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NEUROPEPTIDE RELEASE IN THE RAT DORSAL HORN IN MODELS OF PERSISTENT PAIN – EFFECTS OF OPIOIDS

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ومأأوتيتم من ألعلم إلا قليلا
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Aqoon la'aani waa iftiin la'aan

Abstract

Nerve injury and tissue inflammation may lead to exaggerated responses to both noxious stimuli (hyperalgesia) and innocuous stimuli (allodynia). Altered release patterns of neurotransmitters in the dorsal horn of the spinal cord have been suggested to contribute to hyperalgesia and allodynia. The cholecystokinin (CCK) gene-expression and CCK_2-receptor gene-expression in dorsal root ganglion (DRG) cells is upregulated after sciatic nerve transection (axotomy). Since CCK acts as an anti-opioid peptide, an increased CCK release in the spinal cord following nerve lesions has been proposed to be of importance for the relatively low effectiveness of morphine in the treatment of persistent neuropathic pain. Peripheral inflammation induces increased levels of substance P (SP) in primary afferent neurons and there is also indirect evidence for increased release of SP in inflammation. The present study was undertaken to study the in vivo release of SP- and CCK-like immunoreactivity (-LI) in the dorsal horn of the spinal cord in models of persistent neuropathic (axotomy) and inflammatory pain.

All experiments were performed in the rat and the neuropeptide release was studied by microdialysis combined with radioimmunoassay. In control animals the release of CCK-LI was increased 5-fold by stimulation with potassium (100 mM in the perfusion fluid). In contrast, two to eight weeks after transection of the sciatic nerve, no significant CCK-LI release was detected. Three and seven days after axotomy, a tendency to an increased potassium-induced release of CCK-LI was observed. After subcutaneous administration of morphine (5mg/kg, s.c) to intact rats, an increased spinal release of CCK-LI of the same order of magnitude as observed after potassium stimulation was detected. The morphine-induced CCK-LI release was TTX-sensitive and calcium-dependent and could be blocked by topical application of either the L-type calcium channel blocker verapamil or the N-type calcium channel blocker ω-conotoxin GVIA. The CCK-LI release induced by morphine was also completely inhibited by the δ-opioid antagonist naltrindole, but not by μ-or κ-opioid receptor antagonists. Furthermore, administration of the δ-opioid receptor agonist BW373U86 but not the μ-opioid receptor agonist DAMGO induced a significant increase of the spinal CCK-LI release. After peripheral axotomy, morphine, at doses that induced a release of CCK-LI in normal rats, failed to induce a significant release of CCK-LI. However, the ability of the δ-opioid receptor agonist BW373U86 to evoke a release of CCK-LI was not affected by the axotomy. During induction of long term potentiation (LTP) in single wide dynamic range (WDR) neurons by a conditioning tetanic stimulation of the sciatic nerve, a short lasting significant four-fold increase of the SP-LI release was detected. However, the extracellular level of SP-LI was not altered upon single impulse nerve activation after LTP had been established. Perfusion of the microdialysis probe with capsaicin (50 or 100 μM), an agent known to activate vanilloid receptors on primary afferent C-fibers, induced a significant eight-fold increase of the extracellular SP-LI level. Spinal administration of the NMDA antagonist D-APV, but not the AMPA antagonist NBQX, completely prevented the SP-LI release induced by the low dose of capsaicin (50 μM). Seven days after induction of peripheral inflammation by complete Freund´s adjuvant (CFA), a dose of capsaicin (10 μM), previously insufficient to release SP-LI, induced a significant and D-APV reversible release of SP-LI.

In summary, spinal release of CCK-LI may be of importance for modulation of morphine analgesia and for the altered primary afferent processing during the first week after axotomy. However, the data do not support the concept of an increased spinal CCK release as a mechanism for morphine resistance of "phantom pain". The δ-opioid receptor is likely to mediate the opioid induced release of CCK-LI in intact animals and following sciatic nerve section. A contribution of SP in the induction, but not in the maintenance, of LTP in WDR neurons, is suggested. Moderate doses of capsaicin induce release of spinal SP-LI via an activation of the NMDA receptor. The ability of capsaicin to evoke a release of SP-LI in the dorsal horn is increased one week after CFA-induced inflammation.

Key words: Axotomy, Inflammation, Long term potentiation, Capsaicin, Opioids, Cholecystokinin, Substance P, Glutamate, NMDA, Spinal cord, Microdialysis, Radioimmunoassay.

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List of original papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I **Afrah AW**, Gustafsson H, Olgart L, Brodin E and Stiller CO. Changes in spinal cholecystokinin release after peripheral axotomy. *NeuroReport,* 12: 49-52, 2001.

II Gustafsson H, **Afrah AW** and Stiller CO. Morphine-induced in vivo release of spinal cholecystokinin is mediated by delta-opioid receptors: Effect of peripheral axotomy. *Journal of Neurochemistry,* 78: 55-63, 2001.

III Gustafsson H, **Afrah AW**, Brodin E, Stiller C-O. Pharmacological characterization of morphine-induced in vivo release of cholecystokinin in rat dorsal horn: Effects of ion channel blockers. *Journal of Neurochemistry,* 73: 1145-1154, 1999.

IV **Afrah AW**, Fiskå A, Gjerstad J, Gustafsson H, Tjølsen A, Olgart L, Stiller CO, Hole K and Brodin E. Spinal Substance P release *in vivo* during the induction of long-term potentiation in dorsal horn neurons. *PAIN*, 96: 49-55, 2002

V **Afrah AW**, Stiller CO, Olgart L, Brodin E and Gustafsson H. Involvement of spinal N-methyl-D-aspartate receptors in capsaicin-induced in vivo release of substance P in the rat dorsal horn. *Neuroscience Letters*, 316: 83-86, 2001

VI **Afrah AW**, Svensson C, Gustafsson H, Taylor BK, Olgart L, Brodin E and Stiller CO. Peripheral inflammation facilitates capsaicin-induced release of substance P in the dorsal horn – possible involvement of NMDA receptors. *Manuscript*

Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APV	2-amino-5-phosphonopentanoic acid
BW373U86	(±)-(1[S*],2a,5beta)-4-([2,5-Dimethyl-4-(2-propenyl)-1-piperazinyl][3-hydroxyphenyl]methyl)-N,N-diethyl-benzamide
cAMP	cyclic 3′, 5′-adenosine monophosphate
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide
DAMGO	[D-ala^2-N-Me-Phe^4-Gly^5-ol]- enkephalin
[D-Ala^2] deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly amide
DRG	dorsal root ganglion
EAA	excitatory amino acids
GABA	γ-aminobutyric acid
-ir	immuoreactive
i.t.	intrathecal
i.v.	intravenous
-LI	like immunoreactivity
LTP	long-term potentiation
mRNA	messenger ribonucleic acid
NKA	neurokinin A
NBQX	6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione disodium
NMDA	N-methyl-D-aspartate
ω-CgTX	omega-conotoxin GVIA
RIA	radioimmunoassay
s.c.	subcutaneous
S.E.M.	standard error of the mean
SP	substance P
TTX	tetrodotoxin
WDR	wide dynamic range

Introduction

Background

Persistent pain is a common health problem affecting many people worldwide and leading to decreased quality of life, impaired working ability and high cost of health care.

Pain is defined as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Mersky and Bogduk, 1994). The perception of pain often coincides with an increased sensitivity to sensory information. In general, pain is a normal biological response, which protects the body against tissue damage and facilitates the healing process. Normally, the sensation of pain ceases after the injured tissue has healed. However, in some cases, sustained noxious stimulation of peripheral nociceptors may produce long-lasting conditions with spontaneous pain and/or exaggerated responses to both noxious stimuli (hyperalgesia) and non-noxious stimuli (allodynia).

Persistent pain of neuropathic origin, secondary to injury or to disease of the peripheral or central nervous system (CNS) is often resistant to standard analgesics (e.g. opioids) (Arnér and Meyerson, 1988). The pharmacological treatment options for these conditions include other classes of drugs, such as antidepressants, anticonvulsants and antiarythmic drugs (for review, see McQuay et al. 1996; Sindrup and Jensen, 2000; Martin and Eisenach, 2001). However, the effectiveness of these drugs is often limited by side effects, which may become intolerable. Thus, there is a need for new drugs or approaches for the treatment of neuropathic pain.

In contrast to neuropathic pain, the management of inflammatory pain is often successful with analgesics such as non-steroid anti-inflammatory drugs (NSAIDs) or opioids. Nevertheless, persistent pain states may also arise following peripheral tissue inflammation.

For the rational development of more efficient analgesic drugs, a better understanding of the pathophysiology of neuropathic and inflammatory pain and of the mechanisms responsible for transition of acute pain into persistent pain conditions is of great importance.

Ascending neuronal pathways

Pain transmission is associated with activation of high threshold nociceptors on peripheral nerve endings. Stimulation of these nociceptors generates signals that are conducted along small diameter primary afferent fibers (for review see, Russo and Brose, 1998; Millan, 1999). Many of these fibers are non-myelinated polymodal C-fibers with low conduction velocities. Others are fine myelinated A-delta (A-δ) fibers, which conduct more rapidly. C-fibers mediate dull, diffuse or burning pain, while stimulation of A-δ fibers results in sharp and localized pain (for review, see Millan, 1999). After entering the spinal cord via the dorsal roots, the fibers terminate at different layers of the dorsal horn. The C-fibers and some of the A-δ fibers innervate cell bodies in lamina I and II, while A-beta-fibers penetrate deeper into the dorsal horn at lamina III-IV (for review see, Basbaum, 1984; Rang et al. 1991; Willis and Westlund, 1997). Activation of primary afferent fibers causes a release of excitatory neurotransmitters in the dorsal horn. These neurotransmitters activate secondary neurons, which can be classified into two types. One class, the nociceptive specific (NS) neuron, is excited only by nociceptive stimuli. The other class is the wide dynamic range (WDR) neuron, which responds to both noxious and innocuous stimuli (Mendell 1966; Price and Dubner, 1977; Dubner and Bennett, 1983). NS neurons have small receptive fields and therefore transmit precise information of peripheral location whereas WDR neurons have large receptive fields (Cook and Woolf 1987; Schmidt and Schaible, 1994). The axons of these second order neurons cross the midline of the spinal cord and the majority of these axons ascends in the spinothalamic tract and terminate in the thalamus. From the thalamus, third order neurons send nociceptive signals to the cortex, where the sensation of pain is perceived (Basbaum, 1984; Russo and Brose, 1998).

Descending neuronal pathways

Descending pathways with projections to the spinal cord modulate nociceptive transmission. One of the most important inhibitory pathways consists of neurons in the midbrain periaqueductal gray (PAG), which project to the rostral ventral medulla (RVM). RVM neurons in turn project caudally to the dorsal horn (for review, see Behbehani, 1995; Odeh, 2001). The PAG receives neuronal input from several brain regions including the cerebral cortex, thalamus and hypothalamus (Behbehani, 1995). The descending pain inhibitory pathways terminate at nociceptive sensory projection neurons in the spinal cord and activation of these descending pathways induces release in the dorsal horn of various neurotransmitters with inhibitory properties either via direct synaptic contacts or indirectly by activating inhibitory interneurons (Behbehani, 1995; Willis and Westlund, 1997).

In addition to these inhibitory descending pathways, there are also descending pathways that facilitate nociceptive transmission (Willis and Westlund, 1997; Urban and Gebhart, 1997; Ossipov et al. 2000).

Neurotransmitters and receptors involved in pain transmission and modulation in the spinal cord

Several neurotransmitters are important for the transmission and modulation of pain at the spinal level. Excitatory amino acids (EAA) and neuropeptides are two major categories of neurotransmitters/neuromodulators in primary sensory neurons (for review, see Besson and Chaouch, 1987; Yaksh and Malmberg, 1994; Baranauskas and Nistri, 1998; Hökfelt et al. 2000). Glutamate activates two major classes of receptors: ionotropic and metabotropic receptors. The ionotropic receptors are the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA) and N-methyl–D-aspartate (NMDA) receptors. Glutamate acting on AMPA receptors mediates spinal signaling of non-tissue damaging noxious stimuli, whereas greater stimulus intensities are required to activate the NMDA receptors (Hollmann and Heinemann, 1994; Monaghan et al. 1989). The ionotropic as well as the metabotropic glutamate receptors play a particularly important role for neuronal plasticity in the dorsal horn (for review, see Kidd and Urban, 2001; Mills et al. 2002).

A number of neuropeptides are found in terminals of primary afferent neurons in the dorsal horn (for review, see Hökfelt et al. 1997, 2000). Substance P (SP), neurokinin A (NKA) and calcitonin gene related peptide (CGRP) are co-localized in primary afferents (Wiesenfeld-Hallin et al. 1984; Dalsgaard et al. 1985; Hökfelt et al. 1997). SP and NKA are derived from the same precursor, preprotachykinin A (PPT A), and belong to the tachykinin family of peptides (Snijdelaar et al. 2000; Hökfelt et al. 2001).

Some neuropeptides, including somatostatin, neuropeptide Y (NPY), galanin and opioid peptides inhibit the release of SP and glutamate from primary afferent terminal and have been shown to be antinociceptive at the spinal level (Dray, 1996; Hökfelt et al. 1994, 1997), whereas other neuropeptides, including neuropeptide FF, peptides of the melanocyte-inhibiting factor family and cholecystokinin (CCK), have been shown to counteract opioid analgesia (Cesselin, 1995).

Functional aspects of SP and CCK in the dorsal horn

Substance P (SP)

SP is abundant in terminals of C-fiber primary afferent nerves in the superficial layers (I-II) of the dorsal horn, where most primary afferent fibers terminate (Hökfelt et al. 1975). SP in primary afferents is synthesized in the DRG and transported centrally and peripherally (Hökfelt et al. 1975; Takahashi and Otsuka, 1975; for review, see Basbaum, 1999). In addition, SP has also been demonstrated in the dorsal horn, in spinal interneurons and in terminals of descending neurons (Hökfelt et al. 1975).

