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Nerve Injury Induced Pain and Modulation by Spinal Cord Stimulation

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Man's mind once stretched by a new idea,
never regains its original dimensions.

- Oliver Wendell Holmes

Abstract

Chronic neuropathic pain caused by injury to or disease in the nervous system is relatively common and results in major suffering, poor quality of life and incapacity. Such pain is a therapeutic challenge because a considerable portion of the patients fails to benefit from pharmacotherapy. Therefore, there is a need for alternative treatment modalities. Spinal cord stimulation (SCS) has proven to be effective in the management of some forms of neuropathic pain.

The experimental studies constituting this thesis address various aspects of neuropathic pain of peripheral origin and the mode of action of SCS. Neuropathic pain is generally associated with abnormal responsiveness of the somatosensory system sometimes presenting as increased sensitivity to mechanical stimuli. Animal experimental models supposedly representing neuropathic pain, especially evoked pain, typically exhibit signs of neuropathy in the form of local cutaneous hypersensitivity.

In the present thesis a model of neuropathy (rat) according to Seltzer et. al. (1990) was used. In behavioral tests the withdrawal thresholds in response to von Frey filaments, cold spray and radiant heat were assessed. Possibly attenuating effects of SCS on hypersensitivity were examined in the awake and freely moving animal.

Immunohistochemistry, Western Blot, ELISA, microdialysis and HPLC were employed for the analysis of some transmitters and receptors related to neuropathic pain and/or SCS effects. Various agonists/antagonists were administered intrathecally for the evaluation of the significance of the corresponding spinal receptors in mechanisms underlying SCS effects. Activation of the glutamatergic NMDA receptor in the spinal dorsal horn (DH) is essential for central sensitization and plays an important role in the generation and maintenance of neuropathic pain. Quantification of dorsal horn NMDA receptor subunit expression was based on comparisons between the DHs ipsi- and contralateral to the nerve lesion. The phosphorylation of the NR1 subunit of the NMDA receptor was significantly increased in the ipsilateral DH in hypersensitive, but not in non-hypersensitive nerve injured rats. The non-phosphorylated NR1, NR2A, NR2B, NR2C or the NR2D subtypes were unaffected by the nerve injury as compared to controls.

A dysfunctional spinal GABAergic system is considered to be an important feature of neuropathic pain and this might imply also a reduced synthesis of GABA. The DH levels of the GABA synthesizing enzymes, glutamic acid decarboxylase (GAD) 65 and 67, were analyzed after nerve injury and following application of SCS. The expression of both enzymes appeared to be increased in SCS responding rats subjected to SCS immediately prior to tissue collection as compared to responders without stimulation. In non-responding rats subjected to SCS, a similar increase in GAD67 was also present. Without stimulation, nerve injury per se was not associated with any changes in enzyme expressions regardless of whether or not hypersensitivity was present.

On the basis of preceding observation that clonidine may enhance the SCS effect, experiments with microdialysis of the DH of nerve injured rats and quantitative assessment of extracellular acetylcholine (ACh) release were performed. The basal ACh release was significantly lower in nerve injured than in normal rats. In SCS responding, but not in SCS non-responding rats, application of SCS produced an increased release of ACh. In behavioral experiments, the muscarinic M_4 receptor was identified as the principle one being involved in cholinergic SCS mechanisms. The nicotinic receptor appeared to be of no significance in this study.

There is evidence that the SCS effect is partly exerted via a spinal-supraspinal-spinal loop and most probably comprises descending serotonergic pathways. When SCS was applied in nerve injured rats immediately prior to sacrifice, the 5-HT content in the dorsal quadrant of the spinal cord ipsilateral to the injury was increased in SCS responding rats. There was in these rats also a high density of 5-HT immunoreactive terminals in the DH superficial laminae (I-II) as demonstrated immunohistochemically. The potency to attenuate mechanical hypersensitivity of SCS could be significantly enhanced by a low dose of intrathecal serotonin, and this effect was partially blocked by a $GABA_B$, but not by a muscarinic M_4 receptor antagonist.

There are conflicting results regarding the predictive value of certain neurological symptoms, e.g. cutaneous sensory abnormalities, for the outcome of SCS treatment. In animal models of neuropathic “pain”, the incidence, extent and severity of hypersensitivity is quite variable. A series of rats exhibiting signs of neuropathy were subdivided in groups according to the severity of mechanical hypersensitivity and then subjected to SCS. It appeared that SCS produces a faster and more effective attenuation of hypersensitivity in rats with mild as compared to those with more severe sensory disturbance.

In conclusion, the current studies have shown that in the DH of a rodent model of neuropathy, the expression of non-phosphorylated NMDA receptor subtypes as well as of GABA synthesizing enzymes is not affected by a nerve injury, irrespective of the presence of neuropathic signs. SCS appears to produce a moderate augmentation of the GABA synthesizing enzymes. There is evidence indicating that SCS may activate several pain modulatory systems, and here it has been shown that both cholinergic and serotonergic mechanisms are involved in the SCS effect. The latter may relate to a stimulation-induced restoration of a dysfunctional descending inhibitory and/or facilitatory supraspinal endogenous control. The different SCS mechanisms may operate independently, in parallel or in concert. Finally, the relationship between the outcome of SCS and degree of hypersensitivity demonstrated in an animal model may have clinical implications.

Keywords: Neuropathic pain, Rat, Nerve injury, Spinal cord stimulation, NMDA receptor, GABA, GAD, Acetylcholine, Serotonin, Mechanical hypersensitivity

List of publications

The present thesis is based on the following studies referred to in the text by their roman numerals given below.

- I. Ultenius C., Linderoth B., Meyerson BA., Wallin J.
Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in the rat.
Neuroscience Letters 399 (2006) 85-90
- II. Ultenius C., Song Z., Meyerson BA., Linderoth B.
GABA synthesis in the dorsal spinal cord following peripheral nerve injury and effects of spinal cord stimulation.
Submitted to Brain Research
- III. Schechtmann G., Song Z., Ultenius C., Meyerson BA., Linderoth B.
Cholinergic mechanisms involved in the pain relieving effect of spinal cord stimulation in a model of neuropathy.
Pain 139 (2008) 136-145
- IV. Song Z., Ultenius C., Meyerson BA., Linderoth B.
Pain relief by spinal cord stimulation involves serotonergic mechanisms: An experimental study in a rat model of mononeuropathy.
Pain 147 (2009) 241-248
- V. Smits H., Ultenius C., Deumens R., Koopmans GC., Honig WM., van Kleef M., Linderoth B., Joosten EA.,
Effect of spinal cord stimulation in an animal model of neuropathic pain relates to degree of tactile “allodynia”.
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List of abbreviations

ACh	Acetylcholine
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
BSA	Bovine serum albumin
CCI	Chronic constriction injury
CNS	Central nervous system
DCN	Dorsal column nuclei
DLF	Dorsolateral funiculus
DRG	Dorsal root ganglion
ELISA	Enzyme-linked immunosorbent assay
FG	Fluoro-Gold®
GABA	γ -amino butyric acid
GAD	Glutamic acid decarboxylase
HPLC	High-performance liquid chromatography
5-HT	Serotonin
IASP	International Association for the Study of Pain
IHC	Immunohistochemistry
i.p.	Intraperitoneal
IR	Immunoreactivity
i.t.	Intrathecal
M	Muscarinic (receptor)
MT	Motor threshold
MT-3	Muscarin toxin-3
NeuN	Neuronal nuclei
NMDA	N-methyl-D-aspartate (receptor)
NR	N-methyl-D-aspartate receptor subunit
P	Phosphorylated
PAG	Periaqueductal grey
PBS	Phosphate buffer solution
PNS	Peripheral nervous system

RVM	Rostro ventromedial medulla
SCS	Spinal cord stimulation
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
TENS	Transcutaneous electrical nerve stimulation
WB	Western Blot
WT	Withdrawal threshold

Introduction

The ability to react to noxious stimuli is vital for all animal species. When functioning properly, a warning system activated by threats to the organism is a prerequisite for its survival (Smith and Lewin 2009). The ability to detect and avoid potential injury therefore represents a major selection pressure and may be seen as an important mechanism behind evolution by natural selection. Being able to respond to life threatening mechanical forces is perhaps the most common feature among living organisms (Smith and Lewin 2009).

It is important to differentiate between nociception and pain. Activity of nociceptive pathways is a sensory discriminative physiological process and should not be considered as equivalent to pain perception (Kavaliers 1988; Smith and Lewin 2009). Pain on the other hand, is a subjective sensory experience, which is always associated with emotional and cognitive components.

When “friendly”, pain warns of threatening damage and leads to behavioral responses that protects from injury, but when a “foe”, the sensation of pain is useless and becomes a disease in its own causing persistent suffering (Breivik et al. 2006; Cervero 2009). The line separating pain as a friend and pain as a foe is thin and delicate. Nociceptive pain is a normal response to acute trauma or disease, it is temporary and it gradually diminishes and disappears when healing is complete (Julius and Basbaum 2001). However, following injury or disease pain may become maladaptive and develop into a chronic condition with only weak connection to the initial trauma or the initiating agent (Woolf and Doubell 1994; Costigan et al. 2009).

Whereas acute nociceptive pain as a rule can be effectively relieved, chronic pain may be difficult to control and currently available treatments are often ineffective (for review see (Koltzenburg and Scadding 2001; Jensen and Finnerup 2007; Jensen et al. 2009; Baron et al.). Despite extensive research and efforts to develop new therapies, the management of chronic pain still remains a major challenge.

Pain processing

Nociception starts with the detection of a noxious stimulus. The nociceptive system is able to register very different types of aversive stimuli, e.g. mechanical, thermal or chemical. Some nociceptors are stimulus specific while others are polymodal and respond to several types of stimuli. Given that the stimulus is strong enough, activation of these pain receptors - or nociceptors - triggers impulses that travel along axons of sensory nerves and dorsal root ganglion (DRG) cells, terminating in the spinal cord.

When the neuronal network in the spinal dorsal horn is activated, the processing and integration of the afferent signals lead to spinal responses, often expressed as nocifensive reflexes. The targeted 2nd order neurons in the dorsal horn transmit the information to the brain via ascending pathways, terminating in different relays in the midbrain and thalamus where further integration occurs. From the thalamus, pain signals are forwarded to other structures of the brain or the so called “pain matrix” (Tracey and Mantyh 2007) responsible for the sensory discriminative and the affective aspects of pain (Bushnell 2006; Fields et al. 2006).

The studies in this thesis deal mainly with spinal cord mechanisms of nociception related to some of the important neurotransmitters, the most common excitatory neurotransmitter glutamate (Glu), the major inhibitory transmitter γ -amino butyric acid (GABA), acetylcholine (ACh) and serotonin (5-HT).

Spinal processing

Glutamatergic mechanisms

Activation of nociceptive primary afferents leads to the release of glutamate at the first synapse in the dorsal horn. After presynaptic release, glutamate molecules diffuse over the synaptic cleft to the postsynaptic plasma membrane where they bind to and activate glutamate receptors. These receptors can be divided in two main classes, the ionotropic (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors and the G protein coupled metabotropic (mGlu) receptors.

The NMDA receptor has an important excitatory role in the CNS. At resting potential, the receptor channel is blocked by Mg^{2+} ions requiring both the simultaneous binding of glycine and glutamate and a depolarization of the postsynaptic membrane for the channel to open (review see Hardingham and Bading 2003; Petrenko et al. 2003). When

open, the channel is highly permeable to Ca^{2+} in addition to monovalent cations like Na^{+} and K^{+} . Activation of these receptors may therefore trigger Ca^{2+} -dependent intracellular signaling pathways essential for the induction as well as maintenance of several forms of synaptic plasticity (Cull-Candy et al. 2001; Wu and Zhuo 2009).

In chronic pathological pain activation of the NMDA receptors, receptor-dependent plastic changes occurs at each level of the pain pathway from the periphery to the brain and all seem to be critically involved in the induction and maintenance of neuronal hyperexcitability (Petrenko et al. 2003).

GABAergic mechanisms

Within the dorsal horn, excitatory transmission is controlled by an inhibitory interneuronal network. Here modulation of nociceptive signals occurs via both pre- and postsynaptic mechanisms targeting primary afferent terminals and 2nd order projection neurons (review see Willis 1991; Dickenson et al. 1997; Millan 1999). Among the many different intrinsic inhibitory systems, the principal one is the γ -amino butyric acid (GABA)-ergic (Todd and Sullivan 1990; Enna and McCarson 2006; Todd and Koerber 2006). The GABAergic inhibitory control of spinal dorsal horn neurons may originate from different sources. Input can derive from descending GABAergic and glycinergic pathways, projecting from the rostral ventromedial medulla (RVM) to the dorsal horn (Antal et al. 1996), but presumably most important, from local inhibitory interneurons activated by primary afferents or by descending fiber tracts (Narikawa et al. 2000). GABA and its co-transmitter glycine open ligand-gated ion channels permitting an influx of chloride ions through the plasma membrane. In most neurons, both GABA and Gly inhibit neuronal activation by hyperpolarizing the cell membrane, impairing propagation of excitatory postsynaptic potentials (Zeilhofer 2005). The central terminals of primary afferents are an important exception; under “normal” conditions, opening of the GABA_A receptor channels in these terminals instead induces a depolarization, which inhibits transmitter release (Zeilhofer 2005).

Some evidence suggests that pre- and post-synaptic inhibition in the superficial dorsal horn is mainly mediated by glycine, while GABA primarily acts on presynaptic GABA_B receptors to provide tonic inhibition (Chery and de Koninck 1999; Chery and De Koninck 2000). It has long been known that pharmacological suppression of GABAergic or glycinergic inhibition can induce signs of central sensitization in the spinal cord (Kendall et al. 1982; Sivilotti and Woolf 1994; Malan et al. 2002; Enna and

McCarson 2006). Proper function of this inhibitory control is essential to prevent the generation of painful sensations by normally innocuous stimuli (Millan 1999).

