fully understanding the details of the mechanisms underlying central sensitization and spinal cord LTP. Improved understanding of these underlying mechanisms will provide a better foundation for developing more specific treatment of various forms of pain, such as postoperative-, neuropathic- and chronic inflammatory pain.

5. Conclusion

The electrophysiological recordings confirmed previous observations that LTP can be induced in spinal dorsal horn neurons following a brief HFS applied to the sciatic nerve. The cells became more sensitive to the test-stimuli after the induction of LTP. The LTP always outlasted the duration of the experiments and the proposed early-phase of LTP.

The Zif mRNA expression showed a significant increase 120 minutes after HFS conditioning. At this time, LTP maintenance is at a critical point in transition from early- to late-phase, a process in need of de novo protein synthesis. The increase of the expression of Zif mRNA is an indication that the gene product may be involved in this transition. The Arc mRNA expression showed no obvious change after HFS conditioning.

Upregulation of the mRNA for the transcription factor Zif may possibly be an early step, on a genetic level, of LTP maintenance. The effect of Zif on the activation of other genes related to maintenance of spinal LTP remains to be investigated.

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Appendix

I. RNA Isolation

Procedure for RNA isolation:

1. About 30 mg spinal cord tissue was transfered to a 5 ml tube, pre-cooled on ice.
2. The tissue-tube was heated carefully. 0.8 ml Trizol reagent was added. Tissue was homogenized on Polytron for 30 s.
3. The homogenate was transferred to an eppendorf tube and incubated at room temperature (RT) for 5 min.
4. Tube was centrifuged at 12 000 g for 5 min at 4ºC. 0.7 ml supernatant was transferred to a new eppendorf tube.
5. 0.2 ml chloroform w/lysate was added. The tube was shaken vigorously by hand for 15 s and incubated for 2-3 min at RT.
6. The tube was centrifuged at 12 000 g for 15 min at 4 ºC.
7. The water phase was transferred to a new tube, 150-200 µl at a time. 0.5 ml iso-propanole w/pellet paint (1:1000) was added. The contents were mixed well and incubated at RT for 10 min.
8. The tube was centrifuged at 12 000 g for 15 min at 4 ºC.
9. The supernatant was removed (900 µl at first, then 200 µl) and the RNA pellet was washed with 1 ml 75 % EtOH (in DEPC-water), mixed and vortexed.
10. The tube was centrifuged at 12 000 g for 5 min at 4 ºC.
11. The supernatant was removed, and the pellet was dried for 15-25 min. The pellet had to be dry before water was added. The pellet was dissolved in 12 µl DEPC-water and kept on ice.
12. The tube was incubated at 65 ºC for 10 min, put in ice, spun, put on ice and the content mixed by pipette.
13. A 250 times dilution was made to establish RNA concentration: 2 µl sample + 500ul TE-buffer were mixed and vortexed. The RNA concentration was estimated from the optical density of the solution at 260 nm and 280 nm.
14. Samples were diluted to 1 µg/µl by adding x,x µl DEPC-water: (10 µl * concentration µg/µl)- 10ul = x,x µl to add.
15. The samples were stored at -80ºC.

II. cDNA synthesis

Procedure for cDNA synthesis:

1. The PCR machine was switched on.
2. 1.5 µg RNA was mixed with water to a total volume of 4.5 µl.
3. Mixture 1 was prepared:

Reagent	volume/sample
Random primer p(dN)6	1.5 µl
Deoxynucleotide-mix	1.5 µl
Total	3.0 µl

4. 3 µl of mixture 1 was added per sample (all tubes were spun and vortexed).
5. The tubes were incubated at 65 ºC for 15 min.
6. Mixture 2 was prepared:

Reagent	volume/sample
10xreaction buffer	1.50 µl
25mM $MgCl_2$	3.00 µl
RNase inhibitor 50 u/µl	0.68 µl
AMW reverse transcriptase	0.50 µl
dH_2O	1.80 µl
Total	7.51 µl

7. The PCR machine was heated up.
8. 7.5 µl of mixture 2 was added to each tube (all tubes were spun and vortexed).
9. The reaction was run on the PCR machine [42 ºC for 60 min, 99 ºC for 5 min, 4 ºC for 5 min]
10. The samples were spun down. 135 µl TE-buffer was added per sample. The samples were stored at -80 ºC.

III. Real time PCR

Procedure for the real time PCR:

1. 5 µl β-actin samples were diluted with 95 µl dH_2O
2. The samples used for dilution series for β-actin, were diluted; 5 µl from stock to 20 µl of water. For Zif/Arc; there was no initial dilution. 6,5 µl from dilution nr.1 (the least diluted well) was then added to19.5 µl water to make dilution nr. 2, and so on in order to make four successive dilutions.
3. Mixture 1 and mixture 2 were prepared:

Reagent	volume/sample	final conc.
CDNA 10ng/µl	10.0 µl	
dH_2O	26.0 µl	
10 x Reaction buffer	5.0 µl	1x
MgCl2, 50 mM	4.0 µl	4 mM
DNTP 5 mM	2.0 µl	0.2 mM
Sybr Green 1 (1/2.000 dilution)	1.5 µl	1/66.000
UNG 1u/µl	0.3 µl	0.006 u/µl
Hot Gold Star enzyme 5 u/µl	0.2 µl	0.02 u/µl
Primer UP 25 pmol/µl	0.5 µl	12 pmol
Primer LOW 25 pmol/µl	1.8 µl	12 pmol
Total	50.0 µl	

4. 10 µl of prediluted samples for β-actin and dilution ladders, and 10 µl of stock cDNA for Zif/Arc were added to the PCR set up cooling-plate.
5. 40 µl mixture1 was added to the β-actin wells and 40 µl mixture 2 was added to the Zif/Arc wells on the set up cooling plate.
6. 10 µl dH2O was added to the non template control wells.
7. All samples were mixed and 20 µl of each sample was transferred to the PCR-plate in two parallels.
8. The PCR plate was sealed with plastic film.
9. Samples were spun down.
10. A rubber mat was placed on top and the PCR-reaction was started.