The effect of SP is mediated by neurokinin 1 (NK-1) and neurokinin 2 (NK-2) receptors. The NK-1 receptor, which has the highest affinity for SP (for review, see Basbaum, 1999; Snijdelaar et al. 2000; Hökfelt et al. 2001) is located both pre- and postsynaptically in the dorsal horn (Helke et al. 1986; Hu et al. 1997). Noxious mechanical (Duggan et al. 1988), thermal (Go and Yaksh, 1987) and chemical (Duggan et al. 1987; Calcutt et al. 2000) stimuli as well as peripheral electrical stimulation (Otsuka and Konishi, 1976; Yaksh et al. 1980; Brodin et al. 1987; Go and Yaksh, 1987; Duggan et al. 1988; Linderoth and Brodin, 1988; Yaksh, 1988) have been shown to release SP in the dorsal horn of the spinal cord. Moreover, the irritant ingredient of certain red peppers, capsaicin, which directly and selectively activates vanilloid receptors (VR-1) located on polymodal C-fibers, releases SP in the dorsal horn (Go and Yaksh, 1987). SP-induced activation of NK-1 receptors on dorsal horn neurons causes a prolonged depolarization and an increased response to C-fiber input (Murase and Randic, 1984). Intrathecal injection of SP in animals results in "pain behavior" and increases the magnitude of the spinal flexion reflex elicited by noxious stimuli (Hylden and Wilcox 1981; Yashpal and Henry, 1983). Conversely, NK-1 receptor antagonists can block the responses of spinal cord neurons caused by the various noxious stimuli (Liu et al. 1998). "Knock-out" mice lacking the NK-1-receptor, as well as "Knock-out" mice lacking PPT-A, display a decreased sensitivity to high intensity noxious stimulation (Snijdelaar et al. 2000).

SP interacts with glutamate in the dorsal horn via a variety of mechanisms. *In vivo* microdialysis studies have shown that intrathecal administration of SP causes release of glutamate and aspartate in the cerebrospinal fluid (Skilling et al. 1992). Glutamate acting on NMDA receptors can induce NK-1 receptor internalization (an indirect measure of SP release) in the dorsal horn (Marvisón et al. 1997; Liu et al. 1997). Furthermore, NMDA receptor antagonists can prevent NK1 receptor internalization in the dorsal horn induced by either NMDA stimulation or peripheral electrical nerve stimulation (Marvisón et al. 1997).

In addition to the numerous reports of pronociceptive actions of SP, a limited number of studies suggest that this peptide also has antinociceptive properties. SP has been shown to stimulate the release of endogenous opioids (Tang et al. 1983; Behbehani, 1995) and N-terminal fragments of SP have been suggested to interact directly with opioid receptors (Krumins et al. 1989). In the PAG, SP is present in both cell bodies and terminals (Skirboll et al. 1982, 1983, Barbaresi et al. 1998) and induces a naloxone-reversible antinociceptive effect upon local injection (Malick and Goldstein, 1978).

Cholecystokinin (CCK)

CCK was first identified as a gastrointestinal hormone, contracting the gallbladder and stimulating the secretion of the exocrine pancreas (Jorpes and Mutt, 1966; Mutt and Jorpes, 1968). Later, CCK (or gastrin-like immunoreactivity) was demonstrated in the CNS (Vanderhaeghen et al. 1975). The predominant form of CCK in gastrointestinal tissue is CCK-33, whereas the predominant form of CCK in the CNS is sulphated CCK-8 (Jorpes and Mutt, 1966; Rehfeld, 1978; Larsson and Rehfeld, 1979). The CNS distribution of CCK parallels that of endogenous opioids in the pain processing areas, such as laminae I and II of the spinal cord, the PAG, RVM and the thalamus (for review, see Stengaard-Pedersen and Larsson, 1981; Beinfeld and Palkovits 1982; Wiesenfeld-Hallin et al. 1999).

Early immunohistochemical data indicated that CCK-like immunoreactivity (CCK-LI) was also present in rat DRG cells (Dalsgaard, 1982). Later it was demonstrated that the antibody used cross-reacted with CGRP (Ju et al. 1986). *In situ* hybridisation studies revealed no or only a few CCK-immunoreactive or CCK-mRNA positive neurons in rat DRG (Seroogy et al. 1990; Verge et al. 1993).

The effects of CCK are mediated by at least two types of receptors; the CCK_1 (CCK-A) and the CCK_2 (CCK-B) receptors (for review, see Crawely and Corwin 1994). Initially, the CCK_1-receptors were considered to be limited to the gastrointestinal tract and other peripheral organs, whereas the CCK_2-receptors were considered to be the only CCK-receptor in the CNS (Moran et al. 1986). However, subsequent studies by Hill and colleagues using autoradiography revealed the presence and distribution of both receptor types in the brain (Hill et al. 1987). In the dorsal horn, the highest density of CCK_2-receptors is found in the superficial lamina (Hill and Woodruff, 1990; Ghilardi et al. 1992).

Opioids and opioid receptors

Three major families of opioid peptides have been identified in mammals: enkephalins, endorphins and dynorphins. They are derived from three different precursor molecules: proenkephalin, proopiomelanocortin, and prodynorphin respectively. These opioid peptides are widely distributed in both central (especially in the areas that are associated with pain pathways) and peripheral nervous system (Hughes et al. 1975). Recently, a new class of endogenous opioids, the endomorphins, have been described (Zadina et al. 1997). At least three subtypes of opioid receptors mediate the effects of opioids: μ-, δ- and κ-receptors (Martin et al. 1976). Another receptor of the opioid family is the opioid receptor-like (ORL-1) receptor. The putative endogenous ligand for this receptor is nociceptin/orphanin FQ (Meunier et al. 1995; Reinscheid et al. 1995). Nociceptin/orphanin FQ has negligible affinity to μ-, δ- and κ- receptors, conversely, opioids have no or low affinity for the ORL-1 receptors (Meunier, 1997).

Autoradiographic studies have demonstrated opioid receptors in the brain, spinal cord and peripheral tissues (for review, see Mansour et al. 1988). All receptor subtypes are G-protein coupled (Gi/o), and the activation of these receptors decreases the activity of adenylate cyclase, resulting in a decreased production of cyclic adenosine monophosphate (cAMP). This leads to opening of potassium channels with subsequent membrane hyperpolarization and inhibition of calcium channels. Thus, activation of any of these receptors usually results in decreased neuronal excitability and decreased neurotransmitter release (for review, see Dickenson, 1994; Yaksh, 1987). However, not only inhibitory, but also stimulatory effects of opioid receptor activation have been described (Crain and Shen, 1990; Stiller et al., 1997). An opioid-induced disinhibition of γ-amino-butyric acid (GABA) neurons is generally regarded to be responsible for opioid induced neuronal activation (Stiller et al. 1996). However, this is probably not the only mechanism (Crain and Shen, 1990).

Morphine, extracted from the opium poppy (papaver somniferum), is the most widely used exogenous opioid analgesic substance. Morphine is used for the treatment of acute and chronic severe pain (for review, see Christrup, 1997; O'Callaghan, 2001). The analgesic effect of morphine is mediated by activation of opioid receptors in both the brain and spinal cord. In the dorsal horn, morphine may produce presynaptic inhibition of primary afferent terminals as well as postsynaptic inhibition of neurons projecting to the thalamus in ascending pathways (Jessel and Iversen, 1977; Yaksh and Neueihed, 1985).

In addition, morphine activates descending inhibitory, primarily serotonergic and nordadrenergic pathways (Fang and Proudfit, 1998; Ohsawa et al. 2000).

CCK as an anti-opioid peptide

Substantial evidence indicates that there are peptides suppressing the antinociceptive effects of opioids (for review, see Cesselin, 1995). The most studied anti-opioid peptide is CCK, which has been shown to reduce the analgesic effects of morphine and other opioids in the CNS (for review, see Baber et al. 1989; Cesselin, 1995; Wiesenfeld-Hallin et al. 1999; Wiesenfeld-Hallin and Xu, 2001). CCK decreases opioid antinociception upon systemic or spinal administration (Faris et al. 1983; Xu et al. 1993). CCK antagonists and CCK antiserum prevent the development of morphine tolerance (Ding et al. 1986; Watkins et al. 1984). Since the morphine-induced antinociception is enhanced by CCK_2-receptor-antagonists, an opioid-mediated release of CCK has been suggested (Dourish et al. 1988; Wiesenfeld-Hallin et al. 1990). A stimulation of opioid receptors may stimulate the release of CCK, which in turn reduces the action of opioids. Direct evidence for opioid-induced CCK release has been obtained *in vitro* (Benoliel et al., 1991, 1994) and *in vivo* (Zhou et al. 1993; Lucas et al. 1998). However, the exact mechanism responsible for the morphine-induced *in vivo* release of CCK-LI in the dorsal horn has not yet been determined.

The anti-opioid action of CCK may be due to the mobilization of intracellular calcium storage by CCK, which counteracts the lowering of cytosolic calcium by opioid drugs (Wang et al. 1992). Autoradiographic studies have demonstrated voltage-dependent calcium channels (VDCCs) of the L- and N-type in the dorsal horn (Gandhi and Jones, 1988; Kerr et al. 1988). An influx of calcium at presynaptic terminals through VDCCs is essential for the release of neuropeptides (Smith and Augustine, 1988) and may contribute to opioid-induced release of CCK as well.

Experimental models of persistent pain

A number of animal models of persistent pain have been developed during the last two decades. Most neuropathic pain models are generated by surgical manipulation of the sciatic nerve (for review, see Taylor, 2001). These include complete nerve transection (CNT) a model of deafferentiation or phantom limb pain (Wall et al. 1979), chronic constriction injury (CCI; Bennett and Xie, 1988) and partial sciatic ligation (PSL; Seltzer et al. 1990). Other models of neuropathic pain are based on lesions to spinal nerves or induction of pathological states within the spinal cord itself (Kim and Chung, 1992; Kawakami et al. 1994).

More recently, a photochemical-induced nerve ischemia, secondary to laser activation of the photosensitive dye erythrosin B, has been demonstrated to result in a high incidence of allodynia in rats and mice (Gazelius et al. 1996; Kupers et al. 1998; Hao et al. 2000). The CNT model, first described by Wall (1979), is based on a complete transection of the sciatic nerve (axotomy). Following axotomy, the primary sensory neuron undergoes numerous anatomical and physiological changes, which may persist for months (for review, see Woolf and Doubell, 1994). These changes include an increased gene-expression of CCK-mRNA and of CCK_2-receptor mRNA in DRG neurons (Hökfelt et al. 1994, 1997).

High intensity peripheral electrical stimulation of the sciatic nerve stimulation may induce an increased response to low intensity peripheral stimulation, serving as a model for the transition from acute to persistent pain. Depending on the intensity and frequency of the stimulation on the sciatic nerve, at least two different types of spinal plasticity have been observed in the dorsal horn: Wind up and long term potentiation (LTP) (for review, see Sandkühler, 2000). Wind up, characterized by progressive increase in the number of action potentials in the dorsal horn neurons is produced by repetitive stimulation of dorsal root afferents at low frequency [0.3–5.0 Hz] (Mendell 1966; Jeftinija and Urban, 1994). LTP in WDR neurons and increased field potentials (Svendsen et al. 1997, 1999; Sandkühler, 2000) have been observed in the dorsal horn following tetanic electrical stimulation of the sciatic nerve at high frequency [100 Hz]. In contrast to wind up, which is a short lasting phenomenon, LTP may persist for at least 8 hours (Liu and Sandkühler, 1995, 1997).

Peripheral inflammation can be induced by injection of irritant chemicals into for example a hindpaw. Formalin, carrageenan and complete Freunds adjuvant (CFA) are widely used in models for inflammatory pain. Formalin injection (Dubuisson and Dennis, 1977; Tjølsen et al. 1992; Abbott et al. 1995) induces first phase (0-10 min) and second phase (15-60 min) nocifensive behavior and corresponding electrophysiological (Dickenson and Sullivan 1987a,b) responses. Injection of carrageenan (Winter et al. 1962) into the rat hindpaw produces an acute inflammation associated with significant pain-behavior (Kayser and Guilbaud, 1991) and electrophysiological (Hedo et al. 1999; Rygh et al. 2001) changes appearing 3-20 hours after the injection. CFA injection (Millan et al. 1988) into the rat hindpaw produces a long lasting pain, peaking at 3 days and associated with thermal and mechanical hyperalgesia and allodynia (Ma and Woolf, 1996).

The injection of carragenan or CFA results in an inflammatory response involving, cytokine production, mast cell degranulation, tissue damage and neuropeptide release from sensory neurones (Woolf and Doubell, 1994).

Neuropeptides and persistent pain

Injury to an afferent nerve may result in an altered processing of noxious and non-noxious stimuli. Animal studies have demonstrated that complete section of the sciatic nerve (axotomy), causes complex changes in the gene-expression of many neurotransmitters or neuromodulators and their receptors in the dorsal horn. The gene-expression of excitatory peptides, including SP and CGRP, is downregulated, whereas the gene-expression of some peptides with inhibitory effects (e.g. galanin, vasoactive intestinal polypeptide (VIP) and NPY increases (Hökfelt et al. 1994). Furthermore, CCK mRNA and CCK-B receptor mRNA are upregulated in DRG neurons of the rat (Verge et al. 1993; Zhang et al. 1993). The upregulation of CCK mRNA in the DRG neurons has been suggested to lead to an enhanced release of CCK in the dorsal horn (Stanfa et al. 1994). The enhanced release of CCK in combination with a decrease of μ- and δ -opioid receptor-like immunoreactivity in the dorsal horn after nerve injury (Zhang et al. 1998) may contribute to the decreased efficacy of the opioid in the treatment of neuropathic pain (for review, see Bridges and Thompson, 2001). However, the effect of peripheral nerve injury on the interaction between CCK and opioids, at the spinal level, remains to be elucidated.

High intensity peripheral electrical stimulation induces release of sensory neuropeptides, particularly SP, NKA and CGRP in the dorsal horn (for review, see Urban and Thompson, 1994). These peptides are considered to contribute to the increased sensitivity of dorsal horn neurons following brief high peripheral electrical stimulation (i.e LTP). Indirect evidence suggests that an increased release of SP and glutamate is essential for LTP in dorsal horn neurons. However, the release pattern of SP during the development and maintenance of LTP in the dorsal horn has not yet been studied.