Cholinergic mechanisms

Acetylcholine (ACh) has a pivotal role in an array of physiological process and is present throughout the nervous system subserving the viscera and the musculature as well. Depending on the type of tissue where ACh is released and the type of receptor with which it interacts, ACh can exert either excitatory or inhibitory effects. There are two main types of receptors: muscarinic (mAChRs) and nicotinic (nAChRs). Several subtypes of muscarinic receptors have been identified (e.g. mAChR subtypes 1-5), differing in function with regard to the coupled G-protein and second messenger activity, in turn either activating or inhibiting the cell. The nicotinic receptors instead belong to the family of ligand-gated ion channels, where activation of the channel leads to influx of cations (Na^+ , Ca^{2+}) and subsequently cellular depolarization.

The involvement of the cholinergic system in antinociception is well known (Zhuo and Gebhart 1991; Eisenach 1999), and clinical as well as animal experimental studies have demonstrated that receptor agonists and cholinesterase inhibitors can alleviate pain (review see Flores 2000; Jones and Dunlop 2007).

In both human and rat spinal cord, the muscarinic receptors are mainly present in the superficial laminae (Scatton et al. 1984; Gillberg et al. 1988; Stewart and Maxwell 2003), and they appear to be crucial for nociceptive processing. There is, however, somewhat conflicting evidence as to which of the M_{1-4} subtypes of the receptor that mediates the main analgesic effect of muscarinic agonists. Although M_2 receptor mRNA is the most abundant in the spinal cord, activation of the commonly present M_4 appears to be sufficient to obtain inhibition of noxious pain transmission (review see Jones and Dunlop 2007). In addition to the central effects of the cholinergic system, mAChRs may also have a peripheral site of action in analgesia (Bernardini et al. 2001; Bernardini et al. 2002).

The predominant subtypes of nAChRs in the CNS, referred to as neuronal nicotinic receptors (NNRs), are involved in glutamatergic, GABAergic and monoaminergic synaptic transmission. Both molecular and pharmacological data support the role of these receptors in mediating and processing of pain and have attracted much interest for possible therapeutic use (review see Jones and Dunlop 2007). Implication of these receptors in the generation of neuropathic pain comes from gene expression studies where upregulation of specific subunits have been observed (Yang et al. 2004). On

the contrary, other studies have failed to show changes in NNR subunit expression after nerve injury (e.g. Costigan et al. 2002; Wang et al. 2002). Furthermore, nicotinic agonists administered i.t. may have antinociceptive effects, but there are also studies that failed to show any such effects (Rashid and Ueda 2002).

Clonidine, an α_2 -adrenoreceptor agonist, was initially used as a therapeutic agent in hypertension but was later found also to have antinociceptive properties, and it was first employed as an i.t. adjuvant to morphine in malignant pain (Eisenach et al. 1995; Martin and Eisenach 2001). There is now much evidence indicating that the antinociceptive effect of clonidine mainly is related to cholinergic mechanisms (Eisenach 1999; Xu et al. 2000). One interesting observation is that the antinociceptive effect of clonidine can be abolished by muscarinic receptor antagonists as well as augmented by receptor agonists (Pan et al. 1999; Duttaroy et al. 2002; Kang and Eisenach 2003).

Serotonergic mechanisms

In the dorsal spinal cord, serotonergic pain processing is entirely depending on descending pathways originating mainly from the RVM. The concept of an inherent pain control, of which 5-HT pathways is an important part, was introduced by Fields and Basbaum in 1978 (review see Basbaum and Fields 1978; Fields and Basbaum 1978). This concept was basically derived from the pioneering discovery by Reynolds in the late 1960s of the pain inhibiting properties of the periaqueductal grey (PAG) (Reynolds 1969). Somewhat later, it was demonstrated that the inhibition of pain related activity in the DH produced by electrical stimulation of the PAG was relayed via the nucleus raphe magnus and subsequently via other regions, RVM being the most important (Liebeskind et al. 1982).

Several transmitter systems are involved in the descending modulation, some with an inhibitory and some with a facilitatory effect on nociceptive transmission (Porreca et al. 2002; Heinricher et al. 2009).

The serotonergic pathways descending from the RVM have in recent years been extensively investigated (Mason 2001; Millan 2002). The spinal actions of 5-HT are complex and interfere with synaptic transmission in the DH in several ways (Fields et al. 2006). At least 15 different subtypes of serotonin receptors have been identified in the nervous system, and of these a few have been associated with the processing of pain. Most of them have an inhibitory, antinociceptive net effect. However, at least

one, the 5-HT₃ receptor, is considered to be excitatory and pronociceptive (Suzuki et al. 2004; Suzuki et al. 2004).

Nerve injury induced pain

Neuropathic pain is a form of pain characterized by an almost complete lack of relation between noxious stimuli and the sensation of pain. By definition, neuropathic pain refers to pain originating from pathology (lesion or disease) of the nervous system, specifically comprising the somatosensory system (Merskey 1997; Treede et al. 2008). Neuropathic pain does not represent a specific disease entity but a heterogeneous group of conditions that exhibit more or less distinct features although with different etiologies and locations (Woolf and Mannion 1999; Jensen et al. 2001). Multiple mechanisms contribute to different neuropathic pain syndromes (Bridges et al. 2001; Costigan et al. 2009) and a variety of diseases of the nervous system are associated with, or can cause, neuropathic pain e.g. vascular or metabolic diseases, infection, nerve compression or trauma and autoimmune diseases (Scadding 2006; Baron 2009). In man, the symptomatology of neuropathic pain is varied but often include spontaneously arising continuous or intermittent pain, mechanical and thermal hypersensitivity (allodynia), hyperalgesia and sensory deficits (review see Jensen et al. 2001; Hansson 2002; Backonja 2003; Baron 2006; Jensen et al. 2009; Baron et al. 2010).

Peripheral mechanisms

After nerve injury, multiple sites along the neuron may be functionally altered by changes in thresholds and excitability and transmission properties, resulting in a state of hyperexcitability with increased firing response to both noxious and innocuous stimuli (review see Woolf and Salter 2000; Campbell and Meyer 2006; Devor 2006; Hökfelt et al. 2006; Baron 2009; Costigan et al. 2009; Baron et al. 2010).

Disruption in the myelination of damaged axons may enable interactions, ephaptic cross-talk, between neighboring axons leading to alterations in discharge patterns (Suzuki and Dickenson 2000). The excitability of injured and adjacent uninjured sensory neurons increases and is associated with changes in channel expression and receptor accumulation at the site of injury and in the DRG. Furthermore, the loss

of target innervation disrupts the neurotrophic support from the periphery. These events together with lowered action potential thresholds may result in spontaneous ectopic discharges independent of peripheral stimuli (Suzuki and Dickenson 2000; Devor 2006).

The distal part of the injured axon undergoes Wallerian degeneration, causing a release of various mediators and trophic factors. This change in the local environment exposes the surviving nerve fibers to an altered milieu of cytokines and growth factors. This may in turn cause a phenotypic switch in these neurons, reflected by alterations in gene expression and modifications in the release of several transmitters into the spinal dorsal horn (Hökfelt et al. 1994; Costigan et al. 2002; Hökfelt et al. 2006). The excess of trophic factors from the partly denervated tissue can also lead to sensitization of primary afferent nociceptors (Campbell and Meyer 2006; Baron et al. 2010).

Central mechanisms

The exaggerated and spontaneous input from primary afferents may generate activity-dependent changes in spinal cord excitability, known as central sensitization. This represents a state of hypersensitivity of DH neurons where the threshold of activation is reduced and the responsiveness to synaptic input is augmented. This may result in an expansion of the receptive field. Thus, the gain of the system is turned up (Woolf and Salter 2000; Sorkin 2006). The augmented responsiveness involves several cellular mechanisms: presynaptic, postsynaptic and interneuronal changes, as well as changes in descending modulation and immunological reactions (Basbaum 1999).

Increased release of excitatory neurotransmitters can be triggered by e.g. up- or downregulation of presynaptic autoreceptors or a phenotypic switch of non-nociceptive fibers (Hökfelt et al. 2006).

Additionally, the enhanced synaptic efficacy can be caused by postsynaptic mechanisms, e.g. increased activation and trafficking of the glutamate receptors NMDA and AMPA produce increased ion permeability, i.e. increased synaptic strength. Activation (phosphorylation) of the glutamate receptors initiates an influx of calcium into the postsynaptic neuron causing a cascade of biochemical changes that ultimately contributes to synaptic plasticity and central sensitization (Woolf and Mannion 1999; Woolf and Salter 2000; Fang et al. 2002; Caudle et al. 2003; Petrenko et al. 2003; Galan et al. 2004).

Central sensitization is also associated with increased expression of a variety of immediate-early and late-response genes, which upon activation indirectly regulate intracellular signal pathways and processes (review see Zimmermann 2001).

Excitatory events occurring in the DH are normally modulated by inhibitory interneurons and descending pathways but after nerve injury, the endogenous pain control may become dysfunctional due to disinhibition, and the intrinsic inhibitory control no longer can exert its effect. A dysfunction of the glycinergic and GABAergic inhibitory systems involves several plausible mechanisms, an issue that has been much debated (see further in Discussion) (Sugimoto et al. 1990; Castro-Lopes et al. 1993; Vaello et al. 1994; Ibuki et al. 1997; Moore et al. 2002; Baba et al. 2003; Coull et al. 2003; Coull et al. 2005).

In addition to the changes of peripheral and spinal pain processing, descending modulatory pathways may further contribute to the enhanced responsiveness of dorsal horn neurons (Ossipov et al. 2000). It appears to be a disturbed balance of descending inhibitory and facilitatory control systems following nerve injury (review see Porreca et al. 2002; Suzuki and Dickenson 2005).

Besides neural mechanisms, contribution to central sensitization may arise from non-neuronal cells like spinal microglia and astrocytes, Schwann cells, satellite cells in the DRG and immune cells (reviews see Zimmermann 2001; Marchand et al. 2005; Ren and Dubner 2008; Milligan and Watkins 2009).

Spinal Cord Stimulation (SCS)

The classical gate-control theory presented by Melzack and Wall in 1965 has been considered as “the basis of modern electrotherapy for pain” but the history of this conceptualization of pain generation dates further back. Already in 1906 two different kinds of afferent input were postulated, the epicritic that provided sensory non-painful information about touch, pressure, etc., and the protopathic with painful input warning for potential tissue damage. A lesion in the CNS or PNS could disturb the balance between the two systems implying a decreased epicritic afferent input which eventually could result in chronic pain (Head and Thompson 1906).

It was later proposed that the symptom of hyperalgesia may be due to an altered sensory input to the brain (Zotterman 1939), and that normal non-painful input via rapidly conducting fibers could inhibit painful input mediated via slower fiber systems, “fast blocks slow” (Nordenboos 1959). The idea that an activation of an epicritic

sensory system may have an inhibitory effect on protopathic sensation was already in the early 1960s the basis for clinical trials with electric stimulation of the sensory thalamus as treatment of severe neuropathic pain. However, the first reports on this novel therapeutic approach remained unnoticed for several years (Mazars et al. 1960).

Nonetheless, SCS was directly based on Melzack & Wall's gate concept (1965) implying that activation large diameter fibers peripherally or in the dorsal columns (DCs) could attenuate pain transmission already at the first spinal relay. This implies that the theory should be valid for all types of pain. The first clinical report was published two years later by Shealy et. al. (Shealy et al. 1967). When more centers started to practice SCS it became gradually clear that it was effective primarily for neuropathic forms of pain and that acute and chronic nociceptive pain remained unaffected (Lindblom and Meyerson 1975; Linderöth and Meyerson 1995). Neuropathic pain of peripheral or nerve root origin is often effectively relieved by SCS and has emerged as a cardinal indication (Simpson et al. 2006). About 60-70% of well selected patients may have a satisfactory pain relief ($\geq 50\%$) with SCS, but for unknown reasons about one third of the patients fail to benefit (Linderöth and Meyerson 2009).

As with all surgical therapies, the implantation of an SCS system carries some risk of complications (generally of a mild nature), but at the same time, SCS has comparably few side effects and a superior efficacy in selected pain conditions.

SCS in neuropathic pain

In line with the basic concept of the gate-control theory, it was hypothesized that applying stimulation to the dorsal spinal cord induces antidromic activation of DC fibers that in turn activates segmental inhibitory circuits. Over the years, a better understanding of the mode of action of SCS has emerged, and the importance of the orthodromic DC activation projecting to supraspinal centers has been demonstrated (El-Khoury et al. 2002; Saadé et al. 2006). Recent data further support the view that the pain suppressive effect of SCS is dependent both on spinal segmental mechanisms and on the activation of a spinal-supra-spinal loop (Saadé and Jabbur 2008; Saadé et al. 2009).

The clinical observations that the SCS induced pain relief has a delayed onset (minutes) and may outlast the stimulation period by hours and occasionally days, suggest that SCS is associated with prolonged alteration in release of neurotransmitters/modulators. In animal models of SCS it has been demonstrated that the stimulation may significantly reduce the release of the excitatory transmitters glutamate and

aspartate (Cui et al. 1997) while the release of GABA is augmented (Stiller et al. 1996). Adenosine related mechanisms have been found to participate (Cui et al. 1997), and there is reason to assume that also acetylcholine, serotonin and noradrenalin are involved in the effect of SCS. It has been found that SCS appears to selectively suppress the hyperexcitability of WDR neurons following peripheral nerve injury (Yakhnitsa et al. 1999), and it may be hypothesized that these SCS related events represent the restoration of the impaired balance between the excitatory and inhibitory influences associated with neuropathic pain.

Almost all the knowledge about the mode of action of SCS originates from experimental animal research (Foreman et al. 1976; Saadé et al. 1985; Linderöth et al. 1992; Meyerson et al. 1995; Cui et al. 1996). Some of the findings have led to clinical trials and today combination therapy with SCS and certain drugs appears to be successful (Lind et al. 2004; Lind et al. 2008). The notion of combining the modalities of SCS and pharmacotherapy came from important observations in the animal model of SCS, where a low intrathecal dose of the GABA_B receptor agonist baclofen potentiated a lack of SCS effect (Cui et al. 1996; Cui et al. 1997). This was later confirmed by clinical data where i.t. administered baclofen enhanced the effect of SCS in some patients (Lind et al. 2004). Another example, also derived from animal experiments, is that the α_2 adrenoreceptor partial agonist clonidine, which was later included in a clinical trial to enhance the efficacy of SCS when stimulation by itself was insufficient, demonstrated a similar effect (Schechtman et al. 2004; Schechtman et al. 2010).

SCS most likely induces the release of a cascade of neurotransmitters both in the DHs and in supraspinal relays involving multiple yet unknown mechanisms. However, the fact that SCS may activate spinal inhibitory circuits and descending pathways without affecting normal nociceptive sensations still remains somewhat of a paradox (Lindblom and Meyerson 1975).

Animal models of neuropathic pain

A rough understanding of mechanisms of both acute and chronic pain syndromes has been possible by the use of animal models. These animal models rely on the assumption that the behavioral disorders induced are similar to those observed in clinical pain conditions and that the degree of pain-like behavior displayed by the animals is assessable in a quantitative manner.

A number of animal models have been developed with the aim to study neuropathic pain behavior in animals. Some have been designed to mimic human diseases and others to explore pathophysiological mechanisms. The target of injury in the commonly used rodent models is often the sciatic or spinal nerves. The first established nerve injury model consists of ligation followed by complete transaction of the sciatic and saphenous nerves, causing formation of neuromas (Wall and Gutnick 1974). Since then, several alternative pain models have been developed e.g. the chronic constriction injury (CCI) by Bennett et. al. (Bennett and Xie 1988) and the partial ligation model by Seltzer (Seltzer et al. 1990). A shortcoming of these models is the challenge to produce exactly the same lesion in each animal. Kim and Chung developed a model of spinal nerve ligation (SNL) (Kim and Chung 1992) where standardized procedures have increased reproducibility and the variability in behavioral outcome due to the surgery has been minimized. Also the ischemic (Gazelius) injury model was created in an attempt to decrease variability (Gazelius et al. 1996; Kupers et al. 1998). A further alternative is the spared injury model (SNI) (Decosterd and Woolf 2000), involving a

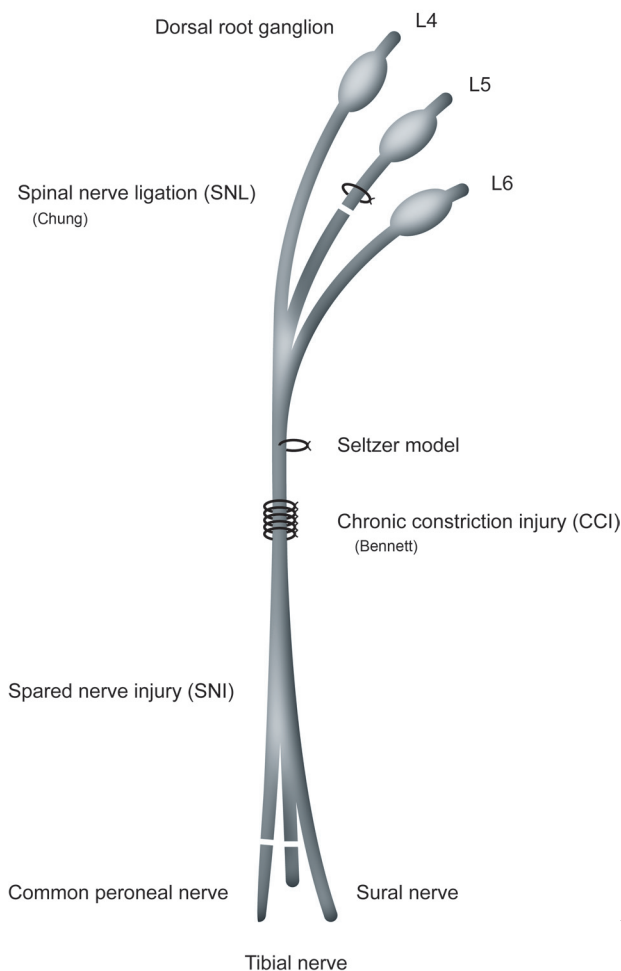


Fig. 1

Commonly used peripheral nerve injury models. The neuroma and the Gazelius models are not illustrated.

lesion of two of the three terminal branches (tibial and peroneal) of the sciatic nerve, leaving the remaining sural branch intact. These models (Fig. 1) result in various pain-like responses with differences in the incidence and degree of hypersensitivity and the sensory modalities involved (review see Zimmermann 2001; Wang and Wang 2003; Mogil 2009). Nerve injured animals generally exhibit behavioral signs of stimulus-evoked pain lasting for months, and only occasionally appear to present signs of ongoing, spontaneous pain (Dowdall et al. 2005).

In animals, pain is monitored and estimated by examining the animal's response to nociceptive/non-nociceptive stimuli. The behavioral tests most often used are procedures to assess static mechanical hypersensitivity with von Frey filaments and methods to examine temperature hypersensitivity/hyperalgesia using cold spray or an acetone drop (for cold) or a movable light source heating the plantar aspect of the hind paw. Thus, what is quantified is a stimulus evoked pain-like response displayed as a withdrawal reaction, which could be interpreted as equivalent to the allodynia or hyperalgesia observed in some patients with neuropathic pain.

Several of these animal models (most commonly the CCI and Seltzer models) and tests have been used to explore the mechanisms behind the effect of SCS. This thesis is based on studies using the Seltzer nerve injury model and responses that are considered to be pain-associated (hypersensitivity to touch, cold, and heat hyperalgesia).

Aims of the thesis

The overall aim of this thesis was to investigate the mechanisms involved in the development of nerve injury-induced pain with special emphasis on the mechanisms behind the pain relieving effect of SCS.

In particular,

1. To study the relationship of the NMDA receptor and the presence of hypersensitivity after nerve injury.
2. To study the involvement of different transmitter systems in the effects of electrical stimulation of the spinal cord (SCS) on symptoms after nerve injury
 - a. GABA synthesis and the effect of SCS
 - b. effect of SCS on the cholinergic system
 - c. effect of SCS on the descending serotonergic system
3. To examine a possible relationship between severity of mechanical hypersensitivity following nerve injury and the outcome of SCS.

Materials and methods

Animals

All experiments within this thesis were carried out in accordance with Guidelines of the Committee for Research and Ethical Issues of the International Association of the Study of Pain (1983). The experimental protocols were examined and approved by the local ethical committee for animal research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Male Sprague–Dawley rats (B&K Universal AB, Sweden) weighing between 180–220g at the time of the initial surgery were used in the experiments. The animals were exposed to a 12:12 hour light:dark cycle and were provided with food and water *ad libitum*. Upon arrival to the animal department, the animals were acclimatized to the environment for at least one week before being exposed to any further experiments. The animals were initially housed in groups of 3–4 per cage. After electrode implantation, the animals were individually housed in order to prevent damage of the contacts.

Surgical procedures and behavioral assessments

Anesthesia

In the present studies, inhalation anesthesia was selected for the reason that the level of anesthesia can be more easily controlled than with the use of sedative drugs administered *intra peritoneally* (i.p.).

In study I, general anesthesia was induced by 4% halothane (Fluothane®, AstraZeneca, Sweden) and maintained with approx 1–2% in a 1:1 mixture of air and oxygen via a face mask at a flow rate of 2 l/min. In study II–V, the rats were anesthetized with Isoflurane (Forene®, Abbot, Sweden), induction 4–5% and maintenance 2–3% at approximately 450 ml/min.

Areflexia to painful pinch stimuli indicated adequate level of anesthesia. A heating pad connected to an automatic temperature controller (CMA/150, CMA

Microdialysis AB, Sweden) was used to keep body temperature at 37 ± 0.5 °C during surgery. Postoperative analgesia following nerve injury and electrode implantation was provided by administering Buprinorphine (Temgesic, 0.05mg/kg i.p., Apoteket, Sweden) or Carprofen (Rimadyl, Apoteket, Sweden (5mg/kg, s.c.) as recommended by the ethical committee. Analgesic treatment in conjunction with surgery is applied not only to reduce pain, but also to improve recovery and reduce morbidity and mortality.

Nerve injury (I-V)

A unilateral partial sciatic nerve lesion, originally described by Seltzer et al. (Seltzer et al. 1990) was used to create a model of nerve injury-induced evoked pain. In brief, the left sciatic nerve was carefully exposed at the proximal thigh level, a curved mini needle with an 8/0 ethilon suture was inserted into the nerve, tightly ligating 1/2–1/3 of the nerve volume. After completion of surgery, the animals were placed in separate cages until they were fully awake and then returned to their home cages.

Behavioral assessments (I-V)

All behavioral tests were carried out in a separate room under standardized conditions with regard to noise level, light intensity and time of day. The animals were placed in a testing cage consisting of a circular plexi glas cage with a wire mesh floor. The rats were allowed to habituate to the testing environment for at least 15 minutes prior to each test session. The sensory tests were performed at 2–4 weeks after the induction of nerve injury, when maximum hypersensitivity generally is observed (Seltzer et al. 1990; Cui et al. 1997), and immediately prior to euthanasia

Mechanical sensitivity (I-V)

Von Frey filaments (OptiHair, Marstock Nervtest®, Germany (in I-IV); North Coast Medical, Inc., Morgan Hill, CA USA (in V)) were used to assess withdrawal thresholds (WT) to static mechanical stimuli. This was done at predetermined intervals before, during and after i.t. administration of drugs and SCS. The OptiHair Marstock Nervtest® type of von Frey filaments are made of optic glass fibres (OptiHair), which are highly elastic and are not influenced by normal temperature and humidity changes. The end of each filament is coated with a small epoxy bead (diameter 0.30 -

0.45 mm) to ensure a fairly constant contact surface for fibers of different diameters. Study V was performed at the Pain Management and Research Center, Dept. of Anaesthesiology, Maastricht University Hospital, where a different type of von Frey filaments was routinely used. However, all comparisons are made solely within animal groups examined with the same filament set.

Fourteen filaments with stiffnesses corresponding to 0.5 to 30 g (I-IV) and in study V 14 filaments 0.16-100 g were applied to the mid-plantar intact (contralateral) and nerve-injured (ipsilateral) hind paws. The test was started with a 4 or 5 g filament and continued in ascending or descending order of stiffness after a negative or positive response, respectively. The softest filament provoking paw withdrawal at least three out of five applications determined the WT, and based on these measurements, the rats were divided into a hypersensitive and a non-hypersensitive group. Rats with a WT of 7-8g or less were considered to be hypersensitive and rats with a WT of 15g or more were defined as non-hypersensitive (Kontinen et al. 2001; Wallin et al. 2002).

In study V, the pre-stimulatory degree of hypersensitivity as related to the outcome of SCS was studied in rats with decreased WT after nerve injury. These rats were divided in three different groups based on defined cut off points, “severe” (WT 0.16-1.0 g), “moderate” (WT 1.4-6.0 g) and “mild (WT 8.0-26 g)” mechanical hypersensitivity.

For studies including drug administration and/or SCS, a baseline WT was established, and the assessment was repeated every 10 min until pre-treatment thresholds were restituted.

Cold sensitivity (I, IV)

In studies I and IV, the response to cold stimulation was assessed with ethyl chloride spray (Rönning's Europa AB, Medikema, Sweden). The responses were scored according to a cold sensitivity rank scale (CS): (0) no response; (1) paw withdrawal; (2) paw shaking; (3) paw licking; (4) vocalization and other generalized aversive reactions (NB, in study IV the scale numbers are reversed). Prior to testing, a few bursts of ethyl chloride were sprayed next to the cage in order to habituate the animal to the noise of the discharge. Cold sensitivity was defined as the mean score response of three quick burst stimuli applied with 5-min recovery periods.

Heat sensitivity (IV)

Heat hyperalgesia was in study IV assessed using the Basile Plantar Test (Ugo Basile, Italy) (Hargreaves test). A beam of a movable infrared generator was focused on the hind paw and the paw withdrawal latencies (PWL) were recorded in seconds (s). The final PWL was based on the mean of three recordings, with 3-min recovery periods. The automatic cut-off time was set to 30 s.

Implantation of spinal electrodes (II-IV)

Animals with sciatic nerve injury showing signs of mechanical hypersensitivity were implanted with a monopolar miniature electrode system for spinal cord stimulation (Fig. 2) (Linderöth et al. 1992; Meyerson et al. 1994; Stiller et al. 1996). After exposure of the spine, a laminectomy of the thoracic vertebra T11 or T12 was performed. The cathode (a solid rectangular silver plate: 3x1.5x0.25 mm; later substituted by a platinum-iridium plate provided by Bakken Research Center, Maastricht) was placed in the dorsal epidural space and the wire was fixed to the musculature with tight sutures. The anode (a solid silver disc 6mm in diameter, later platina/iridium) was implanted in the paravertebral tissue on the left side. The microcontacts and wires connected to the electrodes were tunneled subcutaneously to the neck where they were tightly fixed to the skin. After the electrode implantation the animals were carefully observed for neurological deficits and were allowed to recover for at least 24 hours before further experiments.