Peripheral tissue inflammation also induces specific alterations in primary afferent neurons. The gene-expression of CCK and galanin is downregulated, whereas the gene expression and the levels of other peptides such as CGRP and SP are increased (Galeazza et al. 1995; Minami et al. 1989; Smith et al. 1992; Donnerer et al. 1993; Wiesenfeld and Xu, 2001) in DRG neurons.

In addition, peripheral inflammation induces an increased density NK-1 receptors and NK1 receptor internalization in the dorsal horn (Schäfer et al. 1993; McCarson and Krause, 1994; Abbadie and Basbaum, 1996; Honoré et al. 1999).

Inflammation is also associated with an increased capsaicin-evoked spinal release of glutamate (Sasaki et al. 1998) and of SP (Nanayama et al. 1989; Southall et al. 1998) in the dorsal horn in *vitro*. Moreover, peripheral inflammation increases spinal NMDA receptor function (Rygh et al. 2001) and the central axonal transport of capsaicin sensitive VR-1 receptor mRNA in primary afferents is also increased (Tohda et al. 2001). Since NMDA receptor activation induces internalization of spinal SP receptors in the dorsal horn, presynaptic NMDA receptors have been suggested to release SP from primary afferents (Liu et al. 1997; Marvisón et al. 1997). Capsaicin induces spinal release of glutamate (Sorkin and McAdoo, 1993) and capsaicin-induced SP release from the spinal cord *in vitro* has been suggested to be secondary to activation of NMDA receptors (Malcangio et al. 1998). However, the role of glutamate receptors in capsaicin-induced spinal release of SP-LI has so far not been studied in vivo.

Aims of the study

1. To monitor the effect of a unilateral sciatic nerve section (axotomy) on basal and potassium-induced release of CCK in the rat dorsal horn at different time points after the lesion.

2. To study the effect of systemic and spinal administration of morphine on CCK release in intact animals and to investigate the effect of axotomy on opioid-induced release of CCK in the rat dorsal horn.

3. To determine the opioid receptor mediating morphine-induced release of CCK.

4. To characterize the ion channels involved in morphine-induced release of CCK.

5. To study the release of SP in the spinal cord during induction and maintenance of LTP in dorsal horn neurons following electrical stimulation of the sciatic nerve.

6. To examine the role of glutamate receptors in capsaicin-induced release of SP in the dorsal horn.

7. To examine the effect of peripheral inflammation on capsaicin-induced SP release in the dorsal horn.

Materials and methods

Animals (I-VI)

Most experiments were performed on male Sprague-Dawley (SD) rats (B&K Universal, Sollentuna, Sweden). In paper IV, female SD rats (Møllegaard, Denmark) were used. Animals arrived at least one week before they were used for experiments and were housed under standardized conditions in a 12:12 h light/dark cycle in a temperature and humidity controlled room. Food and water were available ad libitum. All experiments were approved by the regional ethical committee for animal research and followed ethical guidelines for experiments on animals by the International Association for the Study of Pain (Zimmermann, 1983).

Drugs and chemicals (I-VI)

The following drugs and chemicals have been used in these studies: Morphine hydrochloride was obtained from Pharmacia & Upjohn AB (Stockholm, Sweden) and naloxone hydrochloride from Du Pont Pharmaceuticals (Letchworth Garden City, UK). DAMGO, CTOP, [D-Ala^2] deltorphin II, BW373U86 hydrochloride, TTX, verapamil, ω-conotoxin GVIA, bovine serum albumin (BSA, A 4503), bacitracin, NMDA, D-APV, CFA, and carrageenan were obtained from SIGMA, St. Louis, MO, USA. Synthetic sulphated CCK-8 and SP were purchased from Peninsula Labs (CA, USA) and ^{125}I-gastrin-I (specific activity 70 MBq/nmol) was obtained from Euro-Diagnostica AB, Sweden). The CCK/gastrin antiserum (2609/10) was a generous gift from Prof. Jens F. Rehfeld, Rigshospitalet, Copenhagen, Denmark. For SP-radioimmunoassay, ^{125}I-[TYR^8]-SP (*NEN* Life Science Products, Boston, USA) and SP antiserum SP2 (Brodin et al. 1983) were used.

Peripheral nerve injury (axotomy) and induction of inflammation (I, II, VI)

Under halothane anesthesia, the left sciatic nerve was exposed at midthigh level, tightly ligated above the greater trochanter and transected distally to the ligation. The wound was closed with staples. The axotomy was carried out 3-60 days prior to the microdialysis experiments (see below). Peripheral inflammation was induced by subcutaneous injection (150 μl into the left hind paw) of either complete Freund's adjuvant (CFA, 1:1 saline emulsion) or carrageenan (100 μl 2% λ-carrageenan in saline) under halothane anesthesia. The injection was performed 3 and 7 days (CFA group) or 20 h (carrageenan group) before the experiments.

These time points were chosen since an upregulation of SP receptors has been reported in the dorsal horn up to seven days after peripheral inflammation (Abbadie et al. 1996) .

Microdialysis (I-VI)

The microdialysis experiments were performed under halothane anaesthesia. The spinous processes of Th11 and L3 were connected to a stereotaxic spinal device (GH-Medic, Stockholm, Sweden) in order to prevent movement of the spinal cord during dialysis. A hole was drilled through the dorsal part of vertebra Th 13 exposing the dura mater. Using a standard micromanipulator (David Kopf Instruments, Tujunga, Ca, USA), the dialysis membrane of the microdialysis probe was inserted in a rostral-caudal direction into the dorsal horn of the spinal cord at a location medial to the root entry zone of the spinal segments L4-L6. Prior to the experiments, the microdialysis probe (CMA/11, cuprophane membrane, 2 mm length, outer diameter. 0.24 mm, molecular cut-off 6.000 Da) was immersed in a test tube containing 0.5% NaOCl and perfused with NaOCl (0.5%, 15 min, flow rate 3.5 µl/min; microsyringe pump, CMA/102) to eliminate any residual proteins from "sticking" to the tubing walls. Probes were then immersed in a vial, containing 70% ethanol and flushed with ethanol (70%, 15 min, flow rate 3.5 µl/min). The probes were thereafter flushed with deionised H_2O for 15 minutes and perfused (flow rate: 3.5 µl/min) with modified Krebs-Ringer solution (138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 11mM $NaHCO_3$, 1 mM NaH_2PO_4), containing 0.2% bovine serum albumin, 0.2% glucose and 0.03% of the peptidase inhibitor bacitracin (Sigma USA), pH 7.4. The probe was inserted at an angle of 40-45° from the horizontal plane. Following a one-hour wash-out period, dialysate samples (105 µl) were collected at 30 min intervals in Eppendorf vials and stored at -20° C until the assay procedure.

The relative *in vitro* recovery of the microdialysis system for CCK-8 was approximately 2 % (see paper III). The relation between the CCK concentration in the outer medium and the dialysate showed a linear slope. Similar results were obtained for SP (data not shown).

Radioimmunoassay for CCK and SP (I-VI)

CCK-like immunoreactivity (-LI) or SP-LI was determined using a modified radioimmunoassay (RIA) procedure with improved sensitivity developed especially for the determination of neuropeptide concentrations in microdialysates (Brodin et al. 1983, You et al. 1994). The spinal cord dialysate samples and the standard samples (100 µl) were preincubated for 24 h at +4°C with the C-terminal directed CCK/gastrin antiserum (2609/10, in 0.1 M barbital buffer containing 0.2 % BSA, Rehfeld, 1978) or C-terminal directed SP antiserum (SP2, Brodin et al. 1986). Following addition of the radioligand (approximately 1000 cpm per assay tube) of ^{125}I-gastrin-I, (specific activity 70 MBq/nmol) for CCK-LI determination or addition of the radioligand of ^{125}I-[TYR^{8}]-SP (NEN Life Science Products, Boston, USA) for determination of SP-LI, all samples were incubated at +4°C for another 72 hours. Free radioligand was separated from antibody bound radioligand in all RIA samples, except those used for determination of the total radioactivity, by the addition of 250 µl sheep anti-rabbit antibody-coated sepharose suspension. After 30 min of incubation and centrifugation at 2600 g for 10 min, the supernatant is discarded by aspiration and the radioactivity in the pellets containing the bound fraction is measured (10 min/sample) in a gamma-counter. The concentration of CCK or SP in the dialysate samples was calculated from the standard curve (Gustafsson et al. 1999; Brodin et al. 1983).

Combination of in vivo electrophysiology and microdialysis (IV)

The animals were anaesthetized with urethane (1.4-1.8 g/kg body weight i.p.), the dorsal horn surface of the spinal cord was exposed and the vertebral column was fixed (see above). The left sciatic nerve was then dissected free for a length of 8-10 mm and isolated from the surrounding muscles and a bipolar silver hook electrode (1.5 mm between hooks) was placed proximal to the division of the main branches of the nerve. The rats were paralysed by intramuscular injection of 0.2 ml pancuronium bromide (2 mg/ml, Pavulon, Organon) and connected to a ventilator (SAR 830/AP, CWE Inc.) adjusted to normo- or mild hyperventilation by monitoring the end tidal CO_2 concentration. The spinal cord segment with input from the sciatic nerve was localized by means of electrophysiology, and a microdialysis probe was inserted in the left dorsal horn of this segment. A microelectrode with a tip diameter of 2-5 µm was used for single cell recordings.

Following a one-hour period of sampling for the basal level and a one-hour period of sampling in combination with electrophysiological recording of evoked neuronal responses, a high-frequency conditioning stimulus was applied to the sciatic nerve. The high-frequency conditioning stimulation consisted of 20 trains of 2 s duration, 100 Hz and 0.5 ms rectangular pulses with 10 s intervals between trains. Sampling and recording were continued for one hour and 30 min after the conditioning stimulus.

Extracellular electrophysiological recording (IV)

A parylene-coated tungsten microelectrode (2-5 μm Frederick Haer & Co. model 26-05-3) was lowered into the left dorsal horn by means of an electronic stepper with depth control, and extracellular recordings of neurons at a depth of 400-1000 μm were performed. Single cell recording was ensured on the basis of the amplitude and shape of the action potentials. The recorded signals were amplified and band-pass filtered with 1/2 amplitude cut off values of approximately 1000 and 1250 Hz corresponding to the duration of the action potentials (0.8-1 ms). Every 4 min or in another group every 10 sec, a 2-ms-wide rectangular test stimulus with a pulse current of 1.5 x C-fiber response threshold was applied to the sciatic nerve. The number of spikes 0-40 ms after stimulus were defined as A-fiber responses, whereas the number of spikes 40-300 ms after stimulus were defined as C-fiber responses. The data was captured and analyzed using a CED 1401 interface unit connected to an IBM compatible PC. All cells were characterized by their response to natural stimuli (touch, pressure and pinch) before the experiments. Only deep WDR neurons (one in each animal), i.e. cells responding to both non-noxious tactile stimuli and noxious forceps pinch in a graded manner, were studied. A minor increase of the neuronal responses may occur without any tetanic stimulation. Thus, an increased response, indicative of the presence of LTP, was considered to be present if the number of spikes following a test stimulus was more than 25 % higher than at baseline conditions.

Statistical analysis (I-VI)

In the microdialysis studies, the Friedman non-parametric analysis of variance followed by Dunn's test was used to assess the statistical significance within groups. Differences between groups were analyzed by the Mann Whitney test or by the Kruskal-Wallis test followed by Dunn's test, when appropriate. GraphPad PRISM version 2.01 (GraphPad, San Diego CA) was used for statistical analysis.

In the electrophysiological study, the statistical significance within groups were evaluated by one-tailed paired Student's *t*-test. Data between groups were compared by one-tailed unpaired Student's *t*-test. All data are expressed as mean ± SEM. A P-value < 0.05 was accepted as significant.

Results and Discussion

Effect of peripheral axotomy on the potassium-induced release of CCK-LI in the dorsal horn (I)

In intact animals, potassium (100 mM in the perfusion fluid) stimulation induced a significant 5-fold increase of the extracellular CCK-LI level in the dorsal horn. A higher potassium induced release of CCK-LI was observed three days or one week after peripheral axotomy. The magnitude of the potassium-induced release of CCK-LI at these time points did not reach statistical significance when compared to control animals (Fig 1). The enhanced CCK release in response to potassium stimulation, observed during the first week after axotomy, could be due to an increased peptide synthesis or a decreased metabolic breakdown. An upregulation of a membrane protein involved in the regulation of exocytosis of peptide containing vesicles, after nerve lesion (Jacobsson et al. 1998) may also enhance the probability of neurotransmitter release.

From two weeks until at least 2 months after axotomy, potassium stimulation could no longer induce spinal CCK-LI release (Fig 1). These results do not seem to be in line with the an increased CCK-gene expression in some primary sensory neurons two or three weeks after complete sciatic nerve transection (Verge et al. 1993). However, it has to be kept in mind that an increased expression of CCK mRNA does not necessarily lead to an increased translation into the neuropeptide and an increased releasable pool of CCK (Hökfelt et al. 1997). Previous results (Gustafsson et al. 1998) indicate that the failure of potassium stimulation to induce spinal CCK-LI release after axotomy is not due to a loss of releasable CCK in the dorsal horn, but to an increased inhibition involving CCK_2 receptors (Gustafsson et al. 1998). After nerve injury, these receptors are upregulated in the dorsal horn (Brás et al. 1999). Possibly, some CCK_2-receptors, which are upregulated after axotomy, represent inhibitory CCK autoreceptors on dorsal horn interneurons, which are the most likely source of the released CCK (Malcangio et al. 1999; Jacobsson et al. 1998).

Our present results indicate that CCK could contribute to an increased nociceptive signaling during the first week after a nerve lesion, but probably not at later time points.

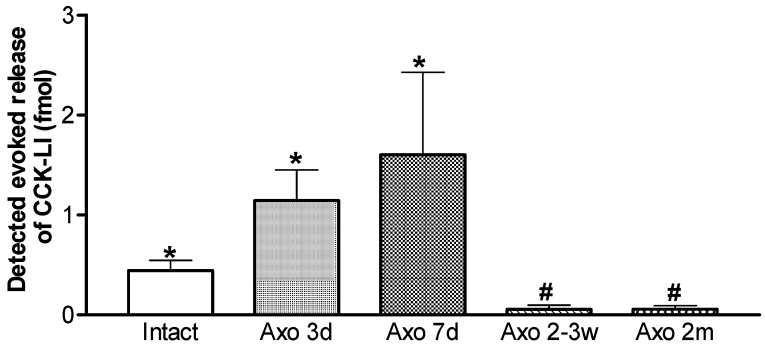

Fig 1. Potassium (100 mM)-evoked CCK-LI release in the dorsal horn of intact rats and 3 days, 7 days, 2-3 weeks and two months after sciatic nerve transection. The experiments were performed under halothane-anesthesia. * $p < 0.05$, (Friedmans test followed by Dunn's test), significant difference compared to baseline within the group. # $p < 0.05$ (Kruskal-Wallis test followed by Dunn's test) significant difference compared to intact rats and 3 and 7 days after axotomy. Data presented as mean ± SEM in fmol. (n = 5-7).