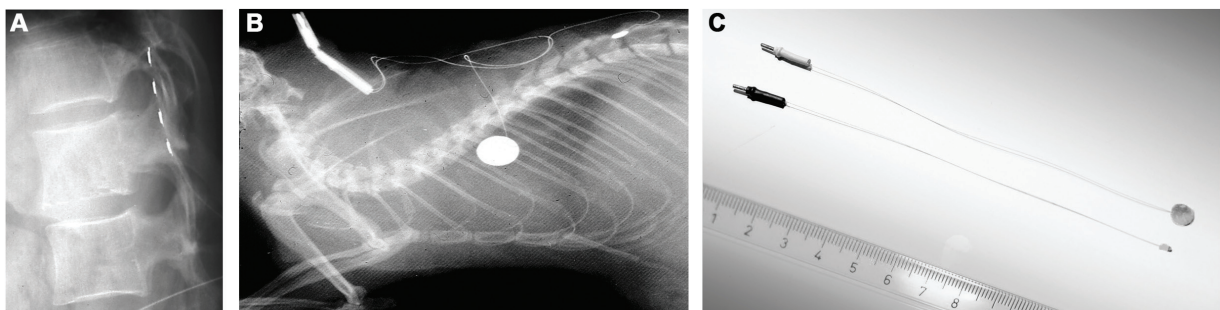


Fig. 2

Radiograph (lateral view) of a patient with a quadripolar plate electrode implanted epidurally in the lower thoracic region (A). Radiograph of a rat with a monopolar miniature electrode system for SCS implanted epidurally in the lower thoracic region (B). The monopolar electrode system for SCS in rats (C).

Implantation of microdialysis probes (III)

Microdialysis is a widely used technique where a probe perfused with a physiological buffer solution is inserted into living tissue, where a substance exchange takes place. Molecules in the extracellular fluid (ECF) and the perfusion buffer diffuse through the semi-permeable membrane of the probe (Fig. 3). The technique is often used to monitor transmitter release in various tissues such as brain, spinal cord, kidneys, etc. (Stiller et al. 2003).

In our experimental model, *in vivo* microdialysis was performed in the dorsal horn (DH) of the spinal cord to monitor the release of ACh (study III) in the tissue over time. The animal was placed in a stereotaxic spinal unit and the vertebrate column was stabilized to minimize interference from breathing movements. The spinal cord was exposed by a laminectomy at T13 and the microdialysis probe (CMA 11; single cuprophane dialysis membrane, length 2 mm, outer diameter 0.24 mm, molecular cut-off 6 kDa; CMA Microdialysis AB, Stockholm, Sweden), carried by a micromanipulator (David Kopf, CA, USA), was inserted in a 45° angle caudally into the dorsal horn ipsilateral to the nerve injury. The estimated depth of the probe tip in the dorsal horn was 1.4 mm.

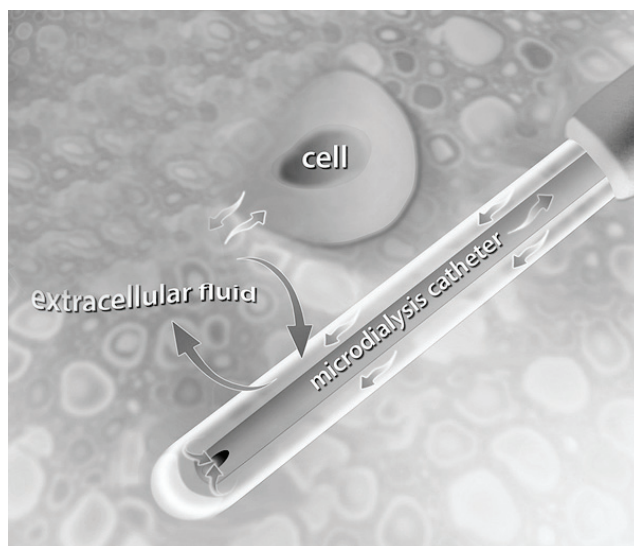


Fig. 3

Schematic representation of microdialysis. Chemical substances from the extracellular fluid diffuse through the dialysis membrane and are transported by the flow of the perfusion fluid and collected in microvials. By courtesy of CMA Microdialysis AB.

Treatment procedures

Spinal cord stimulation (II-V)

By connecting the microcontacts of the implanted SCS electrodes to a Grass S44 stimulator via a constant current unit (CCU 1A) and a stimulus isolation unit (SIU5) (Grass, USA), stimulation was applied with a frequency of 50 Hz, a pulse width of 0.2 ms and a current intensity individually set to 60-80% of the intensity producing a motor response. The motor response or motor threshold (MT), defined as a light twitching of the lower trunk muscles and/or the legs, was determined for each individual rat before each SCS session. The parameters used were chosen to mimic those used in patients (Meyerson and Linderöth 2000) (Linderöth and Meyerson 2009). Each session consisted of 30 min stimulation, during which the rat was allowed to move freely in the observation cage.

Drug administration (III, IV)

A polyethylene catheter (32G, Lynn Scott, USA) was inserted through a 23G cannula between the L5 and L6 vertebrae and advanced rostrally up to the lumbar enlargement. The catheter was fixed to the fascia, tunneled subcutaneously to the neck and sutured to the skin. In order to verify the correct position of the catheter, Lidocaine (10µl, 2% Xylocain®, AstraZeneca, Sweden) was injected. The subsequent transient bilateral flaccid paralysis of the hind limbs was used as physiological confirmation of a properly positioned catheter. In addition, to further confirm site of the catheter tip, methylene blue dye (10µl) was injected and spinal cord staining verified.

Drugs for intrathecal (i.t.) administration (10µl) were dissolved in saline and pre-warmed to approx 37°C. Only one drug per day was tested in each animal. The dosage of the drugs were chosen on the basis of previous studies and adjusted according to pilot experiments. Drugs used for spinal administration and their presumed action are listed in Table 1.

Table 1

Drugs and doses	Mechanism of action	Study
Atropine (30µg)	Non-selective muscarinic receptor antagonist	III
Pirenzepine hydrochloride (10µg)	M ₁ muscarinic receptor antagonist	III
Methoctramine tetrahydrochloride (10µg)	M ₂ muscarinic receptor antagonist	III
4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (10µg)	M ₃ muscarinic receptor antagonist	III
Muscarinic toxin-3 (MT-3) (5µg)	M ₄ muscarinic receptor antagonist	III, IV
Mecamylamine (MCM) (50µg)	Non-selective nicotinic receptor antagonist	III
Neostigmine bromide [10µM]	Acetylcholine esterase inhibitor	III
Serotonin (5-HT) (0.5µg)	Neurotransmitter	IV
CGP-35348 (50µg)	GABA _B receptor antagonist	IV

Assays and analyses

Retrograde labeling and tissue processing (I-IV)

In study I, animals aimed for immunohistochemistry (IHC) were given an intraneural injection of the retrograde axonal tracer Fluoro-Gold® (FG; Fluorochrome Inc, USA) which is taken up by damaged fibers and retrogradely transported to the corresponding cell bodies (i.e. lamina IX motor neurons in the ventral horn). FG labeling was performed in order to localize the spinal cord segments where the sciatic afferent nerve fibers terminate, as well as to distinguish between the ipsi- and contralateral sides (Asato et al. 2000) in the spinal cord sections.

One to two weeks following injection of FG, animals were anesthetized with a lethal dose of pentobarbitone (250 mg/kg, i.p.) and euthanized by transcardial perfusion with 200ml, 37°C saline followed by 500ml, 4°C paraformaldehyd (4%) in phosphate buffer solution (PBS). The spinal cord was excised and the L4-L6 (Fig. 4) post-fixated in 4°C paraformaldehyd (4%) in PBS for 60 min, followed by PBS overnight at 4°C. The tissue was then immersed in 15% sucrose in PBS for 24 h before freezing, sectioning (14µm) and mounting on microscope slides.

For Western Blot and ELISA, the spinal cord was rapidly excised immediately after euthanizing the animal and the dorsal half of the L4-L5 (WB) L4-L6 (ELISA) spinal cord segments divided into quadrants ipsi- and contralateral to the nerve injury. The tissue was immediately frozen on dry ice.

Immunohistochemistry (I, II, IV)

Prior to immunolabelling, the spinal cord sections were examined for FG uptake by fluorescence microscopy and FG negative sections were excluded from further analyses. The FG positive sections were immunolabeled using routine protocols for immunohistochemistry. In brief, sections were incubated overnight at 4°C with primary antibodies and the following day with the corresponding secondary antibody (biotinylated or fluorescent). To reveal immunoreactivity (IR) in the case of biotinylated antibodies (study I), the avidin-biotin complex (ABC, Vectastain Elite ABC kit, Vector Laboratories Inc, USA) and diaminobenzidine-Ni (DAB substrate kit for peroxidase with NiCl₂, Vector Laboratories Inc) protocols were used. Control experiments in which the primary or secondary antibodies were omitted showed no immunostaining.

In study I, the IR of NMDA receptor subunits and Neuronal Nuclei (NeuN) in the spinal dorsal horn was quantified with Scion Image for Windows (Scion Corporation, USA). Areas comprising laminae I–IV of the ipsi- and contralateral dorsal horns were photographed and the amount of immunostaining analyzed (for details see study I). In study II and IV, antibody staining was visualized with fluorescently conjugated secondary antibodies and examination of the slides was performed blindly. Where double labeling was performed, the procedure was repeated for the next antibody.

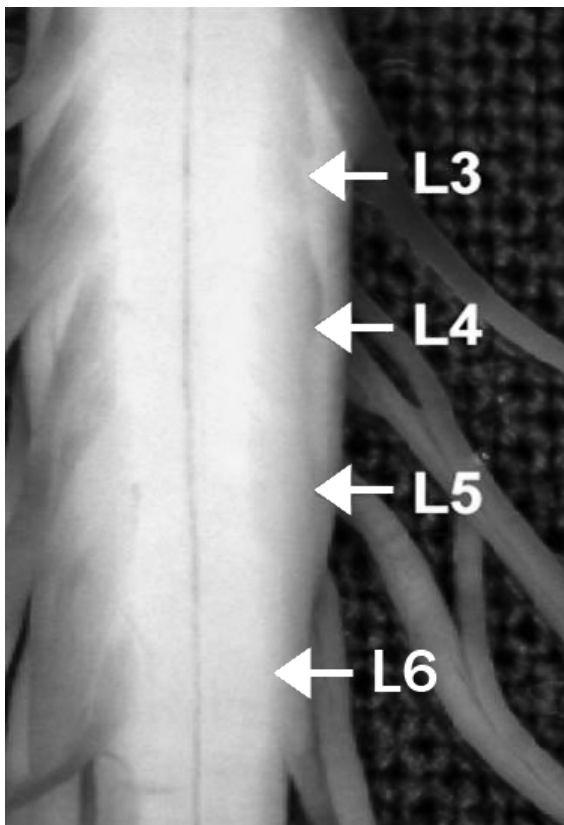


Fig. 4

Photograph of the ventral side of the rat spinal cord. Arrows indicate the entries of the sciatic nerve roots L3-L6. Adapted from Lidman et al. 2003.

Western Blot (I, II)

In order to analyze protein content, tissue from the ipsi and contralateral spinal dorsal quadrants was homogenized in solubilization buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1mM EDTA, 1%NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1mM Na_3VO_4) containing protease inhibitor (Protease Inhibitor Cocktail, Sigma, St Louis, MO, USA) and centrifuged twice at 10 000 x g for 10 min at 4°C. Equal amounts of protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, UK). Non-specific binding was blocked by incubation in dry-milk. Following incubation with blocking buffer, the membranes were incubated with primary antibody followed by horseradish peroxidase conjugated IgG Amersham™, GE Healthcare, UK). Immunoreactive bands were detected using Enhanced Chemiluminescence (Amersham ECL™ and ECL Advanced™, GE Healthcare, UK) and exposing autoradiographic film to the membranes (Amersham Hyperfilm ECL, GE Healthcare, UK).

Microdialysis and HPLC (III)

Briefly, the High-Performance Liquid Chromatography (HPLC) system consists of a microbore column liquid chromatography linked to a post-column immobilized enzyme reactor and electrochemical detection on a peroxidase-redox polymer-coated electrode. The detection limit for ACh was 10 fmol/20µl (review see Lunte and Lunte 1996). Before the in vivo µ-dialysis experiments, in vitro recovery for five different concentrations of ACh was analyzed (50, 100, 250, 500 and 1000 nM) (to ensure stable recovery and that the HPLC could detect the changes).

For the in vivo experiments, the probe was perfused with modified Ringer solution (NaCl 148 mM; KCl 2.7 mM; CaCl_2 1.2 mM; MgCl_2 0.85 mM) containing 10 µM of neostigmine. The inclusion of the acetylcholine esterase inhibitor is essential to prevent degradation of ACh and thereby enabling detection (Barber et al. 1984; Hoglund et al. 2000; Abelson and Hoglund 2002). In the experiment, dialysates collected in consecutive 30 min intervals were analyzed to determine the levels of ACh under three different settings: basal levels prior to SCS, during and after stimulation. The dialysates were immediately frozen, before subsequent HPLC analysis. The microdialysis session was set up so that each experiment was ended with inclusion of 100 nM KCl in the perfusion solution, in order to induce a massive depolarization and total ACh depletion in the vicinity of the probe.

The experimental set-up of study III was: (a) microdialysis and HPLC analysis of spinal ACh release following SCS; (b) influence of ACh receptor antagonists on the effect of SCS in behavioral experiments.

ELISA (IV)

In study IV, 5-HT content in the spinal tissue was quantitatively assessed using a commercially available ELISA kit (IBL, Germany). The L4-L6 ipsilateral and contralateral dorsal quadrants were homogenized in ice-cold lysis buffer (137 mM NaCl, 20 mM Tris HCl (pH 8.0), 1% NP40, 10% l glycerol, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vanadate). Homogenates were centrifuged at 10 000 x g for 10 min and the supernatant collected and stored at -70 °C. Standards, acylated control serum, and acylated samples were loaded into appropriate wells and serotonin-biotin and antiserum was added. The plate was sealed and incubated for 16 h at 4°C. After washing the wells with wash buffer, enzyme conjugate was added and incubated for 120 min at room temperature. Following this step, the plate was washed and substrate buffer was added and incubated for 60 min at room temperature until color development was achieved. The substrate reaction was terminated with stop solution and optical absorbance was recorded at 450 nm with a microplate reader (ELx808 Absorbance Microplate Reader, BioTek Instruments, Inc). The average of duplicated data was obtained and sampled concentrations were determined from the standard curve.