Effect of systemic and spinal administration of morphine on CCK-LI release in intact animals (III)

Systemic (2.5 -5 mg/kg, s.c.) and spinal (1 or 100 μM in perfusion fluid) administration of morphine induced a significant release of CCK-LI in the rat dorsal horn. The CCK-LI release induced by morphine (5 mg/kg) was blocked not only by systemic, but also spinal, pretreatment with naloxone. The CCK-LI release induced by systemic administration was dose dependent. Doses of 1 mg/kg had no effect on the CCK-LI release, whereas 2.5 mg/kg increased the CCK-LI release more than 6-fold and 5 mg/kg more than 10-fold (Fig 2).

These results demonstrate that morphine, given systemically at doses within the range required to induce antinociception in behavioral tests, is able to induce CCK-LI release in the dorsal horn that was of the same order of magnitude as potassium stimulation (100 mM in the

perfusion fluid) (Fig 1). The release of CCK-LI in the dorsal horn after systemic morphine treatment coincided in time with the peak of the antinociceptive effect of morphine (Zhou et al. 1993).

Considering the anti-opioid effects of CCK, the present data are in accordance with the concept of endogenous CCK counteracting morphine antinociception (Wiesenfeld-Hallin et al. 1999).

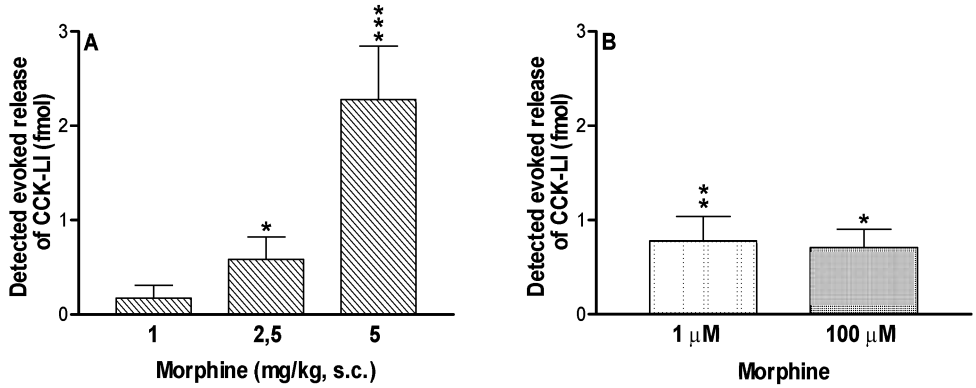

Fig 2. Evoked release of CCK-LI induced by systemic (1-5 mg/kg ,s.c) (**A**) and spinal (1 or 100 μM) (**B**) administration of morphine. Data presented as mean ± SEM in fmol. (n=5-7, * $p < 0.05$, * *$p < 0.01$, *** $p < 0.001$, Kruskal-Wallis test followed by Dunn's test).

Effects of opioid receptor agonists and antagonists on the release of CCK-LI in the dorsal horn of intact and axotomized animals (II-III)

The release of CCK-LI in response to systemic administration of morphine (5 mg/kg, s.c.) was inhibited by spinal administration of the δ-opioid receptor antagonist naltrindole. In contrast, neither spinal administration of the μ-opioid receptor selective antagonist CTOP nor the κ-opioid receptor antagonist nor-BNI induced any significant inhibition of the morphine- (5 mg/kg, s.c.) evoked release of CCK-LI. Systemic administration of the selective δ-opioid receptor agonist BW373U86 (1 mg/kg, s.c.) induced a significant increase of the spinal CCK-LI release, an effect almost completely prevented by spinal administration of the δ-opioid receptor antagonist naltrindole (10 μM in the perfusion medium) (Fig 3B). The δ-selective agonist BW373U86 (1 mg/kg, s.c.) induced a release of CCK-LI of the same order of magnitude as systemic administration of morphine (5 mg/kg, s.c) (Fig 4).

The ability of this δ-selective agonist to release CCK-LI was also maintained two to three weeks after axotomy, at a time point when morphine (5 mg/kg, s.c) failed to induce release of CCK-LI. Systemic administration of the μ-opioid receptor agonist DAMGO (1 mg/kg, s.c.) failed to alter the extracellular level of CCK-LI in the dorsal horn in control animals as well as after axotomy (Fig 4A-F). Thus, the delta-opioid receptor seems to be the opioid receptor responsible for opioid-induced release of CCK. In addition, the present data indicate that the morphine-induced release of CCK in the spinal cord is mediated by activation of the δ-opioid receptor. These findings are in agreement with a previous *in vivo* microdialysis study in the rat frontal cortex, showing a δ-opioid receptor-mediated release of CCK (Becker et al. 1999).

The present finding of a decreased ability of morphine to induce release of CCK-LI after axotomy may be partly related to the decreased density of the δ-opioid receptor in the experimental pain condition (Zhang et al. 1998). In a previous study, morphine (10 mg/kg) was found to induce a significant release of spinal CCK-LI in axotomized animals (Lucas et al. 1998) and it may be suggested that the dose-effect curve for the CCK releasing effect of morphine is shifted to the right after axotomy. The finding that systemic or spinal administration of the selective μ-opioid receptor agonist DAMGO failed to alter the extracellular CCK-LI level seems to be in conflict with the report of a μ-opioid receptor induced reduction of stimulated spinal CCK release *in vivo* (Rodriguez and Sacristan, 1989) and *in vitro* (Benoliel et al. 1991, 1994).

However, since the effect of μ-opioid receptor agonists on stimulated release was not investigated in the present study and the basal CCK-LI level were close to the detection limit of the RIA, we cannot exclude the possibility of a decreased release of CCK by DAMGO.

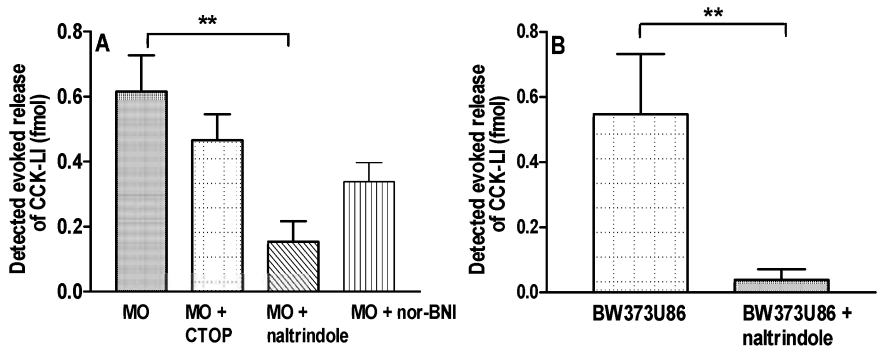

Fig 3. Release of CCK-LI evoked by morphine (5 mg/kg, s.c.) (MO) given alone and in the presence of the μ-antagonist CTOP (10 μM in the perfusion fluid), the δ-opioid receptor antagonist naltrindole (10 μM in the perfusion fluid) or the κ- receptor antagonist nor-BNI (10 μM in the perfusion fluid) **(A)**. CCK-LI release evoked by the δ-opioid receptor agonist BW373U86 given alone and in the presence of the δ-opioid receptor antagonist naltrindole (10 μM in the perfusion fluid) **(B)**. Data presented as mean ± SEM in fmol. (n=5-7, ** = $p < 0.01$, Kruskal-Wallis test followed by Dunn's test).

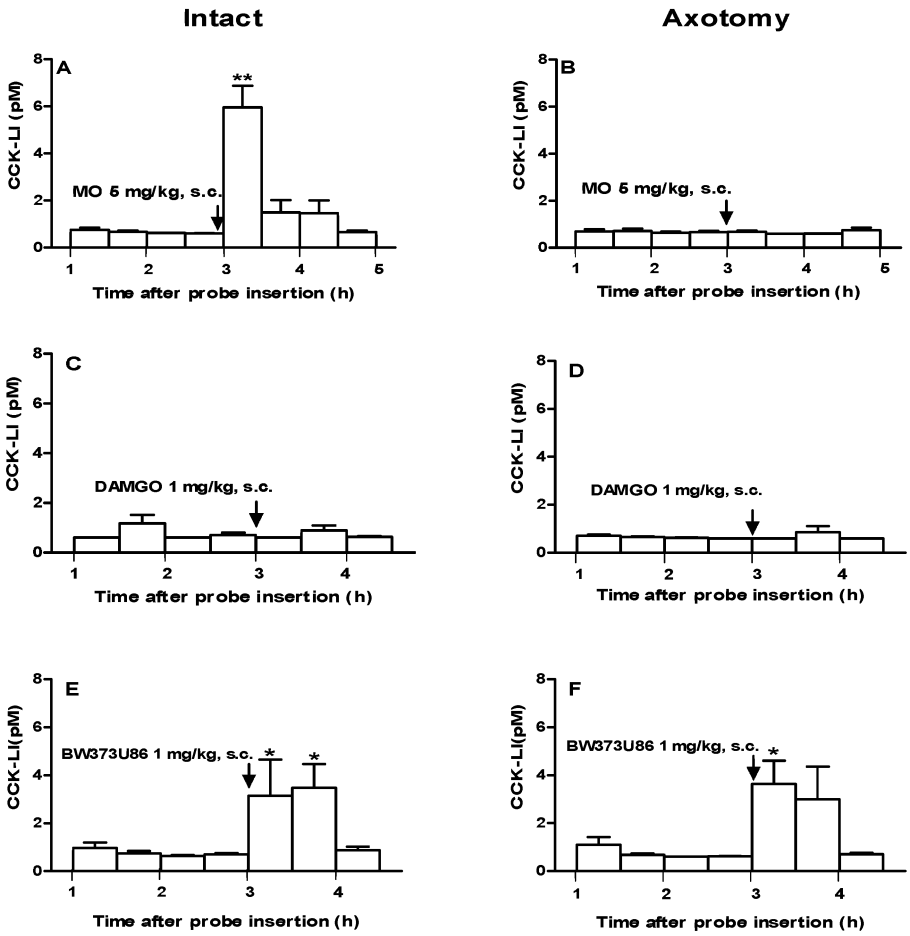

Fig 4. Extracellular concentration of CCK-LI in the dorsal horn anesthetized rats: Effect of systemic administration of morphine (MO, 5 mg/kg, s.c.) in intact animals (**A**) and 2-3 weeks after axotomy of the sciactic nerve (**B**). Effect of the selective μ-opioid agonist DAMGO (1 mg/kg, s.c.) in intact animals (**C**) and 2-3 weeks after axotomy (**D**). Effect of the δ-opioid receptor agonist BW373U86 (1 mg/kg, s.c.) in intact animals (**E**) and 2-3 weeks after axotomy (**F**). Data presented as mean ± SEM in pM. (n=5-7, * = $p < 0.05$, ** = $p < 0.01$, Friedmans test followed by Dunn's test).

Effects of sodium channel blockers and calcium channel blockers on morphine-evoked release of CCK-LI in intact animals (III)

Local administration the sodium channel blocking agent TTX prevented the stimulatory effect of morphine on the release of CCK-LI. When the dialysis probe was perfused with calcium-free medium, morphine failed to alter the CCK-LI level. Pretreatment with either the L-type calcium channel blocker verapamil or the N-type calcium channel blocker ω-CgTX by topical application onto the dorsal surface of the spinal cord completely inhibited the release of CCK-LI induced by morphine (Fig 5A-D). Since pretreatment with TTX prevented the morphine-evoked CCK-LI release in our *in vivo* experiments and previous *in vitro* studies (Benoliel et al. 1991, 1994), the release CCK-LI seems to require propagation of action potentials along axons in the dorsal horn and is probably not due to a direct activation of δ-opioid receptors on CCK-containing nerve terminals.

Activation of opioid receptors generally induces inhibitory effects on voltage-dependent calcium channels (Mulder et al. 1990; Cruciani et al. 1993). On the other hand, opioid receptor-mediated increase of intracellular calcium levels in neuronal cell lines via L-type calcium channels has also been reported (Jin et al. 1992). Even though L- and N-type channels may occur on the same neurons (Tsien et al. 1991; Snutch and Reiner, 1992; Westenbroek et al. 1992), it is not known if L- and N-type calcium channels are colocalised on CCK-containing neurons.