Statistics

Statistical analysis was performed with Graph Pad Prism (Graph PadPrism Software Inc., San Diego, CA, USA). The Mann-Whitney U-test (study I, II) or the Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc test (study III, IV) was used for analysis of differences between groups. The Wilcoxon signed ranks test was employed used for analysis of differences between two paired observations (study III, IV) and the Friedman test for repeated measures with multiple treatments.

In study V, parametric statistical tests were used. Student's paired t-test (with post hoc correction) was used comparing values during and after SCS as well as maximal therapeutic effect compared to baseline within the same group. In general, a p-value ≤ 0.05 was considered as significant.

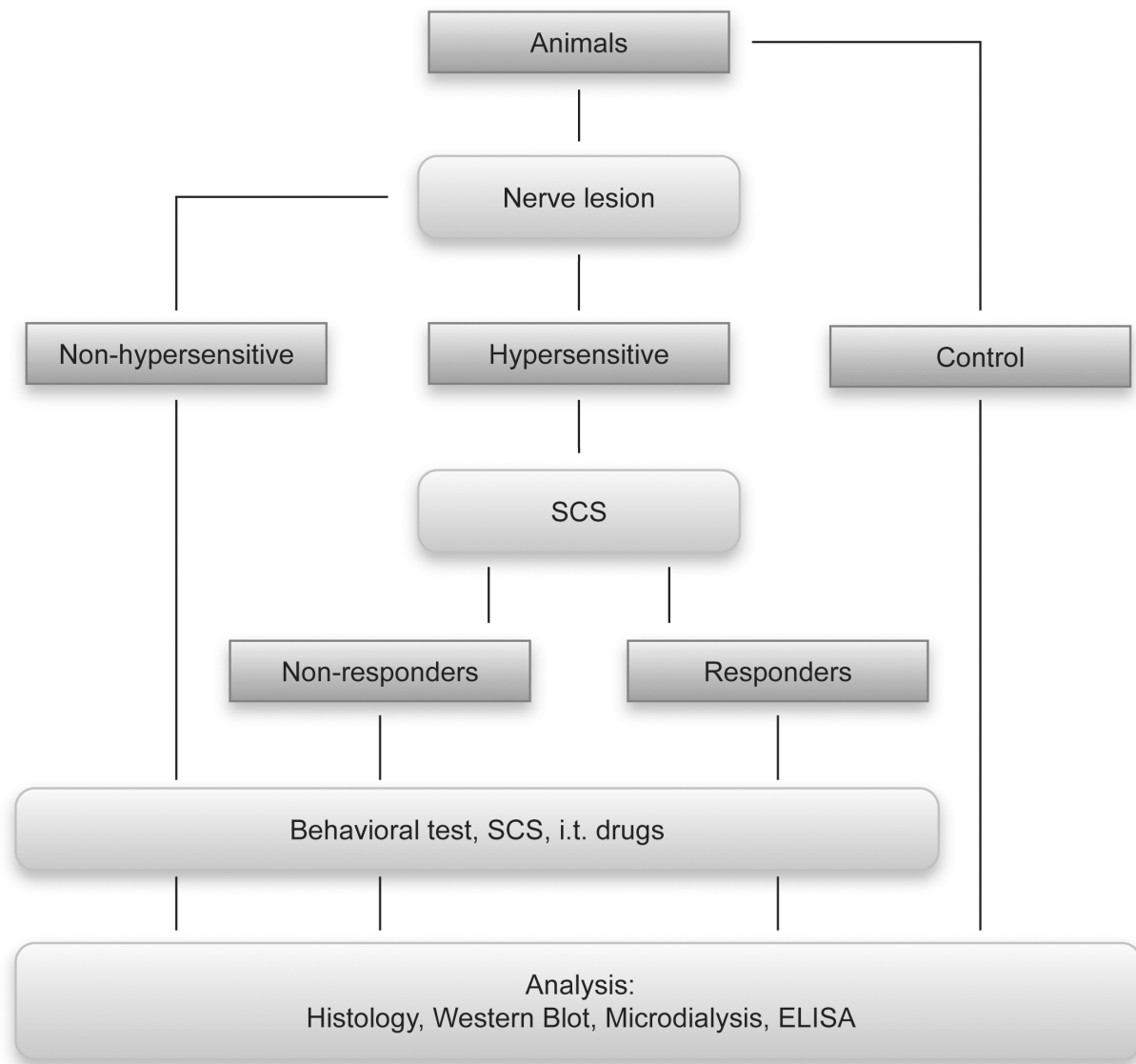


Fig. 5

General outline of the experimental design of the projects included in the thesis. Application of techniques and analyses varied in the studies.

Methodological considerations

Animal models of neuropathic pain and SCS

For obvious reasons, the inability to communicate with animals limits the chances of valid pain evaluations. There is an on-going debate regarding the usefulness and relevance of the currently available animal models for chronic pain and the limited success in translating research on nociception in animals into new and more effective

pain treatments (Hansson 2003; Vierck et al. 2008; Mogil 2009; Mogil et al. 2010). The criticism is focused on the extensive usage of innate reflex responses (reflex withdrawal), assessing only the sensory discriminative aspect of the pain experience, and several of the existing models of pain are by many clinicians considered as not having enough relevance for human conditions.

Although the most commonly used models of chronic nerve injury usually present with diverse behavioral neuropathic pain-like responses, they differ clearly from clinical nerve injury-associated pain. Few of the animal models are able to mimic the most frequent symptoms of chronic neuropathic pain in humans where the dominating complaint generally is spontaneous, on-going pain (incl. numbness, paraesthesias and dysesthesias) (Otto et al. 2003; Scadding 2006; Backonja and Stacey 2004; Scholz et al. 2009). Instead, most studies have focused on assessing evoked pain responses like thermal and mechanical hypersensitivity that is present in maximally 20-40% of the neuropathic pain patients (Hansson 2003; Mogil and Crager 2004).

To increase the knowledge about the mechanisms underlying nerve injury induced pain and in our case, effects of SCS, there is a need to perform studies in vivo. In this thesis mechanical hypersensitivity was evaluated in terms of withdrawal thresholds to von Frey filaments. However, since pain is not a simple phenomenon but a multidimensional experience with major components as anxiety, depression and anger, it is impossible for it to be represented or described by a single parameter or number. While this method of evaluation assesses what may correspond to static mechanical allodynia, increased sensitivity to cutaneous brush-like stimuli (i.e. dynamic mechanical allodynia) is both more common and regarded to be more incapacitating in human nerve injury induced pain, but difficult to assess reliably in rats (Woolf and Mannion 1999; Hansson 2002; Backonja and Stacey 2004; Mogil and Crager 2004).

The incidence of pain-like signs in these models is generally high (50-90%), while it is estimated that only about 5% of the patients with peripheral nerve injuries develop neuropathic pain (Hansson 2002; Hansson 2003).

Moreover, from an epidemiological aspect, the chronic pain prevalence in a human population and the choice of animal models do not always match. Although experimental and clinical evidence demonstrates that women have lower pain thresholds/tolerance, and that the patients suffering from chronic pain are mostly women and the prevalence is higher in the middle-aged and elderly (Berkley 1997; Barrett et al. 2002; Greenspan et al. 2007), the experiments in this thesis, like in many other pain studies, were performed in young male rats.

Despite the discussion on relevance, there is a great value with animal models and an advantage when exploring basic physiological mechanisms of pain. There is a possibility of standardization of genetic and environmental backgrounds and they offer access to fine characterization of neurochemistry and anatomy. It is, however, indisputable that there are considerable advantages of using humans as subjects, especially when trying to investigate pain conditions that have no obvious cause (Mogil et al. 2010). Nonetheless, animal models are indispensable both in pain research in general and for development of new therapies.

Immunohistochemistry

The antibodies used were visualized with either fluorescently or enzymatically conjugated antibodies. The former has the advantage of easily illustrating co-localization when using two or more antibodies, but at the same time it has the disadvantage of fading. When performing enzymatic labeling, the system permits use of light microscopy and structures within the tissue may be more easily observed and the risk of losing the results (due to fading) is low.

A false negative reaction due to low antigen retrieval is an often encountered problem when performing immunohistochemistry, but also false positive reactions may occur. The use of proper controls will help eliminate these reactions and may also be a way of troubleshooting in the experimental process.

Immunohistochemistry protocols applied in this thesis include BSA to counteract nonspecific binding, NaN_3 to reduce microbial activity and a detergent to facilitate primary antibody tissue penetration. Both positive and negative controls were generally included. Although immunochemical techniques benefit from an extreme specificity, it should be kept in mind that there is always the risk of a faulty representation of true immunoreactivity/protein presence. In addition, although IHC can produce information about precise localization of a specific target, quantification may be problematic. Therefore, quantification in the present studies was performed by the use of other techniques.

Western Blot

In the present studies (I-II), WB was used to quantify the levels of proteins in spinal cord tissue. The method of detection in WB depends on the label that has been conjugated to the (in this case) secondary antibody. We used a horseradish peroxidase conjugated antibody which was visually detected by a chemiluminescent substrate, emitting light when conversion of the enzyme takes place and captured on x-ray film. The chemiluminescent antibody detection system is extremely sensitive, but just as in immunohistochemistry, the result is dependent on the chosen antibody (primary and secondary) and its binding characteristics. With some antibodies, especially monoclonal generated against native antigen, there may be problems with recognizing proteins that have been fractioned under reduced and denatured conditions as in SDS-PAGE. For meaningful results, the antibodies must bind only to the protein of interest and not to the membrane. Non-specific binding was in the current studies reduced using non-fat dry milk as blocking agent. It has been shown that including Tween-20 detergent may have a renaturing effect on antigens, resulting in improved recognition by specific antibodies (Van Dam et al. 1990; Zampieri et al. 2000). In study I, phosphorylated proteins were the target of detection. Protease inhibitors were added to the tissue homogenate to prevent dephosphorylation of the proteins, but could possibly also have been added to the blocking solution since addition of phosphatase inhibitors to the blocking solution has been shown to increase the signal with phospho-specific antibodies (Sharma and Carew 2002).

Microdialysis

Microdialysis has for a long time been a valuable tool for monitoring chemical communication between cells. With this technique it is possible to in vivo continuously study substances that are filtered across the semi-permeable membrane with the driving force of passive diffusion (review see Stiller et al. 2003; Lee et al. 2008).

When using microdialysis to monitor synaptic transmitter release over time, there are some aspects to consider. The concentration of transmitters in the extracellular fluid is not only a result of release, but also by other processes like cellular re-uptake, diffusion and degradation. In order to minimize the degradation and ensure maximal recovery of a neurotransmitter, enzyme inhibitors augmenting the available amount of a substance, can be added to the perfusion solution. In study III, neostigmine was used to counteract the degradation of ACh. Previous microdialysis studies have

shown that the use of neostigmine is essential to achieve measurable ACh levels and that it does not otherwise interfere with the results (Billard et al. 1995; Hoglund et al. 2000). The diffusion of substances through the probe membrane depends on the size of the molecules and low recovery is a constant problem in microdialysis (Stiller et al. 2003). A common strategy is therefore to provoke release of the investigated transmitter at the end of the experiment by an increase of the potassium concentration in the perfusion solution. The potassium stimulation causes a massive depolarisation of all cells and terminals in the vicinity of the probe, and rather than reflecting the physiological synaptic release of a certain transmitter, it represents the releasable pool in the local tissue. It also serves as a validation of the viability of the neuronal tissue at the end of the experiment and of the experimental set-up.

ELISA

The ELISA is a fast and accurate assay, and if a purified antigen standard is available, it is possible to determine the absolute amount of antigen in an unknown sample. In study IV, the amount of serotonin present in the spinal dorsal quadrant was measured. Since serotonin also is present in blood (concentrated in platelets), the tissue was rinsed clear of all blood at the time of animal euthanasia and tissue dissection. The quantitative determination of the serotonin concentration in our samples was performed by plotting the extinction values measured from provided standards against the corresponding concentrations, producing the calibration curve from which the concentration of serotonin could be determined.

Results

Nerve injury and NMDA receptor phosphorylation (I)

Following a partial sciatic nerve lesion (Seltzer et al. 1990) with subsequent development of hypersensitivity, rats were in this study tested for sensitivity to mechanical stimuli and to cold. Based on WT's, the animals were categorized as hypersensitive (41%) or non-hypersensitive (43%). The remaining animals (16%) that could not be classified to fit into any of these groups were excluded from further experiments. There was a relationship between the degree of mechanical and cold hypersensitivity.

The animals were euthanized at peak behavioral change, two to three weeks post injury, and spinal cord tissue was collected and processed for immunohistochemistry and Western Blot. The superficial laminae of the lumbar spinal dorsal horn exhibited a moderate to strong immunoreactivity of the labeled NMDA subunits (NR1, P-NR1, NR2A, NR2B, NR2C, NR2D) and NeuN (study I, Figs. 2 A-H). In animals with a pronounced hypersensitivity, the immunoreactivity of the phosphorylated NR1-subunit was increased in the DH ipsilateral to the nerve injury as compared to the contralateral one. This side difference in phosphorylation was only present in the hypersensitive group (Fig. 6). On the contrary, we could not detect an alteration of any of the other NR-subunits, neither between the ipsi- and contralateral side nor between the two groups.

These data suggest that signs of neuropathy are associated with increased NMDA receptor phosphorylation and that this prolonged phosphorylation may represent one mechanism of central sensitization following nerve injury.