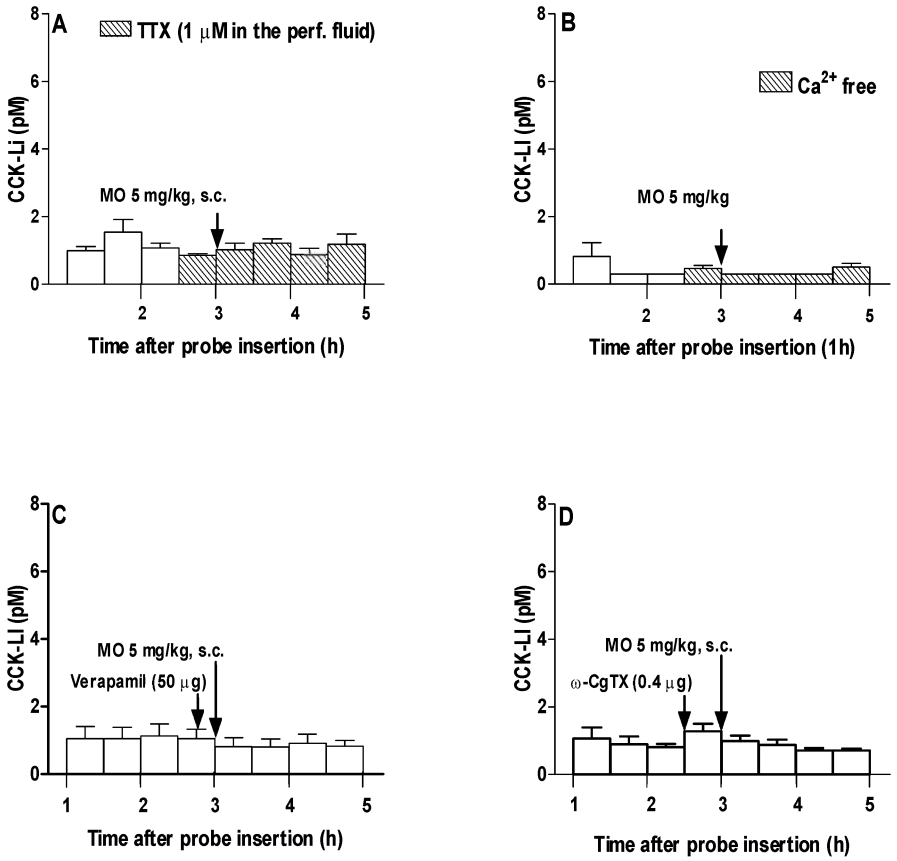

Fig 5. Effect TTX (1μM in perfusion fluid) (hatched bars) (50 μg) **(A)** or perfusion with calcium free medium (hatched bars) **(B)** or topical application verapamil (50 μg) **(C)** or omega conotoxin GVIA (ω-CgTX, 0.4 μg) **(D)** on CCK-LI release induced by morphine (MO, 5 mg/kg, s.c.). Data are presented as the mean ± SEM in pM. (n=5-8, ** = $p < 0.01$, Friedman test followed by Dunn's test).

Effect of tetanic stimulation on C-fiber evoked responses and release of SP-LI in the dorsal horn (IV).

The release of SP-LI and the development of long-term potentiation (LTP) were simultaneously monitored in the spinal cord of anaesthetized rats. A significant increase in the C-fiber induced responses (LTP) was recorded in single WDR neurons after the conditioning tetanic stimulation (Fig 6A). The A-fiber mediated responses were not affected (Fig 6B).

A significant release of SP-LI in response to tetanic stimulation of the sciatic nerve was observed in the dorsal horn as compared to basal levels within the group (Fig 6C) and compared to the control group (Fig 6D). These results suggest an involvement of SP in the induction of LTP, which is in agreement with a previous study showing that intravenous application of the NK-1 receptor antagonist RP 67580 inhibits the induction of LTP (Liu and Sandkühler, 1997). However, this SP antagonist failed to block an established LTP (Liu and Sandkühler, 1997). In the current study, the extracellular level of SP-LI was not altered upon single impulse nerve activation after LTP had been established. One interpretation of these findings is that once LTP is developed, SP has no or little role in maintaining the facilitated transmission.

Our data thus indicate that a profound SP release during a brief high frequency tetanic stimulation may be of importance for initiation, but not for maintenance of LTP in WDR neurons.

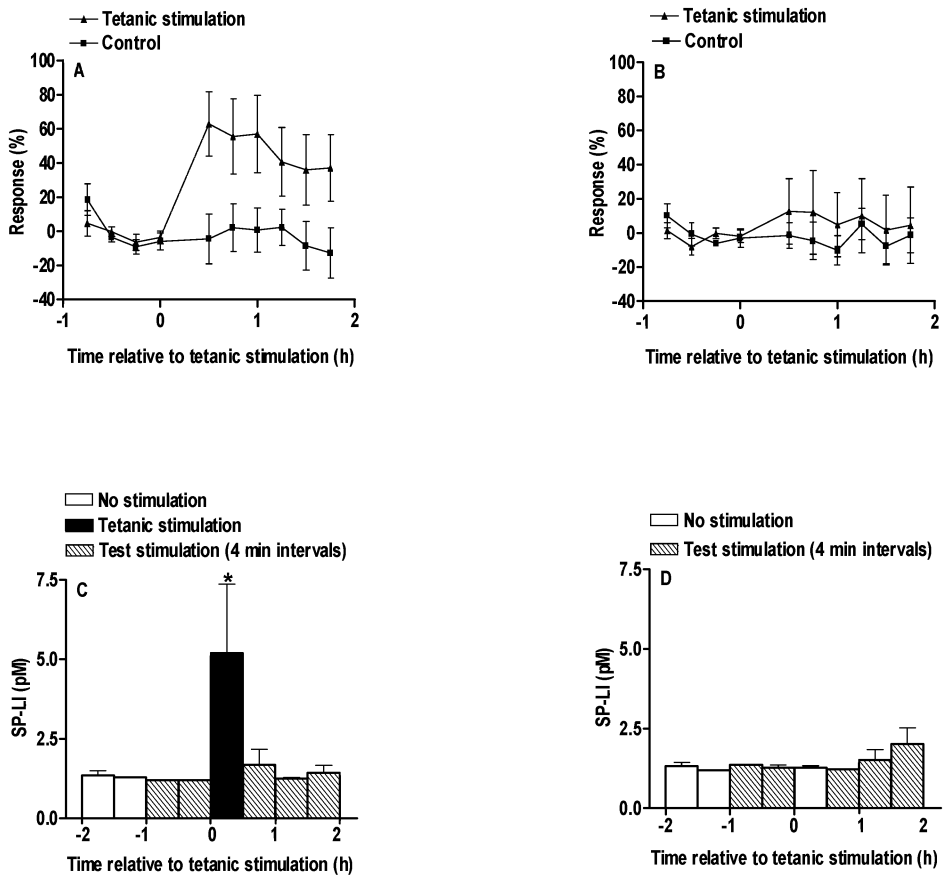

Fig 6. The effect of tetanic stimulation applied to the sciatic nerve on C-fiber **(A)** and A-fiber **(B)** responses to single pulse stimulation. The number of spikes are given as per cent of baseline. A significant increase in the C-fiber induced responses (LTP) was recorded (mean after tetanus compared to baseline, $p< 0.05$, one-tailed paired Student's t-test, mean after tetanus compared to control, $p< 0.05$, one-tailed unpaired Student's t-test). The release of SP-LI in the dorsal horn in experiments with single test stimulations and tetanic stimulation **(C)** and in experiments with only single test stimulations **(D)**. Data are presented as mean ± SEM in pM (n=5-6, *$p<0.05$ compared to baseline, Friedman's test and compared to control group (experiments with only single test stimulations), Mann Whitney test).

The role of glutamate in capsaicin-evoked release of SP-LI in the dorsal horn (V-VI)

Local perfusion of the dialysis probe with 50, but not 1 or 10 μM capsaicin, for 30 minutes, induced a significant eight-fold increase of the SP-LI release in the dorsal horn. A similar response was observed during perfusion with 100 μM capsaicin. (Fig 7 A-D). Spinal administration of the selective NMDA antagonist D-APV (5 mM in the perfusion fluid), but not the AMPA/KA antagonist NBQX (0.5 mM), prevented the release of SP-LI evoked by 50μM capsaicin (Fig 8A). However, neither D-APV (10 mM) nor NBQX (0.5 mM) prevented the SP-LI release induced by the higher concentration of capsaicin (100 μM) (Fig 8B).

These results support the notion that capsaicin-induced release of SP-LI in the dorsal horn is mediated via an activation of NMDA receptors (Malcangio et al. 1998). The AMPA/KA receptors, on the other hand, do not appear to contribute to the release of SP-LI evoked by capsaicin, which is in line with previous studies in vitro (Malcangio et al. 1998). The exact location of the spinal NMDA receptors mediating capsaicin-induced SP-LI release is not known. NMDA receptors are present on dorsal horn neurons (Petralia et al. 1994) as well as presynaptically on terminals of C-fibers (Liu et al. 1994). Considering 1) the present results, 2) the abundance of SP in C-fiber primary afferents (Hökfelt et al. 1975), 3) the presence of vanilloid receptors also on primary afferent neurons not containing SP (Guo et al. 1999), 4) the existence of NMDA autoreceptors on C-fiber primary afferents (Liu et al., 1994) and 5) the indirect evidence of an NMDA-induced release of SP provided by studies of the internalization of the NK-1 receptor (Liu et al., 1997; Marvisón et al. 1997), it seems reasonable to suggest the following: Capsaicin, at moderate concentration, induces a release of glutamate from C-fiber primary afferents, which in turn activates NMDA receptors located on SP-containing primary afferents leading to release of SP. Capsaicin at higher concentration, on the other hand, seems to activate SP-release by a direct activation of vanilloid receptors on SP-containing terminals/neurons.

The release of SP by capsaicin from primary afferents may also partly occur indirectly via postsynaptic mechanisms involving diffusible messengers, such as nitric oxide (NO) (Meller and Gebhart, 1993; Wu et al. 2001). In spinal cord slices; synthesis of NO has been reported to be of importance for the capsaicin-induced release of CGRP (Garry et al. 2000), which coexists with SP in DRG neurons.

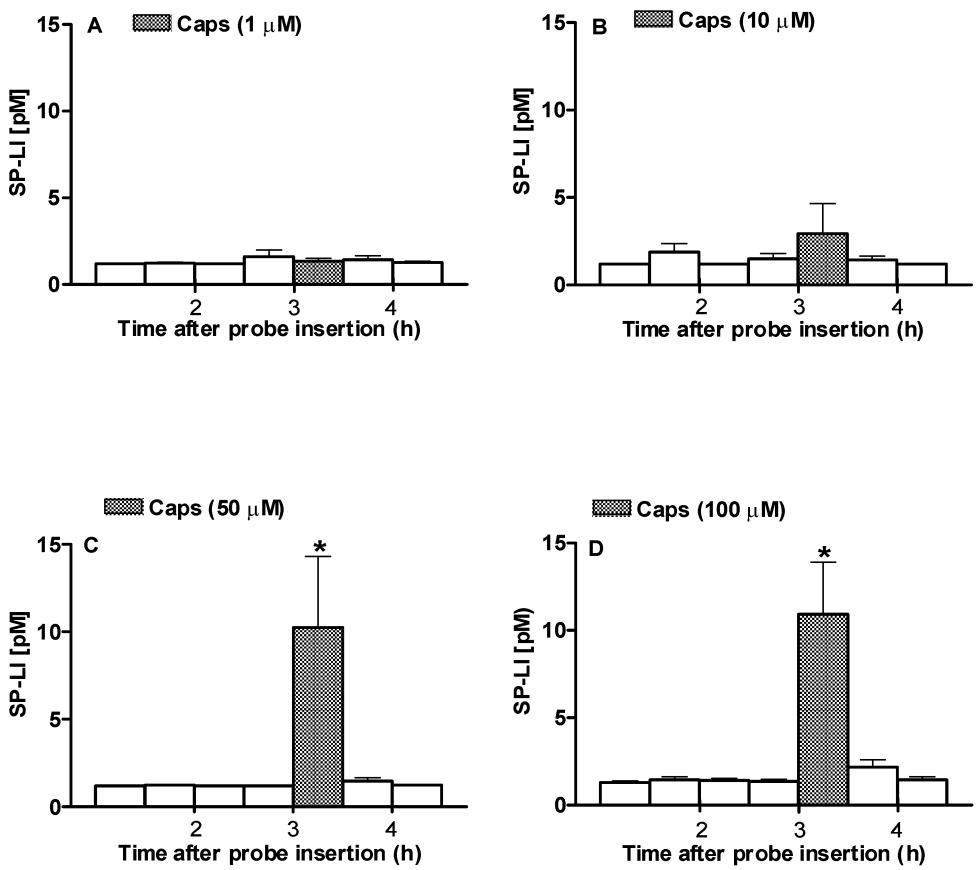

Fig 7. The effect of spinal capsaicin (in the perfusion fluid for 30 min) on the extracellular levels of SP-LI in the dorsal horn of control rats. 1 μM **(A),** 10 μM **(B)**, 50 μM **(C)** and100 μM **(D)** capsaicin (Caps). Data are expressed as mean ± SEM in pM. n= 7-8,* = $P < 0.05$, Friedman test followed by Dunn's test.

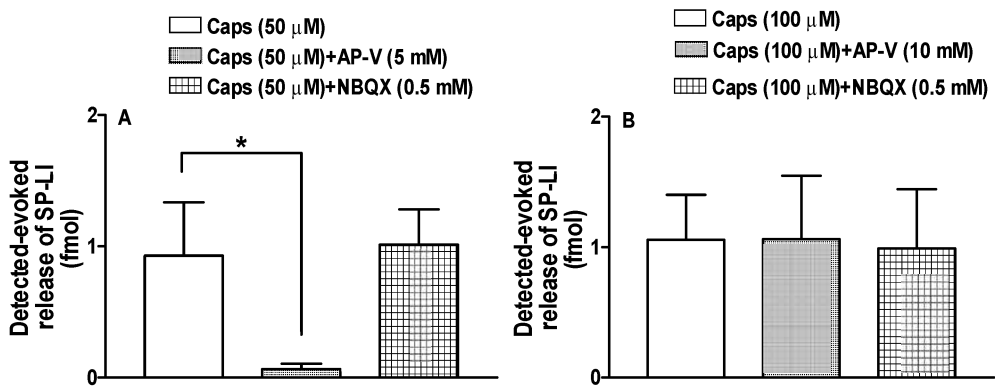

Fig 8. The effect of the selective NMDA-antagonist D-APV (5 or 10 mM in the perfusion fluid) and the AMPA/KA receptor antagonist NBQX (0.5 mM in the perfusion fluid) on SP-LI release (fmol) evoked by 50μM **(A)** or 100 μM **(B)** capsaicin (Caps) in perfusion fluid for 30 min. Data expressed as mean ± SEM, n=5-8, * = $P < 0.05$, Kruskal-Wallis test followed by Dunn's test, significant difference compared to control.

The effect of peripheral inflammation on the capsaicin-evoked SP-LI release (VI)

One week after the induction of peripheral inflammation by CFA, perfusion of the dialysis probe with 10 μM capsaicin for 30 min significantly increased the extracellular level of SP-LI as compared to baseline and to the control group. However, 10 μM capsaicin had no effect on the release of SP in A) control animals, B) 3 days after CFA-injection or C) 20 h after carrageenan injection. Stimulation with 50 μM capsaicin significantly increased the extracellular level of SP-LI in all 4 experimental groups (Fig 9). There was a tendency to a decreased response to 50 μM capsaicin 20 h after carrageenan. This may be explained by a transient depletion of SP in primary afferent terminals after injection of carrageenan. The capsaicin (10 μM)-evoked release of SP-LI one week after CFA injection was completely blocked by co-administration of D-APV (5 mM) in the dorsal horn (see Fig 3 in paper VI). The following mechanisms may contribute to the potentiation of capsaicin-evoked SP-LI release during inflammation:

A) An increased sensitivity of vanilloid receptors (VR1) that then mediate SP release.