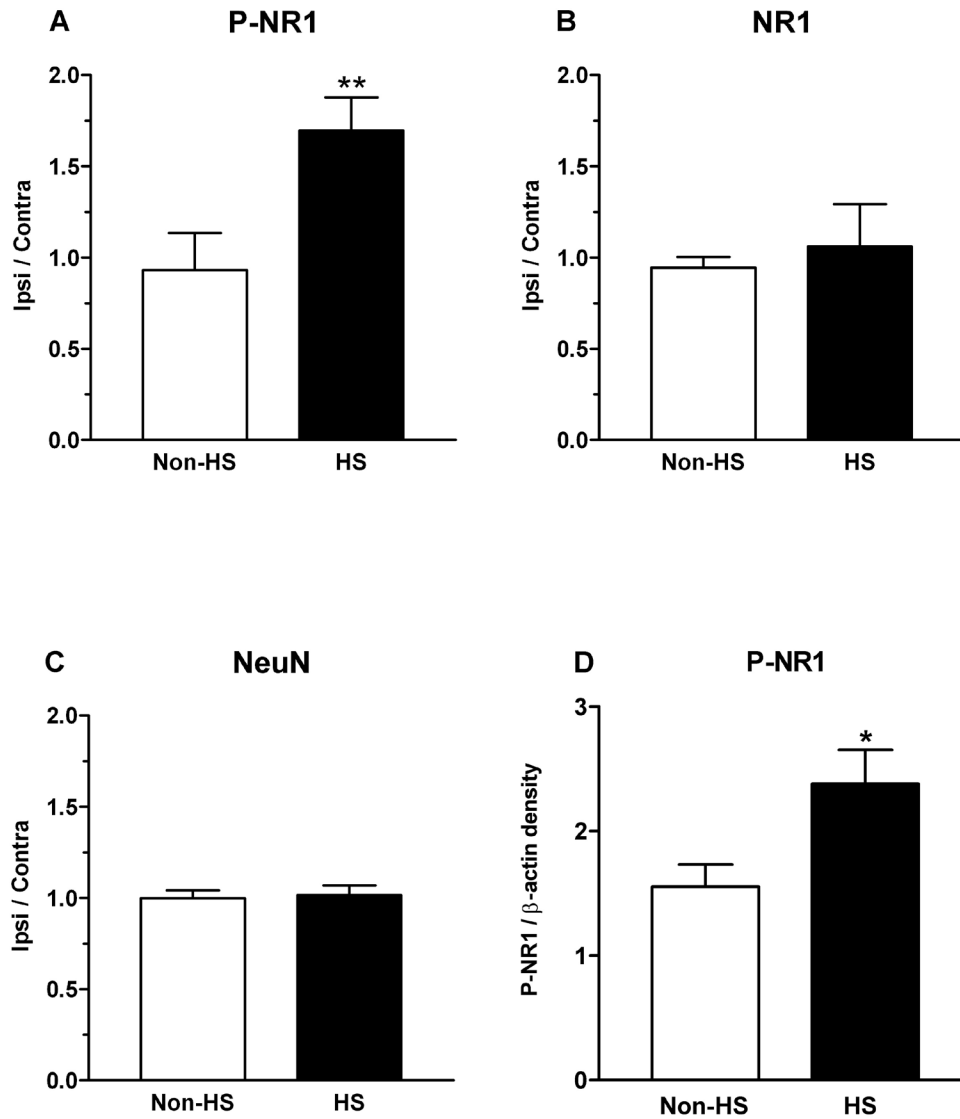


Fig. 6

Quantification of P-NR1 (A), NR1 (B) and NeuN (C) immunoreactivity (IR) in the dorsal horn of hypersensitive (HS) and non-hypersensitive (Non-HS) rats. Immunostaining is presented as the ratio between the ipsi- and contralateral side in the labeled pixel area. The ipsi/contra ratio of P-NR1 IR was significantly higher in hypersensitive as compared to non-hypersensitive rats. (D) Western Blot analysis of ipsilateral dorsal horn tissue (L4-L5) in hypersensitive and non-hypersensitive rats. The amount of P-NR1 was significantly increased in the ipsilateral dorsal horn of HS compared to non-HS rats (approximately 14 days post-nerve injury). (* $p \leq 0.05$, ** $p \leq 0.01$, Mann-Whitney U-test)

GABA synthesizing enzymes and SCS (II)

In this study we demonstrated changes of the GABA synthesizing enzymes GAD65 and GAD67 in the DH following SCS.

Nerve injured animals were tested for mechanical sensitivity with von Frey filaments and subsequently classified as either hypersensitive ($WT \leq 7-8$ g) or non-hypersensitive ($WT \geq 15$ g). Once reaching a stable level of hypersensitivity, a group of hypersensitive rats received SCS systems. During 30 min of stimulation, mechanical sensitivity was evaluated and the rats subsequently divided into SCS responders and SCS non-responders (study II, Fig 1).

Following behavioral testing, spinal cord tissue was collected from the groups hypersensitive, non-hypersensitive, SCS non-responders and SCS responders with or without stimulation applied immediately prior to euthanasia. The lumbar dorsal spinal segments were either processed for Western Blot or sectioned for immunohistochemistry and the levels of the two isoforms of the GABA synthesizing enzymes, GAD65 and GAD67 were analyzed (Fig. 7).

GAD-IR was present throughout the grey matter in the lumbar spinal dorsal horn with a more intense staining in lamina I-III as compared to lamina IV-VI and X. A tendency to a differential distribution of the GAD-IRs could be seen, with the GAD67 antibody appearing as a band with relatively more intense staining in lamina IV-V while the GAD65 antibody had a more widespread distribution.

No apparent differences in spinal GAD staining could be detected, neither between the ipsi- and contralateral dorsal horns nor when comparing the groups of nerve injured and control animals. In contrast, there was a marked increase in GAD65-IR in the group responding to SCS and subjected to stimulation immediately prior to tissue collection, while responders without this stimulation did not show the same increase. The effect of SCS on GAD67-IR, was much less obvious, but there seemed to be a widening of the immunoreactive band in the stimulated groups. As with GAD65, the GAD67-IR was again less prominent in the group of SCS responders without stimulation.

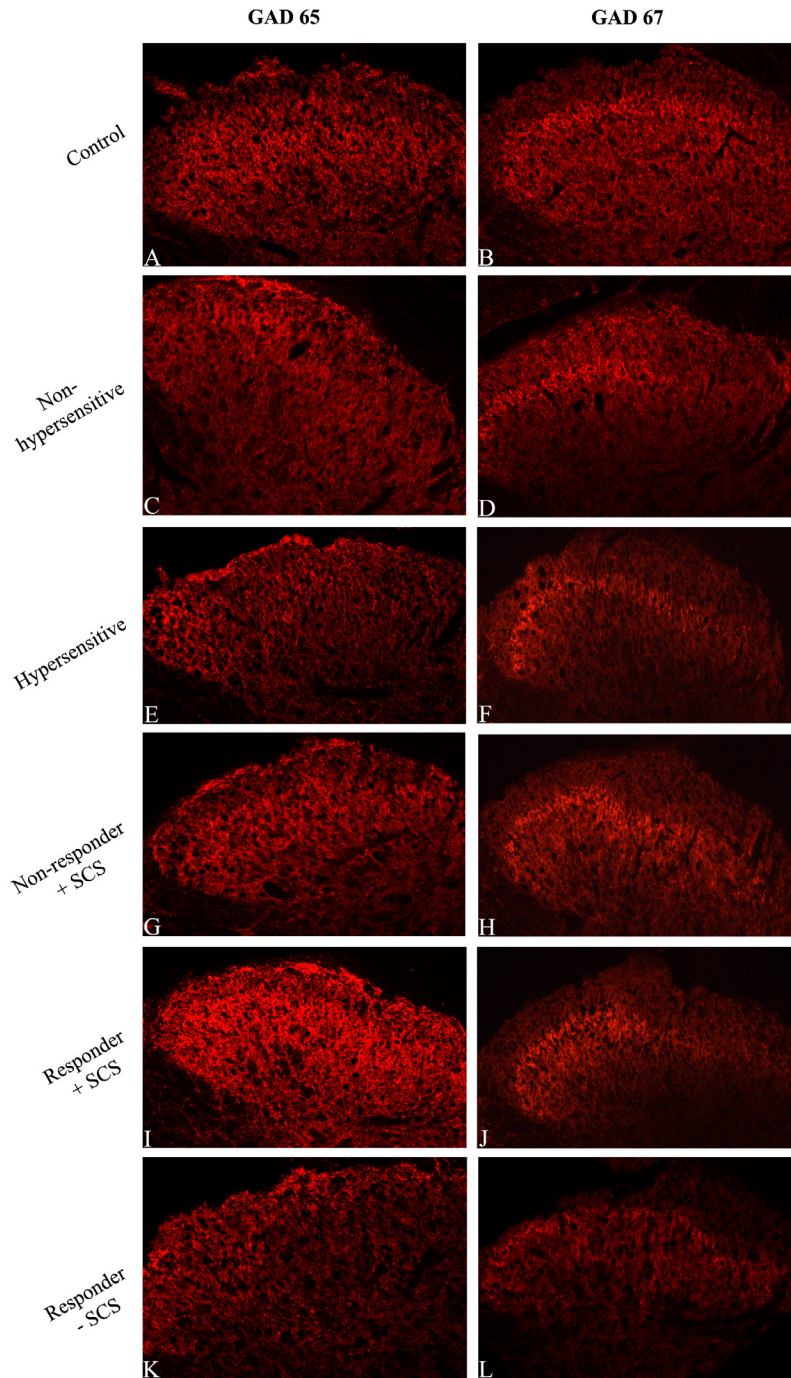


Fig. 7

Glutamic acid decarboxylase (GAD) immunoreactivity (IR) in the lumbar dorsal horn, photographed with 10x magnification. Examples of GAD65 and GAD67 IR on the side ipsilateral to nerve lesion. Control (A, B), Non-hypersensitive (C, D), Hypersensitive (E, F), SCS non-responder with stimulation (G, H), SCS responder with stimulation (I, J) and SCS responder without stimulation (K, L). GAD65-IR appeared to be higher in responders + SCS (I) compared to the other groups. A similar tendency was observed for GAD67 both in non-responders and responders (H, J).

Also with Western Blot, an increase in GAD levels following SCS could be detected (Fig. 8). This increase in GAD67 expression was present in both responders and non-responders with stimulation applied immediately prior to tissue collection. However, there appeared to be a more prominent SCS induced increase in GAD65 in rats responding to stimulation compared to those that did not. No differences, however, reached statistical significance. In contrast, there were no changes in GAD expression when SCS had not been applied immediately prior to tissue collection.

Although the levels of GABA synthesizing enzymes appeared to be augmented by SCS in stimulated rats, no relationship between consistent and prominent changes in responsiveness to innocuous mechanical stimuli after nerve injury and the levels of the GABA synthesizing enzymes could be confirmed in spite of a decreased basal release of GABA in these animals as demonstrated in a previous study (Stiller et al. 1996).

The present results thus emphasize the importance of the GABAergic system in the effect of SCS suggesting that it deserves further exploration and that the stimulation induced effects seem to be more general and complex than previously believed.

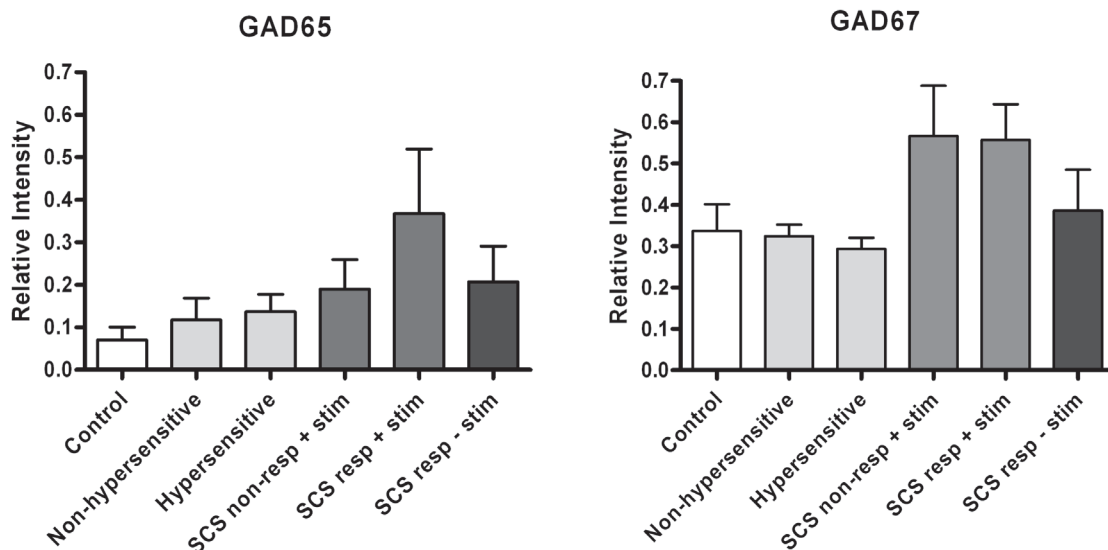


Fig. 8

Graphs showing Western Blot analyses of GAD65 and GAD67 in the dorsal quadrant of the spinal cord ipsilateral to the nerve lesion. The level of GAD65 intensity was increased in the group of rats responding to SCS and receiving stimulation immediately prior to tissue collection (+ stim), as compared to non-responders with stimulation and responders without stimulation (- stim). The level of GAD67 intensity increased both in non-responders and responders with stimulation immediately prior to tissue collection. None of the differences reached statistical significance.

Acetylcholine release and the effect of SCS (III)

In order to elucidate whether and how the spinal cholinergic neurotransmitter system is involved in the pain relieving effect of SCS, nerve injured hypersensitive rats were implanted with an electrode system and subjected to dorsal horn microdialysis to assess a possible spinal release of ACh produced by SCS.

It was observed that during SCS, there is an increased release of ACh in the dorsal horn ipsilateral to the nerve injury in animals responding to SCS, whereas no changes were detected in the group of non-responding animals (Fig. 9).

An unexpected and significantly lower basal dorsal horn release of ACh was observed in rats with signs of neuropathy as compared to control rats. Also the K^+ induced ACh release was significantly lower in the group of hypersensitive rats than in the control group.

However, no evident differences in basal ACh levels were observed between the responder and non-responder groups.

In the second part of the study, the aim was to identify nicotinic and/or muscarinic receptor subtypes that might be involved in the SCS effects. For this purpose specific and non-specific ACh receptor antagonists were administered i.t. before SCS application in SCS-responding rats and the resultant behavior changes during SCS were recorded.

The tests revealed that the SCS effect could be completely eliminated by i.t. atropine and a selective muscarinic M_4 receptor antagonist. The M_1 and M_2 receptor antagonists only produced a partial attenuation. No changes of the SCS effect were observed with the administration of saline, an M_3 antagonist or with the non-selective nicotinic receptor antagonist (Fig. 10).

This investigation provides evidence that the SCS effect involves a stimulation induced ACh release and activation of especially spinal M_4 receptors. This observation suggests that the pain relieving effect of SCS at least partly relies on spinal cholinergic mechanisms.

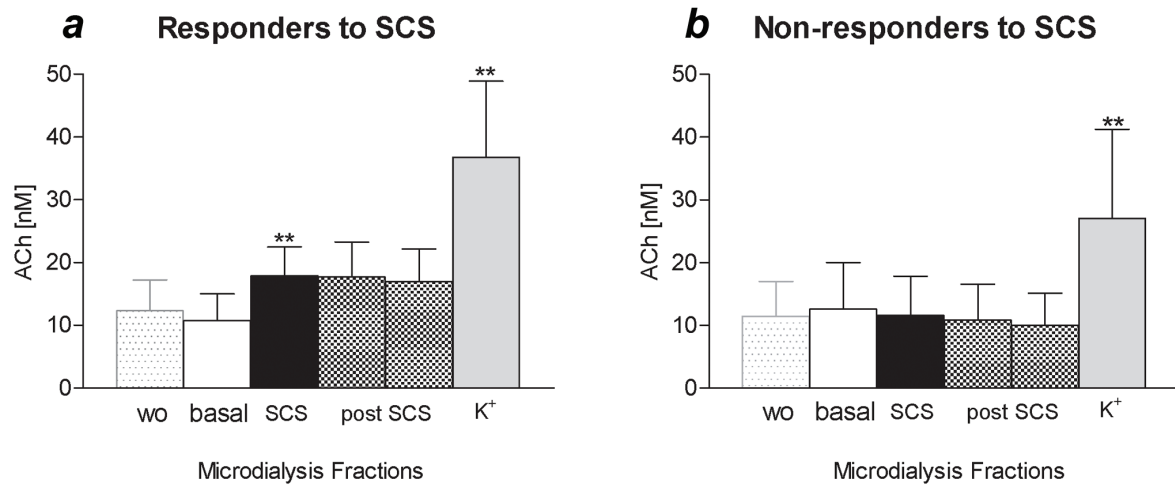


Fig. 9

Acetylcholine microdialysis. Graphs showing ACh release in the dorsal horn, before, during, and after SCS, in (A) SCS responders and (B) SCS non-responders. SCS induced a significant increase in ACh release in responders during stimulation as compared to the basal level of release. SCS did not induce an increase in ACh in the non-responding group. K⁺ stimulation induced a significant ACh release in both responding and non-responding rats. (wo = wash-out fraction) (**p≤0.01, Wilcoxon signed rank test).

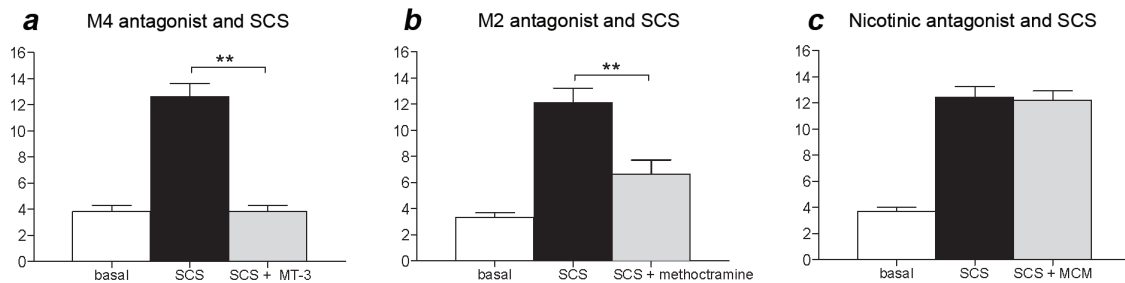


Fig. 10

Graphs showing the effect of SCS per se on tactile hypersensitivity and in combination with ACh receptor antagonists. A selective M₄ receptor antagonist, completely abolished the effect of SCS (a) while a selective M₂ receptor antagonist partially reduced the SCS effect. The non-selective nicotinic receptor antagonist produced no changes of the SCS effect. (**p≤0.01, Wilcoxon signed rank test).

Serotonergic mechanisms and SCS (IV)

In order to examine a possible interaction between the spinal systems involved in the inherent pain control and the effect of SCS, serotonin was targeted in study IV. Following partial sciatic nerve injury, approximately 65% of the rats in this study developed signs of pain related behavior in the form of mechanical and cold hypersensitivity as well as heat hyperalgesia. About 50% of these rats were subsequently classified as SCS-responders.

SCS significantly augmented the 5-HT content, as analyzed by ELISA, in the ipsilateral dorsal quadrant of the spinal cord in responding rats when SCS was applied immediately prior to sacrifice. This increase was not observed in SCS non-responders or in responders without preceding stimulation (Fig. 11). Similar, but bilateral, changes were found with IHC.

By administrating a sub-effective dose of serotonin it was possible to markedly enhance the pain relieving effect of SCS on mechanical and cold hypersensitivity in the animals where SCS per se was ineffective, i.e. to transform non-responders into responders. This, however, was not effective for heat hyperalgesia. The enhancing effect of serotonin on SCS could be partially blocked by a GABA_B receptor antagonist. In contrast, administration of an M₄ receptor antagonist had little or no effect on the serotonin enhancement.

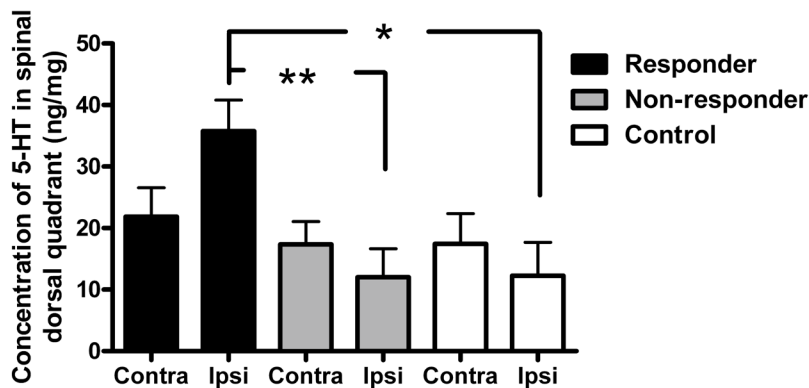


Fig. 11

ELISA analysis of serotonin (5-HT) content in the dorsal quadrant of the L4-L6 spinal segments, ipsilateral (ipsi) and contralateral (contra) to the nerve injury. Both in nerve injured and control animals, ipsi and contra refer to the left and right sides, respectively. SCS significantly increased the 5-HT content in the ipsilateral side in responding animals. This increase was not present in non-responding nor in control rats. SCS was applied immediately prior to tissue collection. (* $p \leq 0.05$, ** $p \leq 0.01$, Kruskal-Wallis ANOVA).

Data presented in this study provides evidence for an important role of spinal 5-HT in the mode of action of SCS, involving the activation of descending serotonergic pathways that may be inhibitory to spinal nociceptive processing, partially via a GABAergic link.

SCS effect as related to degree of hypersensitivity (V)

In this study, the effect of SCS in an experimental model of neuropathy was investigated and related to the severity of mechanical hypersensitivity following nerve injury.

Rats that developed a decrease of the WT after partial the nerve injury were subdivided into three groups representing mild, moderate and severe hypersensitivity (8.0-26 g, 1.4-6.0 g and 0.16-1.0 g, respectively). All rats were subjected to 30 min of SCS. The WTs during and after SCS were compared to the pre-stimulation WTs in the three groups. In the group with severely hypersensitive rats no statistically significant increase in WTs was found, while SCS in both the moderately and mildly hypersensitive rats significantly elevated the WTs as compared to pre-stimulation values. In the group defined as “mild” hypersensitivity, the WTs even reached pre-injury levels as early as 15 min after the initiation of SCS.

This study demonstrates that the response to SCS appears to differ with the severity of mechanical hypersensitivity, and that also the time course of the response is altered.

Discussion

This thesis deals with neuropathic pain studied in an animal model that exhibits stimulus-evoked pain related symptoms. Since presently available analgetic drugs often are ineffective and other types of substances (e.g. antiepileptics and antidepressants) provide pain relief only in a small portion of cases, the use of alternative methods, like SCS, has increased.

The studies span from excitatory receptors in the DH to activation of endogenous control systems operating at segmental spinal levels and supraspinally.

NMDA receptors

The activation of spinal cord NMDA receptors is associated with the development and maintenance of central sensitization (e.g. Woolf and Thompson 1991; Zou et al. 2000). Receptor initiated events, where the net effect is increased intracellular calcium, lead to an increased number of effective synapses on DH neurons and enhanced neuronal excitability (Woolf and Thompson 1991).

Results from both experimental and clinical studies show that NMDA receptor antagonists can suppress nerve injury induced hypersensitivity (thermal and mechanical hyperalgesia) (Kristensen et al. 1992; Burton et al. 1999; Chizh and Headley 2005; Wilson et al. 2005). However, NMDA receptor-dependent synaptic plasticity plays a role not only in pathological conditions such as chronic pain, but also in cognition and functions such as learning and memory (McMahon et al. 1993). The wide spread localization of the receptor and the complex role of glutamate signaling in the CNS implies that nonselective NMDA receptor blockers may cause serious and unacceptable side effects (Carpenter and Dickenson 2001; Zeilhofer 2005).

In study I it was investigated whether it would be possible to correlate NMDA receptor phosphorylation (activation) and the expression of different receptor subunits to the presence of neuropathic pain-related signs. Our immunohistochemical and Western Blot results indicated that the level of the phosphorylated NMDA NR1

subunit was increased in nerve injured hypersensitive rats as compared to animals lacking or displaying less pronounced hypersensitivity.

In line with our results, other studies with various experimental approaches have demonstrated enhanced dorsal horn expression or phosphorylation of NMDA receptor subunits, both of the NR1 and the NR2B (Zou et al. 2000; Guo et al. 2002; Caudle et al. 2003; Brenner et al. 2004; Gao et al. 2005). At the time of our study commercially available antibodies specific for phosphorylated NMDA receptors included those recognizing either the NR1 or the NR2B subunit (Mandell 2003). Also the P-NR2B antibody was tested, but in our hands, tissue staining was not satisfactory and a quantification not achievable. It has been reported that peripheral nerve injury can induce changes also in net NMDA receptor expression (Wang et al. 2005; Wilson et al. 2005). However, in study I, the overall dorsal horn expression of the examined receptor subunits was unaffected after nerve injury regardless of whether or not mechanical and cold hypersensitivity was present.

While the distribution of NR1 in the CNS is ubiquitous other subunits of the NMDA receptor exhibit a distinct regional expression pattern and have been suggested to be of particular importance for pain signaling (Gurwitz and Weizman 2002; Petrenko et al. 2003; Wu and Zhuo 2009). Among the NR2 subunits, the NR2B exhibits the largest expression with a restricted distribution in the superficial dorsal horn, and is present also in DRG cells (Boyce et al. 1999; Karlsson et al. 2002). An increasing number of reports implicate the NR2B as being of particular importance in central sensitization and generation of neuropathic pain (Zhuo 2007; Qu et al. 2009; Wu and Zhuo 2009; Zhuo 2009).

NR2B antagonists have proven to be effective both in animal models of neuropathic pain and in patients, demonstrating better efficacy and fewer adverse effects than nonselective NMDA receptor blockers (Claiborne et al. 2003; Childers and Baudy 2007).

Although NMDA receptors are critically involved in activity dependent changes in spinal nociceptive processing, their contribution to the pathogenesis of pain originating from peripheral nerve injury is not yet clear.

SCS mode of action: spinal segmental circuits and descending supraspinal inhibition

GABA

GABA is one of the more important inhibitory transmitters and interest has been focused on this substance since it was realized that endogenous opioids were not involved in the effects of SCS (Meyerson 1983).

A multitude of experimental data indicates that a deficient GABAergic or glycinergic inhibition contributes to central sensitization in the spinal cord (Yaksh 1989; Castro-Lopes et al. 1993; Sivilotti and Woolf 1994; Hwang and Yaksh 1997; Zeilhofer 2005). Besides exaggerated excitation (NMDA activation) as the major cause of neuropathic and inflammatory pain, the GABAergic system has been in the focus of interest because of a presumed reduction of the inhibitory tone in the spinal dorsal horn possibly underlying the development of neuropathic pain.

In animals with behavioral signs of neuropathy after nerve injury, the *in vivo* basal extracellular release of GABA in the DH is significantly lower than in intact animals. SCS has been shown to trigger a release of GABA in the DHs of nerve injured hypersensitive rats responding to the stimulation (Stiller et al. 1996; Cui et al. 1997). Could these observations, together with other findings indicative of a deficient GABAergic inhibition, reflect also a more general dysfunction in the GABA system?

With the hypothesis that changes in GABA synthesis occur after nerve injury leading to development of hypersensitivity, the aim of study II was to examine the relationship between expression of the GABA synthesising enzymes GAD65 and GAD67, reflecting GABA synthesis, and the presence of nerve injury induced hypersensitivity. The intention was also to compare possible differences in the levels of these enzymes in animals responding and not responding to SCS. Although we were not able to detect any major changes in GAD levels in hypersensitive or non-hypersensitive animals as compared to controls we could demonstrate that stimulation *per se* tended to augment the DH enzyme levels.