B) An increased glutamatergic transmission activating presynaptic NMDA receptors on SP-containing primary afferent terminals in the dorsal horn. The capsaicin-induced release of glutamate in the dorsal horn in vitro has been shown to be increased after adjuvant-induced inflammation (Sasaki et al. 1998).

C) An increased spinal prostaglandin (PG) production leading to a sensitization of SP-containing sensory nerve terminals in the dorsal horn. PGE_2 has previously been shown to induce SP-release from DRG cells in vitro (White, 1996).

D) An increased availability of SP in primary afferent terminals during inflammation. An increased PPT-A mRNA expression (McCarson and Krause, 1994) and increased content of SP (Donnerer et al. 1992) in DRG has been reported 4-5 days after induction of inflammation with CFA.

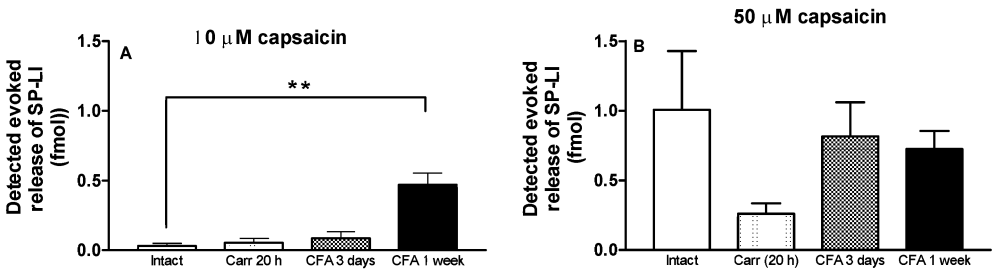

Fig 9. The capsaicin evoked (Caps)-evoked (5 mg/kg, s.c.) SP-LI release in intact animals and animals with 20h carrageenan (Carr) inflammation or 3 days and 1 week after CFA injection. 10 μM capsaicin (in the perfusion fluid) **(A)**. 50 μM capsaicin (in the perfusion fluid) **(B)**. Data presented as mean ± SEM in fmol. (n=5-7, * = $p < 0.01$, Kruskal-Wallis test followed by Dunn's test).

General Discussion

The first part of this thesis has addressed the hypothesis that an increased release of CCK in the spinal cord contributes to neuropathic pain and that morphine induces release of CCK, which then counteracts the analgesic effect of morphine (Dickenson et al. 1994, Xu et al. 1993,1994). Since the CCK gene expression as well as the CCK_2-receptor gene expression is upregulated after axotomy of the sciatic nerve (Hökfelt et al. 1994), we used this model. Our observation of a loss of the ability of potassium, as well as of morphine, to induce a significant release of CCK-LI in the dorsal horn 2 weeks after axotomy does not support the hypothesis of an increased CCK release as being responsible for neuropathic or deafferentiation (or phantom) pain. The enhanced morphine-induced antinociception by CCK_2-receptor antagonists (Xu et al. 1993; Nichols et al. 1995) in models of neuropathic pain seems more likely to be due to the increased density of spinal CCK_2-receptors, which has been observed after peripheral nerve lesion (Brás et al. 1999). However, the possibility that the release of CCK in the dorsal horn is elevated in other animal models of neuropathic pain, cannot be excluded.

Previous reports indicate that the lack of the ability of potassium to induce release of CCK after axotomy is due to activation of the CCK_2 receptor (Gustafsson et al. 1998). The ability of the CCK-2 antagonist to enable potassium induced release of CCK after axotomy is, hard to conceptualize with the antiopioid effect of CCK and the potentiation of opioid analgesia by CCK_2-antagonists (Dourish et al. 1990, Wiesenfeld-Hallin and Xu, 1996). However, the potassium induced depolarization is unspecific and stimulates the release of both excitatory and inhibitory neurotransmitters. Morphine and the other opioid agonists used to release CCK-LI in the present study, are more specific in their effect on CCK release. Thus it cannot be concluded, that activation of CCK_2 receptors also counteracts the morphine-induced CCK-release two weeks after axotomy. Instead, a decreased sensitivity of δ-opioid receptors after axotomy is a more likely explanation. A decreased density of δ-opioid receptors in DRG cells has been reported after peripheral sciatic nerve injury (Zhang et al. 1998). Since the data presented in this thesis indicate that δ-opioid receptor agonists stimulate the release of CCK also in neuropathic pain states, selective μ-opioid agonists or morphine in combination with δ-opioid receptor antagonists, may be of value in relieving neuropathic pain.

The present results also demonstrate that the blockers of L-type (Carta et al. 1990) and N-type (Omote et al. 1996) VDCCs prevent the morphine-induced release of CCK-LI.

Thus, these agents may be suitable as adjuvants to morphine. In fact, calcium channel antagonists have been shown to augment morphine-induced antinociception in animals (for review, see Chaplan, 2000) and the L-type calcium channel blocker nimodipine significantly increased the sensitivity to morphine in patients with cancer and pain (Santillan et al. 1998). It is of importance to further assess the clinical value of this drug combination in neuropathic pain in man.

The focus of the second part of the thesis is the role of SP in central sensitization after high intensity peripheral stimulation and the interaction of SP and glutamate in inflammation. During inflammation, increased syntheses of SP (Galeazza et al. 1995) and increased capsaicin-evoked spinal release of SP *in vitro* (Southall et al. 1998) have been observed. This is in line with the present results showing that peripheral inflammation increases the ability of capsaicin to evoke a release of spinal SP-LI *in vivo*. The involvement of SP in nociception has been demonstrated in numerous behavioral studies showing that spinal administration of SP produces pain-like responses (Yashpal et al. 1993) and that NK-1 receptor-antagonists have antinociceptive properties (Iyengar et al. 1997, Okano et al. 1998). In addition, electrophysiological recordings of spinal neurons have demonstrated that application of SP elicits firing of nociceptive specific dorsal horn neurons (De Koninck and Henry, 1991) and intravenous application of the NK-1 receptor antagonist RP 67580 inhibits the induction of LTP in dorsal horn neurons (Liu and Sandkühler, 1997). However, NK-1 receptor antagonists fail to inhibit established LTP (Liu and Sandkühler, 1997), thus NK-1 receptor antagonists are not likely be suitable for the treatment of pain states generated by high intensity nociceptive stimulation. Instead, glutamate seems to be the principle neurotransmitter maintaining LTP, since NMDA antagonists suppressed established LTP by approximately 80 % (Svendsen et al. 1998).

NMDA- as well as NK1-receptor antagonists attenuate inflammation-induced behavioral hyperalgesia (Ren et al. 1992a,b; Traub, 1996; Okano et al. 1998). Our finding of an enhanced capsaicin induced spinal SP release via NMDA-receptor activation one week after induction of inflammation with CFA, further support the idea that SP and glutamate interact in inflammatory pain processing. A combination of NMDA- and SP-receptor antagonists could therefore be a possible strategy for the treatment of chronic inflammatory pain.

Summary and conclusions

1. Spinal release of CCK-LI may be of importance for the altered primary afferent processing during the first week after axotomy.

2. Systemic and spinal administration of morphine induces a dose-dependent release of CCK-LI in the dorsal horn. The CCK-LI release induced by systemic morphine is of the same magnitude as the potassium (100 mM) induced release.

3. The most important opioid receptor for morphine induced CCK-LI release in the dorsal horn is the δ-opioid receptor.

4. Activation of L- and N-type calcium channels and TTX-sensitive sodium channels contribute to morphine-induced CCK-LI release.

5. The present data do not support the concept of an increased spinal CCK release as a mechanism for morphine resistance of "phantom pain".

6. SP may be involved in the induction, but not the maintenance, of LTP in WDR neurons in the dorsal horn.

7. Administration of submaximal doses of capsaicin-induces spinal release of SP-LI via activation of NMDA receptors.

8. The ability of capsaicin to evoke a release of SP-LI in the dorsal horn is increased one week after CFA-induced inflammation.

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References

Abbadie C, Brown JL, Mantyh PW, Basbaum AI. Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neuroscience.* 1996, 70:201-209

Abbott FV, Franklin KB, Westbrook RF. The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain.* 1995, 60:91-102.

Arnér S, and Meyerson BA. Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. *Pain,* 1988, 33: 11-23.

Baber N.S., Dourish C.T., and Hill D.R. The role of CCK caerulein, and CCK antagonists in nociception. *Pain.* 1989, 39: 307-328.

Barbaresi P.Immunocytochemical localization of substance P receptor in rat periaqueductal gray matter: a light and electron microscopic study. *J Comp Neurol.* 1998, 398:473-490

Baranauskas G, Nistri A. Sensitization of pain pathways in the spinal cord: cellular mechanisms.*Prog Neurobiol.* 1998, 54:349-365.

Basbaum AI. Anatomical substrates of pain and pain modulation and their relationship to analgesic drug action. In: M. Kuhar and G. Pasternak (Eds.), *Analgesic: Neurochemical behavioral and clinical perspectives.* Raven Press, New York, pp. 1984, 97-123.

Basbaum A.I. Distinct neurochemical features of acute and persistent pain. *Proc. Natl. Acad. Sci. U S A.* 1999, 96:7739-7743.

Becker C, Hamon M, Cesselin F, and Benoliel JJ. Delta (2)-opioid receptor mediation of morphine-induced CCK release in the frontal cortex of the freely moving rat. *Synapse.* 1999, 34**:** 47-54.

Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. *Prog Neurobiol.* 1995, 46:575-605.

Beinfeld MC, Palkovits M. Distribution of cholecystokinin (CCK) in the rat lower brain stem nuclei. *Brain Res.* 1982, 238:260-265

Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain.* 1988, 33:87-107

Benoliel JJ, Bourgoin S, Mauborgne A, Legrand JC, Hamon M, and Cesselin F. Differential inhibitory/stimulatory modulation of spinal CCK release by mu and delta opioid agonists, and selective blockade of mu-dependent inhibition by kappa receptor stimulation. *Neurosci. Lett.*, 1991, 124: 204-207.

Benoliel JJ, Collin E, Mauborgne A, Bourgoin S, Legrand JC, Hamon M, and Cesselin F. Mu and delta opioid receptors mediate opposite modulations by morphine of the spinal release of cholecystokinin-like material. *Brain Res.*, 1994, 653:81-91.

Besson J-M. and Chaouch A. Peripheral and spinal mechanisms of nociception. *Physiol. Rev.* 1987, 67:67-186.

Brás JMA, Laporte AM, Benoliel JJ, Bourgoin S, Mauborgne A, Hamon M, Cesselin F and Pohl M. Effects of peripheral axotomy on cholecystokinin neurotransmission in the rat spinal cord. *J. Neurochem.* 1999,72:858-867.

Bridges D, Thompson SW, Rice AS. Mechanisms of neuropathic pain. *Br J Anaesth.* 2001, 87:12-26.

Brodin E, Lindefors N, and Ungerstedt U. Potassium evoked in vivo release of substance P in rat caudate nucleus measured using a new technique of brain dialysis and an improved substance P-radioimmunoassay. *Acta Physiol. Scand. Suppl.* 1983, 515: 17-20.

Brodin E, Lindefors N, Dalsgaard CJ. Theodorsson-Norheim E, Rosell S. Tachykinin multiplicity in rat central nervous system as studied using antisera raised against substance P and neurokinin A. *Regul Pept* 1986, 13:253-272

Brodin E, Linderoth B, Gazelius B, and Ungerstedt U. In vivo release of substance P in cat dorsal horn studied with microdialysis. *Neurosci. Lett.* 1987, **76:** 357-362.

Calcutt NA, Stiller CO, Gustafsson H, Malmberg AB. Elevated substance-P-like immunoreactivity levels in spinal dialysates during the formalin test in normal and diabetic rats. *Brain Res.* 2000, 856:20-27.

Carta F, Bianchi M, Argenton S, Cervi D, Marolla G, Tamburini M, Breda M, Fantoni A, Panerai AE. Effect of nifedipine on morphine-induced analgesia. *Anesth Analg.* 1990, 70:493-498.

Cesselin F. Opioid and anti-opioid peptides. *Fundam. Clin. Pharmacol.* 1995, 9:409-433.

Chaplan SR. Neuropathic pain: role of voltage-dependent calcium channels. *Reg. Anesth. Pain. Med.*, 2000, 25:283-285.

Christrup LL. Morphine metabolites. *Acta Anaesthesiol Scand.* 1997, 41:116-122.

Cook AJ, Woolf CJ, Wall PD, McMahon SB.Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. *Nature.* 1987, 325:151-153.

Crain SM and Shen KF. Opioids can evoke direct receptor-mediated excitatory effects on sensory neurons. *Trends Pharmacol. Sci.* 1990, 11: 77-81.

Crawley JN, Corwin RL. Biological actions of cholecystokinin. *Peptides.* 1994, 15:731-755

Cruciani RA, Dvorkin B, Morris SA, Crain SM, and Makman M.H. Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proc. Natl. Acad. Sci.USA.* 1993, 90:3019-3023.

Dalsgaard CJ, Vincent SR, Hökfelt T, Lundberg JM, Dahlström A, Schultzberg M, Dockray J and Cuello AC. Coexistence of cholecystokinin- and substance P-like peptides in neurons of the dorsal root ganglia of the rat. *Neurosci. Lett.*, 1982, 33:159-163.

Dalsgaard CJ, Haegerstrand A, Theodorsson-Norheim E, Brodin E, Hokfelt T.Neurokinin A-like immunoreactivity in rat primary sensory neurons; coexistence with substance P. *Histochemistry.* 1985, 83:37-39.

De Koninck Y, Henry JL. Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons in vivo by noxious stimulation. *Proc Natl Acad Sci U S A.* 1991, 88:11344-11348.

Dickenson AH, Sullivan AF. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. *Neurosci Lett.* 1987*a*, 83:207-211.

Dickenson AH, Sullivan AF. Subcutaneous formalin-induced activity of dorsal horn neurones in the rat: differential response to an intrathecal opiate administered pre or post formalin. *Pain.* 1987*b*, 30:349-360

Dickenson AH. Where and how do opioids act?. In: G.F. Gebhart and T.S. Jensen (Eds.), *Proceedings of the 7th World Congress on Pain*, vol. **2**, IASP press, Seattle, 1994, pp. 525-552.

Ding XZ, Fan SG, Zhou JP, Han JS. Reversal of tolerance to morphine but no potentiation of morphine-induced analgesia by antiserum against cholecystokinin octapeptide. *Neuropharmacology.* 1986, 25:1155-1160

Donnerer J, Schuligoi R, Stein C. Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. *Neuroscience.* 1992, 49:693-698

Donnerer J, Schuligoi R, Stein C, Amann R. Upregulation, release and axonal transport of substance P and calcitonin gene-related peptide in adjuvant inflammation and regulatory function of nerve growth factor. *Regul Pept.* 1993, 46:150-154.

Dougherty P.M. and Willis W.D. Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-iontophoretic application of N-methyl-D-aspartic acid and substance P. *Pain.* 1991, 47: 85-93.

Dourish CT, Hawley D, Iversen SD. Enhancement of morphine analgesia and prevention of morphine tolerance in the rat by the cholecystokinin antagonist L-364,718. *Eur J Pharmacol*. 1988, 147:469-472

Dourish CT, O'Neill MF, Coughlan J, Kitchener SJ, Hawley D., and Iversen S.D. The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur. J. Pharmacol*. 1990, 176: 35-44.

Dray A.Neurogenic mechanisms and neuropeptides in chronic pain. *Prog Brain Res* 1996, 110:85-94

Dubner R, and Bennett GJ. Spinal and trigeminal mechanisms of nociception. *Ann. Rev. Neurosci*. 1983, 6: 381-418.

Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*. 1977, 4:161-74.

Duggan AW, Morton CR, Zhao ZQ, Hendry IA. Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res* 1987, 403:345-349.

Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res.* 1988, 451:261-273.

Duggan AW. Release of neuropeptides in the spinal cord. In: Nyberg F, Sharma HS, Wiesenfeld-Hallin Z, editors. Progress in Brain Research, Vol. 104. *Elsevier.* 1995, pp. 197-223.

Fang F, Proudfit HK. Antinociception produced by microinjection of morphine in the rat periaqueductal gray is enhanced in the foot, but not the tail, by intrathecal injection of alpha1-adrenoceptor antagonists. *Brain Res*. 1998, 790:14-24

Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science.* 1983, 219:310-312.

Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS. Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. *Neuroscience.* 1995, 66:443-458

Gandhi VC, and Jones DJ. Identification and characterization of [3H]nitrendipine binding sites in rat spinal cord. *J. Pharmacol. Exp. Ther*. 1988, 247: 473-480.

Garry MG, Walton LP, Davis MA. Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from the spinal cord is mediated by nitric oxide but not by cyclic GMP. Brain Res. 2000, 861:208-219

Gazelius B, Cui JG, Svensson M, Meyerson B, Linderoth B. Photochemically induced ischaemic lesion of the rat sciatic nerve. A novel method providing high incidence of mononeuropathy. *Neuroreport. 1996*, 7:2619-2623

Ghilardi J.R., Allen C.J., Vigna S.R., McVey D.C., and Mantyh P.W. () Trigeminal and dorsal root ganglion neurons express CCK receptor binding sites in the rat, rabbit, and monkey: possible site of opiate-CCK analgesic interactions. *J. Neurosci*. 1992, 12: 4854-4866.

Go V.L. and Yaksh T.L. Release of substance P from the cat spinal cord. *J. Physiol.*, 1987, 391: 141-167.

Guo, A., Vulchanova, L. Wang J, Li X. and Elde R., Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur. J. Neurosci*. 1999,11: 946-958.

Gustafsson H, de Araujo Lucas G, Schott E, Stiller CO, Alster P, Wiesenfeld-Hallin Z, Brodin E. Peripheral axotomy influences the in vivo release of cholecystokinin in the spinal cord dorsal horn-possible involvement of cholecystokinin-B receptors. *Brain Res.* 1998, 790:141-150.

Gustafsson H, de Araujo Lucas G, Schott E, Stiller CO, Alster P, Wiesenfeld-Hallin Z, Brodin E. Measurement of cholecystokinin release in vivo in the rat spinal dorsal horn. Brain *Res Brain Res Protoc*. 1999, 4:192-200.

Hao JX, Blakeman KH, Yu W, Hultenby K, Xu XJ, Wiesenfeld-Hallin Z. Development of a mouse model of neuropathic pain following photochemically induced ischemia in the sciatic nerve. *Exp Neurol*. 2000, 163:231-8.

Hedo G, Laird JM, Lopez-Garcia JA. Time-course of spinal sensitization following carrageenan-induced inflammation in the young rat: a comparative electrophysiological and behavioural study in vitro and in vivo.Neuroscience. 1999, 92:309-318

Helke CJ, Charlton CG, Wiley RG. Studies on the cellular localization of spinal cord substance P receptors. *Neuroscience.* 1986,19:523-533.

Hill DR, Campbell NJ, Shaw TM, Woodruff GN. Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. *J Neurosci*. 1987, 7:2967-2976.

Hill D.R. and Woodruff G.N. () Differentiation of central cholecystokinin receptor binding sites using the non-peptide antagonists MK-329 and L-365,260. *Brain Res*., 1990, 526:276-283.

Hollmann M, Heinemann S.Cloned glutamate receptors.Annu Rev Neurosci. 1994, 17:31-108

Honoré P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM, Mantyh PW. Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J Neurosci* 1999, 19:7670-7678.

Hu HZ, Li ZW, Si JQ. Evidence for the existence of substance P autoreceptor in the membrane of rat dorsal root ganglion neurons.Neuroscience. 1997, 77:535-541.

Hughes J. Smith T.W., Kosterlitz H.W., Fothergill L.A., Morgan B.A., and Morris H.R., Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*, 1975,58: 577-580.

Hylden JL. and Wilcox GL. Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. *Brain Res.* 1981, 217, 212-215.

Hylden JL, Thomas DA, Iadarola MJ, Nahin RL, Dubner R. Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement ofnoradrenergic mechanisms.Eur J Pharmacol. 1991, 194:135-143.

Hökfelt T, Kellerth JO, Nilsson G, and Pernow B. Substance p: localization in the central nervous system and in some primary sensory neurons. *Science.* 1975,190: 889-890.

Hökfelt T., Zhang X, and Wiesenfeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci.* 1994; **17:**22-30.

Hökfelt T, Zhang X, Xu Z-Q, Ru-Rong J, Shi T, Corness J, Kerekes N, Landry M, Rydh-Rinder M, Broberger C, Wiesenfeld-Hallin Z, Bartfai T, Elde R, and Ju G. Cellular and synaptic mechanisms in transition of pain from acute to chronic. In: T.S. Jensen, J.A. Turner and Z. Wiesenfeld-Hallin (Eds.), *Proceedings of the 8th World Congress on Pain*, IASP Press, Seattle, pp. 1997, 133-153.

Hökfelt T, Broberger C, Xu Z-Q, Sergeyev V, Ubink R, and Diez M. (2000) Neuropeptides - an overview. *Neuropharmacology*, 2000, 39: 1337-1356.

Hökfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. *J Intern Med*. 2001, 249:27-40.

Iyengar S, Hipskind PA, Gehlert DR, Schober D, Lobb KL, Nixon JA, Helton

DR, Kallman MJ, Boucher S, Couture R, Li DL, Simmons RM. Related Articles LY303870, a centrally active neurokinin-1 antagonist with a long duration of action. *J Pharmacol Exp Ther*. 1997, 280:774-785

Jacobsson G, Piehl F, Meister B. VAMP-1 and VAMP-2 gene expression in rat spinal motoneurones: differential regulation after neuronal injury. *Eur J Neurosci*. 1998, 10:301-316.

Jeftinija S, Urban L. Repetitive stimulation induced potentiation of excitatory transmission in the rat dorsal horn: an in vitro study. J Neurophysiol. 1994, 71:216-228

Jessell TM, Iversen L.L. () Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature*. 1977, 268: 549-551.

Jin W, Lee NM, Loh HH, Thayer SA. Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma x glioma hybrid NG108-15 cells. *Mol Pharmacol*. 1992, 42:1083-1089

Jorpes E. and Mutt V. Cholecystokinin and pancreozymin, one single hormone?. *Acta Physiol. Scand*. 1966, 66: 196-202.

Ju G, Hökfelt T, Fischer JA, Frey P, Rehfeld JF, and Dockray GJ. Does cholecystokinin-like immunoreactivity in rat primary sensory neurons represent calcitonin gene-related peptide?. *Neurosci. Lett,* 1986, 68:305-310.

Kawakami M, Weinstein JN, Chatani K, Spratt KF, Meller ST, Gebhart GF. Experimental lumbar radiculopathy. Behavioral and histologic changes in a model of radicular pain after spinal nerve root irritation with chromic gut ligatures in the rat. Spine. 1994, 19:1795-802

Kayser V. and Guilbaud G. Physiological relevance and time course of a tonic endogenous opioid modulation of nociceptive messages, based on the effects of naloxone in a rat model of localized hyperalgesic inflammation. *Brain Res.,* 1991, 567:197-203.

Kerr LM, Filloux F, Olivera B.M, Jackson H, and Wamsley JK. Autoradiographic localization of calcium channels with $^{[125I]}$omega-conotoxin in rat brain. *Eur. J. Pharmacol.* 1988, 146: 181-183.

Kidd BL, Urban LA. Mechanisms of inflammatory pain.Br J Anaesth. 2001,87:3-11.

Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain. 1992, 50:355-363.

Krumins SA, Kim DC, Seybold VS, Larson AA. Modulation of [3H]DAGO binding by substance P (SP) and SP fragments in the mouse brain and spinal cord via MU1 interactions.Neuropeptides. 1989, 13:225-233.

Kupers R, Yu W, Persson JK, Xu XJ, Wiesenfeld-Hallin Z. Photochemically-induced ischemia of the rat sciatic nerve produces a dose-dependent and highly reproducible mechanical, heat and cold allodynia, and signs of spontaneous pain. Pain. 1998, 76:45-59

Larsson LI. and Rehfeld JF. Localization and molecular heterogeneity of cholecystokinin in the central and peripheral nervous system. *Brain Res*. 1979, 165:201-218.

Linderoth B. and Brodin E. Tachykinin release from rat spinal cord in vitro and in vivo in response to various stimuli. *Regul. Pept*., 1988, 21:129-140.

Liu H, Wang H, Sheng M, Jan LY, Jan YN. and Basbaum, A.I., Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn, Proc. *Natl. Acad. Sci. USA*. 1994, 91:8383-8387.

Liu H, Mantyh PW, Basbaum AI. NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature*. 1997, 386:721-724.

Liu XG, Sandkühler J. Long-term potentiation of C-fiber-evoked potentials in the rat spinal dorsal horn is prevented by spinal N-methyl-D-aspartic acid receptor blockage. *Neurosci Let*t 1995, 191:43-46.

Liu XG, Sandkühler J. Characterisation of long-term potentiation of c-fiber-evoked potentials in spinal dorsal horn of adult rat: essential role of NK1 and NK2 receptors. J Neurophysiol 1997, 78:1972-1982.

Liu XG, Sandkühler J. Activation of spinal N-methyl-D-aspartate or neurokinin receptors induces long-term potentiation of spinal C-fiber-evoked potentials. Neuroscience 1998 86:1209-1216.

Lucas GA, Alster A, Brodin E, and Wisenfeld-Hallin Z. Differential release of cholecystokinin by morphine in rat spinal cord. *Neurosci. Lett.* 1998, 244: 13-17.

Ma QP, Woolf CJ. Progressive tactile hypersensitivity: an inflammation-induced incremental increase in the excitability of the spinal cord. Pain. 1996, 67:97-106.

Magnan J, Paterson SJ, Tavani A, Kosterlitz HW. The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn Schmiedebergs Arch. Pharmacol.* 1982, 319:197-205.

Magnuson DS, Sullivan AF, Simonnet G, Roques BP, and Dickenson A.H. Differential interactions of cholecystokinin and FLFQPQRF-NH2 with mu and delta opioid antinociception in the rat spinal cord. *Neuropeptides*. 1990, 16, 213-218.

Malcangio M, Fernandes K, Tomlinson DR. NMDA receptor activation modulates evoked release of substance P from rat spinal cord. Br J Pharmacol. 1998, 125:1625-6.

Malcangio M. and Bowery S.G. Peptide autoreceptors: does an autoreceptor for substance P exist?. *Trends Pharmacol. Sci.,* 1999, 20: 405-407.

Malick JB, Goldstein JM. Analgesic activity of substance P following intracerebral administration in rats. *Life Sci.* 1978, 23:835-44

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. Anatomy of CNS opioid receptors. Trends Neurosci. 1988, 11:308-314

Marvisón JC, Martinez V, Grady EF, Bunnett NW, Mayer EA. Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. J Neurosci 1997, 17:8129-8136.

Martin WR, Eades CG, Thompson JA, Huppler R.E., and Gilbert P.E. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther*. 1976, 197: 517-532.

Martin TJ, Eisenach JC.Pharmacology of opioid and nonopioid analgesics in chronic pain states.J Pharmacol Exp Ther. 2001,299:811-817.

McQuay HJ, Tramer M, Nye BA., Carroll D, Wiffen P.J, and Moore R.A. A systematic review of antidepressants in neuropathic pain. *Pain*, 1996, 68:217-227.

McCarson KE, Krause JE. NK-1 and NK-3 type tachykinin receptor mRNA expression in the rat spinal cord dorsal horn is increased during adjuvant or formalin-induced nociception. J Neurosci 1994, 14:712-720.

Mendell LM.Physiological properties of unmyelinated fiber projection to the spinal cord. Exp Neurol. 1966, 16:316-32.

Meller ST and Gebhart GF. Nitric oxide (NO) and nociceptive processing in the spinal cord, Pain, 1993, 52:127-136

Merighi A., Polak J.M., and Theodosis D.T. () Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. *Neuroscience*, 1991, 40, 67-80.