Possible causes of a disinhibition are reduced release of glycine or GABA from inhibitory neurons and/or a selective death of GABAergic or glycinergic interneurons (Sugimoto et al. 1990; Castro-Lopes et al. 1993; Ibuki et al. 1997). It has been suggested that peripheral nerve injury induces loss of GABAergic input in the DH due to death of GABAergic interneurons (Moore et al. 2002; Scholz et al. 2005). However, we

were not able to demonstrate a reduction of GAD after nerve injury (study II) nor to detect any substantial change in number of dorsal horn neurons (study I, Fig. 3C). Furthermore, several subsequent studies have found no evident signs of loss of GABAergic neurons and concluded that this is not a necessary condition for the development of neuropathic symptoms like thermal hyperalgesia and mechanical hypersensitivity (Polgar et al. 2003; Polgar et al. 2004; Polgar et al. 2005; Polgar and Todd 2008).

Trauma to peripheral nerve tissue induces a reduction in the expression of the potassium chloride exporter KCC2 in DH neurons, shifting the chloride equilibrium towards depolarization (Coull et al. 2003; Coull et al. 2005). This reduction in transmembrane chloride gradient causes GABAergic and glycinergic synaptic inhibition to become less effective, which is therefore not necessarily reflected by a change in GABA synthesis alone.

Acetylcholine

The spinal cholinergic system has since long been known to be involved in pain modulation (Eisenach 1999; Flores 2000) and might possibly also participate in the effect of SCS (Schechtmann et al. 2004).

With the principal aim to explore the possible involvement of the cholinergic system in the effect of SCS, microdialysis experiments were performed. The finding of a significantly lower basal release of ACh in the DH in the hypersensitive animals was somewhat unexpected, although Dussor and colleagues (Dussor et al. 2005) have previously reported a reduced in vitro spinal ACh release in animal models of nerve injury. The pathophysiological changes underlying the reduction in ACh release following nerve injury and the development of hypersensitivity are not known, but it has been suggested that it may be due to degeneration of cholinergic afferents or a result of conformational change of interneuron synapses (Dussor et al. 2005). However, in order to determine the functional significance of decreased basal levels of ACh for the development and maintenance of signs of neuropathy, and perhaps also nerve injury induced pain, it would be necessary also to examine the basal levels of ACh in a group of nerve injured animals not displaying such signs.

Increased release of ACh induced by SCS

In this study it was also demonstrated that SCS induces a release of ACh in the dorsal horn and that this was related to the outcome of the stimulation on the pain-like behavioral response to mechanical stimuli. This effect was present only in animals responding to SCS, while no changes were detected in the group of non-responders. Both the nerve injury induced decrease and the subsequent SCS triggered release of ACh were remarkably similar to alterations of GABA release earlier described (Stiller et al. 1996), indicating that SCS modulates several different transmitter systems acting in parallel or in concert.

The subsequent behavioral experiments confirmed and further substantiated the importance of the cholinergic system in the effect of SCS on pain related behavioral signs. Both atropine and a selective M_4 receptor antagonist completely eliminated the effect of SCS. The prominent role of the M_4 receptor could not be reproduced with any of the other tested receptor antagonists although an M_2 antagonist demonstrated a partially attenuating effect. Although not selective for a certain subtype, the muscarinic receptor agonist oxotremorine proved to potentiate the effect of SCS in non-responding rats (Song et al. 2008), further supporting the involvement of muscarinic receptors in the effect of SCS. Also the effect of TENS seems to be related to activation of spinal muscarinic receptors (Radhakrishnan and Sluka 2003), but for this type of peripheral stimulation, the M_1 and M_3 subtypes appeared to be of greater importance. Although the role of nicotinic receptor antagonists in antinociception is well established (review see Flores 2000; Rashid and Ueda 2002), the nicotinic receptor appeared not to be involved in the effect of SCS in the present animal model.

It still remains unclear from where the ACh release in the dorsal horn originates, but there are multiple neuronal ACh sources. Some data suggest that descending pathways from supraspinal structures may contribute to the ACh release (Jones et al. 1986; Bouaziz et al. 1996). Others propose that the major DH ACh release originates from local interneurons (Barber et al. 1984; Ribeiro-da-Silva and Cuello 1990; Hoglund et al. 2000) and possibly also, but to a lesser extent, from primary sensory nerve terminals (Sann et al. 1995; Dussor et al. 2005).

Serotonin

Electrical stimulation of the brain stem nuclei, dorsolateral funiculus (DLF) or peripheral nerves may attenuate spinal nociceptive transmission by activation of descending pathways and an increased release of spinal 5-HT (Tyce and Yaksh 1981; Liu et al. 1988; Sluka et al. 2006). This finding together with the observations that SCS induces both GABA and ACh release in the DH, called for an exploration of the functional role of serotonin in the effect of SCS in a model of nerve injury induced pain.

Spinal 5-HT was examined with immunohistochemistry and quantified with ELISA. Following nerve injury, the basal 5-HT content remained the same in both dorsal quadrants of the spinal cord and no difference was detected between normal and hypersensitive rats. However, similarly to the transmitters GABA and ACh, the content of 5-HT in the ipsilateral dorsal quadrant of the L4-L6 spinal segments was found to be augmented by SCS in animals responding to the stimulation. Again, this increase was not present in non-responders or in responders without stimulation immediately prior to tissue collection.

Earlier studies have show that SCS applied also in decerebrated cats increases the release of 5-HT in the DH (Linderöth et al. 1992). A confounding factor may be that in the rat the relationship between the size of the electrode and the size of the spinal cord, could perhaps enable activation not only of the DCs but also of the DLF. Thus, it might be that the increase in serotonin release is produced not solely by DC activation, relayed via the RVM, but also by a direct activation of the DLF. However, abundant data where SCS has been applied at the dorsal column nuclei (DCN) level clearly demonstrate that an effective inhibition can be obtained, indistinguishable from that by SCS as performed in our experiments (Saadé et al. 1986; Saadé et al. 1999; El-Khoury et al. 2002). In fact, DCN stimulation as well as low thoracic SCS applied in the same animal produce similar effects in behavioral tests (Saadé et al. 2009). In addition, on-going experiments with recording of neuronal activity in the RVM demonstrate that SCS applied at the lower thoracic levels activates 5-HT neurons in the brain stem in SCS responding rats (Song et al. unpublished data).

The fact that SCS non-responders can be converted to responders by i.t. administration of GABA, 5-HT, a muscarinic receptor agonist, as well as adenosine, indicates that several spinal transmitter systems are involved in the SCS effect. In the present study it was shown that while a GABA_B receptor antagonist partially blocked the enhancing effect of exogenous 5-HT on SCS in non-responding rats, a muscarinic

M₄ antagonist did not, although the same M₄ antagonist was shown to eliminate the effect of SCS in responders (study III). This observation is somewhat unexpected considering that the “antiallodynic” effect of i.t. 5-HT₂ receptor agonists is partly linked to cholinergic mechanisms, in particular muscarinic (Obata 2002; Obata et al. 2003). These differences in effects indicate that the 5-HT mediated effect of SCS works in multiple and complex ways, which might be difficult to selectively target pharmacologically.

Outcome of SCS related to degree of mechanical hypersensitivity

Although SCS has a well documented capacity of providing good relief of neuropathic pain, there is still a considerable portion of the patients that do not benefit from the treatment. Therefore, there is an urgent need for methods to enable identification of patients with a high probability to respond favorably. It might be that the severity and nature of sensibility abnormalities can have a predictive value. In clinical experience, symptoms of severe denervation, hypoesthesia/anesthesia are considered as negative predictors. Conversely, positive symptoms, like moderate allodynia, appear to have a positive value. This background and the observations that in animals displaying severe hypersensitivity SCS could rarely increase the WT's up to pre-injury (“normal”) levels was an incentive for us to use double criteria for a positive SCS response in study III. Study V was performed in order to examine, in more detail, the possible relationship between the severity of mechanical hypersensitivity following nerve injury and the effect of SCS.

Stratification of animals into groups depending on degree of mechanical hypersensitivity (mild, moderate, and severe), revealed that the response to SCS varied with the severity of the displayed hypersensitivity, and further, the response to SCS differed in time in the various groups. The conclusion was that SCS leads to a faster and more effective relief of the pain-like behavior in animals classified as mildly hypersensitive as compared to those with a more severe hypersensitivity. Similar results were found by Li et. al. who reported a tendency of that efficacy of SCS in suppressing hypersensitivity was inversely related to its severity (Li et al. 2006).

The observation of a differential effect of SCS, as demonstrated in this study, may have important clinical implications. Clinically, there is a large variation in the degree of allodynia and hyperalgesia. It might be that a more thorough, and quantitative

evaluation of the type and severity of mechanical allodynia can help to identify patients who are most likely to benefit from SCS treatment (Landerholm 2010). In fact, in a recent study by van Eijs et. al. 2010 (van Eijs et al. 2010), it was reported that brush-evoked allodynia was a negative predictor of the outcome of SCS in complex regional pain syndromes.

Conclusions

1. Signs of pain-like behavior following a peripheral nerve injury are associated with an increased phosphorylation of the NMDA receptor 1 subunit on neurons in the spinal dorsal horn, with no concomitant changes of non-phosphorylated subunits.
2. The mechanisms by which SCS provides its pain suppressing effect involve several endogenous transmitters and systems such as the GABAergic, cholinergic and serotonergic. In each system some of the receptor subtypes appear more important than others, e.g. antagonizing the M_4 receptor affected the SCS effect more than an M_2 antagonist. The effect involving the descending serotonergic system could be partially blocked by a GABA_B antagonist; in contrast, antagonizing the M_4 receptor had a limited or no effect. This implies that these systems appear to operate in parallel and in concert, and that the relation between the systems is complex.
3. If present, allodynia in neuropathic pain may appear with a large variation in severity. Results from experiments in an animal model of neuropathy suggest that the presence of advanced allodynia could be a predictor of a low responsiveness to SCS.

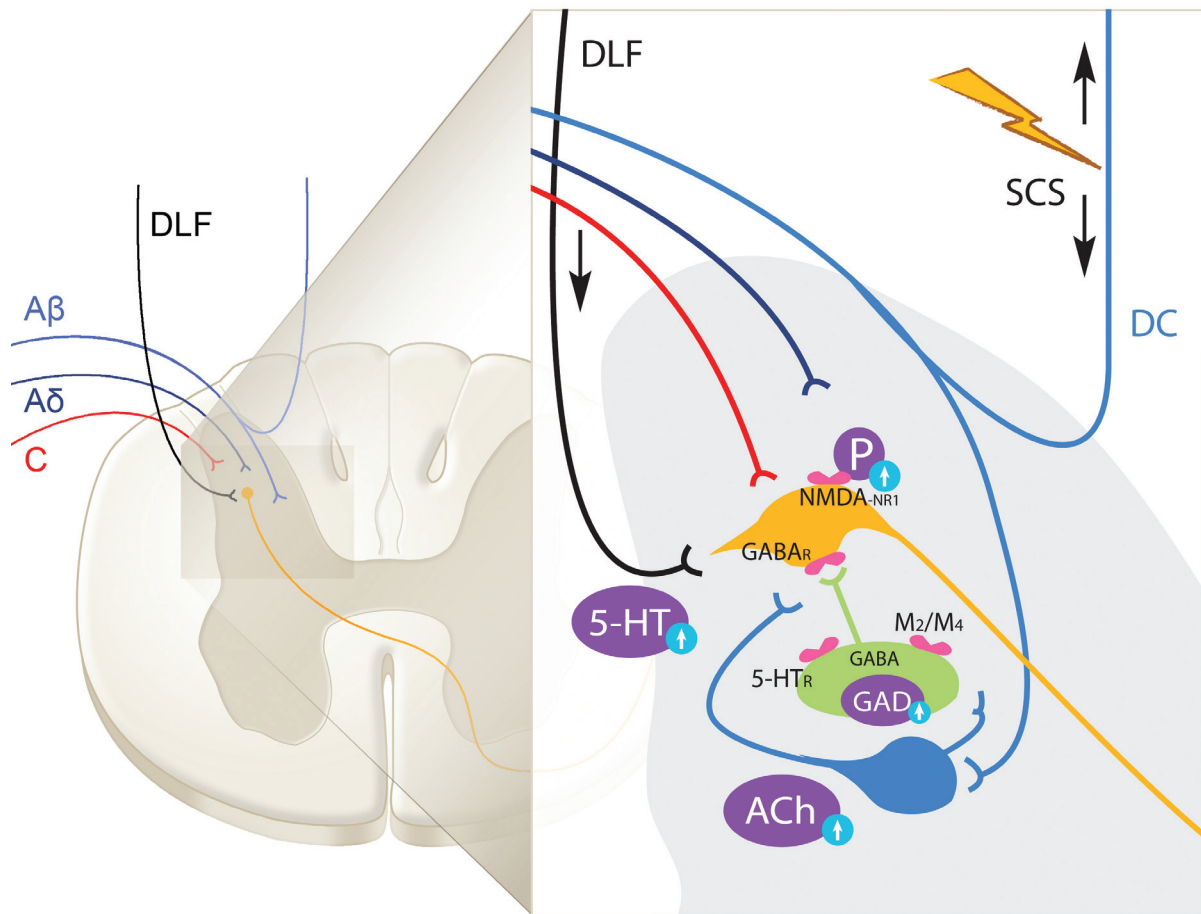


Fig. 12

A schematic overview of the findings in the studies included in this thesis. The drawing to the right represents a close-up of the dorsal horn and illustrates findings following peripheral nerve injury and spinal cord stimulation (SCS).

The presence of hypersensitivity following peripheral nerve injury is associated with an increased phosphorylation of NMDA receptor subunit 1 (NMDA-NR1-P). In earlier studies, an increased release of GABA in the dorsal horn was observed following SCS, and here, also the enzymes responsible for the GABA synthesis (GAD) were found to be augmented. Both acetylcholine (ACh) and serotonin (5-HT) release was found to increase in animals responding to the stimulation. This increase in transmitter release was not observed in non-responding rats.

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