Merskey H and Bogduk N. IASP Task Force on Taxonomy. In: H. Merskey and N. Bogduk (Eds.), *Classification of Chronic Pain,* IASP Press, Seattle, pp. 1994, 209-214.

Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B, Mazarguil H, Vassart G, Parmentier M, and Costentin J.

Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature. 1995, 377:532-535.
Meunier JC.Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. J Pharmacol 1997, 340:1-15
Millan MJ, Czlonkowski A, Morris B, Stein C, Arendt R, Huber A, Hollt V, Herz A. Inflammation of the hind limb as a model of unilateral, localized pain: influence on multiple opioid systems in the spinal cord of the rat. *Pain.* 1988, 35:299-312
Millan MJ. The induction of pain: an integrative review. *Prog Neurobiol.* 1999, 57:1-164.
Mills CD, Johnson KM, Hulsebosch CE. Group I metabotropic glutamate receptors in spinal cord injury: roles in neuroprotection and the development of chronic central pain. *J Neurotrauma.* 2002, 19:23-42.
Minami M, Kuraishi Y, Kawamura M, Yamaguchi T, Masu Y, Nakanishi S, Satoh M. Enhancement of preprotachykinin A gene expression by adjuvant-induced inflammation in the rat spinal cord: possible involvement of substance P-containing spinal neurons in nociception. *Neurosci Lett.* 1989, 98:105-110
Monaghan DT, Bridges RJ, Cotman CW. The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu Rev Pharmacol Toxicol.* 1989, 29:365-402.
Moran TH, Robinson PH, Goldrich MS, and McHugh PR. Two brain cholecystokinin receptors: implications for behavioral actions. *Brain Res.* 1986, 362: 175-179.
Mulder AH, Schoffelmeer AN, and Stoof JC. On the role of adenylate cyclase in presynaptic modulation of neurotransmitter release mediated by monoamine and opioid receptors in the brain. *Ann. N. Y. Acad. Sci.* 1990, 604: 237-249.
Murase K, Randic M. Actions of substance P on rat spinal dorsal horn neurones. *J Physiol* 1984, 346:203-217.
Mutt V. and Jorpes J.E. Structure of porcine cholecystokinin-pancreozymin. 1. Cleavage with thrombin and with trypsin. *Eur. J. Biochem.*, 1968, 6:156-162.
Nanayama T, Kuraishi Y, Ohno H, Satoh M. Capsaicin-induced release of calcitonin gene-related peptide from dorsal horn slices is enhanced in adjuvant arthritic rats. *Neurosci Res.* 1989, 6:569-572.
Nichols ML, Bian D, Ossipov MH, Lai J and Porreca F. Regulation of morphine antiallodynic efficacy by cholecystokinin in a model of neuropathic pain in rats. *J. Pharmacol. Exp. Ther.* 1995, 275:1339-1345.
O'Callaghan JP. Evolution of a rational use of opioids in chronic pain. *Eur J Pain.* 2001, 5 Suppl A:21-26.
Odeh F, Antal M. The projections of the midbrain periaqueductal grey to the pons and medulla oblongata in rats. *Eur J Neurosci.* 2001, 14:1275-1286.
Ohsawa M, Mizoguchi H, Narita M, Chu M, Nagase H, Tseng LF. Differential mechanisms mediating descending pain controls for antinociception induced by supraspinally administered endomorphin-1 and endomorphin-2 in the mouse. *J Pharmacol Exp Ther.* 2000, 294:1106-1111
Okano K, Kuraishi Y, Satoh M. Involvement of spinal substance P and excitatory amino acids in inflammatory hyperalgesia in rats. *Jpn J Pharmacol.* 1998, 76:15-22.
Omote K, Kawamata M, Satoh O, Iwasaki H, and Namiki A. Spinal antinociceptive action of an N-Type voltage-dependent calcium channel blocker and the synergistic interaction with morphine. *Anesthesiol.* 1996, 84: 636-643.
Ossipov MH, Lai J, Malan TP Jr, Porreca F. Spinal and supraspinal mechanisms of neuropathic pain. *Ann N Y Acad Sci.* 2000, 909: 12-24.
Otsuka M, Konishi S. Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. Nature. 1976, 264:83-84

Petralia RS, Yokotani N and Wenthold, RJ. Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody, *J. Neurosci.* 1994, 14:667-696

Price D.D. and Dubner R. Neurons that subserve the sensory-discriminative aspects of pain. *Pain*, 1977, 3:307-338.

Przewlocki R, Labuz D, Mika J, Przewlocka B, Tomboly C, Toth G.Pain inhibition by endomorphins. *Ann N Y Acad Sci.* 1999, 897:154-164

Rang HP, Bevan S, and Dray A. Chemical activation of nociceptive peripheral neurones. *Br. Med. Bull.* 1991, 47: 534-548.

Rehfeld JF and Hansen HF. Cholecystokinin and gastrin peptides in the nervous system: post-translational processing of the precursors. In: J. De Belleroche and GJ. Dockray GJ. (Eds.), Cholecystokinin (CCK) in the nervous system, Ellis Horwood, Chichester, 1978, pp. 31-40.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ Jr, Civelli O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science.* 1995, 270:792-794.

Ren K, Hylden JL, Williams GM, Ruda MA, Dubner R. The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal horn neuronal activity in rats with unilateral inflammation. Pain. 1992*a*, 50:331-344.

Ren K, Williams GM, Hylden JL, Ruda MA, Dubner R. The intrathecal administration of excitatory amino acid receptor antagonists selectively attenuated carrageenan-induced behavioural hyperalgesia in rats. *Eur J Pharmacol.* 1992*b*, 219:235-243.

Rodriguez RE. and Sacristan MP. In vivo release of CCK-8 from the dorsal horn of the rat: inhibition by DAGOL. *FEBS Lett.* 1989, 250:215-217.

Russo CM, Brose WG.Chronic pain. *Annu Rev Med.* 1998, 49:123-133.

Rygh LJ, Svendsen F, Hole K, Tjolsen A. Increased spinal N-methyl-D-aspartate receptor function after 20 h of carrageenan-induced inflammation. *Pain.* 2001, 93:15-21.

Sandkühler J, Liu X. Induction of long-term potentiation at spinal synapses by noxious stimulation or nerve injury. *Eur J Neurosci* 1998, 10:2476-2480.

Sandkühler. Learning and memory in pain pathways. *Pain.* 2000, 88:113-118.

Santillan R, Hurle MA, Armijo JA, de los Mozos R, Florez J. Nimodipine-enhanced opiate analgesia in cancer patients requiring morphine dose escalation: a double-blind, placebo-controlled study. *Pain.* 1998, 76:17-26

Sasaki M, Tohda C, Kuraishi Y. Region-specific increase in glutamate release from dorsal horn of rats with adjuvant inflammation. *Neuroreport.* 1998, 9:3219-3222.

Schäfer MK, Nohr D, Krause JE, Weihe E. Inflammation-induced upregulation of NK1 receptor mRNA in dorsal horn neurones. NeuroReport 1993, 4:1007-1010.

Schiffmann S.N. and Vanderhaeghen J.J. Distribution of cells containing mRNA encoding cholecystokinin in the rat central nervous system. *J. Comp. Neurol.*, 1991, 304:219-233.

Schmidt RF, Schaible HG, Silent and Active nociceptors: Structure, Functions and Clinical implications. In proceeding of the 7th word congress on pain, Jensen GH, *IASP Press* 1994, 213-250

Seltzer Z, Dubner R, Shir Y. A novel behavioural model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain. 1990, 43:205-218.

Seroogy KB, Mohapatra NK, Lund PK, Rethelyi M, McGehee DS and Perl ER. Species-specific expression of cholecystokinin messenger RNA in rodent dorsal root ganglia. *Mol. Brain Res.* 1990, 7:171-176.

Sindrup SH, Jensen TS. Pharmacologic treatment of pain in polyneuropathy. *Neurology.* 2000, 55:915-20.

Skilling SR, Harkness DH, Larson AA. Experimental peripheral neuropathy decreases the dose of substance P required to increase excitatory amino acid release in the CSF of the rat spinal cord. *Neurosci Lett.* 1992, 139:92-96

Skirboll L, Hokfelt T, Rehfeld J, Cuello AC, Dockray G. Coexistence of substance P- and cholecystokinin-like immunoreactivity in neurons of the mesencephalic periaqueductal central gray. *Neurosci Lett.* 1982, 28:35-39.

Skirboll L, Hokfelt T, Dockray G, Rehfeld J, Brownstein M, Cuello AC. Evidence for periaqueductal cholecystokinin-substance P neurons projecting to the spinal cord. *J Neurosci.* 1983, 3:1151-1157

Smith S.J. and Augustine G.J. Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci.* 1988, 11:458-464.

Smith GD, Harmar AJ, McQueen DS, Seckl JR. Increase in substance P and CGRP, but not somatostatin content of innervating dorsal root ganglia in adjuvant monoarthritis in the rat. *Neurosci Lett* 1992, 137:257-260.

Smullin DH, Skilling SR, and Larson AA. Interactions between substance P, calcitonin gene-related peptide, taurine and excitatory amino acids in the spinal cord. *Pain.* 1990, 42: 93-101.

Snijdelaar DG, Dirksen R, Slappendel R, Crul BJ. Substance P. *Eur J Pain.* 2000, 4:121-135.

Snutch T.P. and Reiner P.B. Ca2+ channels: diversity of form and function. *Curr. Opin. Neurobiol.* 1992, **2**:247-253.

Southall MD, Michael RL, Vasko MR. Intrathecal NSAIDS attenuate inflammation-induced neuropeptide release from rat spinal cord slices. *Pain.* 1998, 78:39-48.

Sorkin LS, McAdoo DJ. Amino acids and serotonin are released into the lumbar spinal cord of the anesthetized cat following intradermal capsaicin injections. *Brain Res.* 1993, 607: 89-98.

Stanfa L, Dickenson A, Xu XJ, and Wiesenfeld-Hallin Z. Cholecystokinin and morphine analgesia: variations on a theme. *Trends Pharmacol. Sci.* 1994, 15: 65-66.

Stengaard-Pedersen K, Larsson LI. Localization and opiate receptor binding of enkephalin, CCK and ACTH/beta-endorphin in the rat central nervous system. *Peptides.* 1981, 2 Suppl 1:3-19.

Stiller C-O, Bergquist J, Beck O, Ekman R, Brodin E. Local administration of morphine decreases the extracellular level of GABA in the periaqueductal gray matter of freely moving rats. *Neurosci Lett.* 1996, 209:165-168

Stiller C-O, Gustafsson H, Fried K, Brodin E. Opioid-induced release of neurotensin in the periaqueductal gray matter of freely moving rats. *Brain Res.*, 1997, 774:149-158.

Svendsen F, Tjølsen A, Hole K. LTP of spinal A beta and C-fiber evoked responses after electrical sciatic nerve stimulation. *NeuroReport.* 1997, 8:3427-3430.

Svendsen F, Tjølsen A, Hole K. AMPA and NMDA receptor-dependent spinal LTP after nociceptive tetanic stimulation. *NeuroReport.* 1998, 9:1185-1190.

Svendsen F, Tjølsen A, Rygh LJ, Hole K. Expression of long-term potentiation in single wide dynamic range neurons in the rat is sensitive to blockade of glutamate receptors. *Neurosci Lett.* 1999, 259:25-28.

Tang J, Chou J, Yang HY, Costa E. Substance P stimulates the release of Met5-enkephalin-Arg6-Phe7 and Met5-enkephalin from rat spinal cord. *Neuropharmacology.* 1983, 22:1147-50.

Takahashi T, Otsuka M.Regional distribution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section.Brain Res. 1975, 87:1-11.

Takemori AE, and Portoghese PS. Evidence for the interaction of morphine with kappa and delta opioid receptors to induce analgesia in beta-funaltrexamine-treated mice. *J. Pharmacol. Exp. Ther.* 1987, 243: 91-94.

Tohda C, Sasaki M, Konemura T, Sasamura T, Itoh M, Kuraishi Y. Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. *J Neurochem.* 2001, 76:1628-1635.

Taylor BK. Pathophysiologic mechanisms of neuropathic pain. *Curr Pain Headache Rep.* 2001, 5:151-161

Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. *Pain.* 1992, 51:5-17

Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. Pain. 1996, 67:151-161

Tsien R.W., Ellinor P.T., and Horne W.A. Molecular diversity of voltage-dependent Ca2+ channels. *Trends Pharmacol. Sci.* 1991, 12:349-354.

Urban L, Thompson SW, Dray A.Modulation of spinal excitability: co-operation between neurokinin and excitatory amino acid neurotransmitters. *Trends Neurosci.* 1994, 17:432-438.

Urban MO, Gebhart GF. Characterization of biphasic modulation of spinal nociceptive transmission by neurotensin in the rat rostral ventromedial medulla. *J Neurophysiol.* 1997, 78:1550-1562

Vanderhaeghen JJ, Signeau JC, Gepts W. New peptide in the vertebrate CNS reacting with antigastrin antibodies. *Nature.* 1975, 257: 604-605.

Verge VMK, Wiesenfeld-Hallin Z, Hökfelt T. Cholecystokinin in mammalian primary sensory neurons and spinal cord: in situ hybridizaton studies in rat and monkey, *Eur. J. Neurosci.* 1993, 5: 240-250.

Wall PD, Devor M, Inbal RAU, Scadding JW, Schonfeld D, Seltzer Z. Tomkiewicz M.M. Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. *Pain.* 1979, 7:103-111.

Wang J, Ren M, Han J. Mobilization of calcium from intracellular stores as one of the mechanisms underlying the antiopioid effect of cholecystokinin octapeptide. *Peptides.* 1992, 13: 947-951.

Watkins LR, Kinscheck IB, Mayer DJ. Potentiation of opiate analgesia and apparent reversal of morphine tolerance by proglumide, *Science*, 1984, 224:395-396.

Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Catterall WA. Biochemical properties and subcellular distribution of an N-type calcium channel alpha 1 subunit. *Neuron.* 1992, 9:1099-1115

Wiesenfeld-Hallin Z, Hokfelt T, Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA, Fischer JA. Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neurosci Lett. 1984, 52:199-204

Wiesenfeld-Hallin Z, Xu XJ, Hughes J, Horwell DC, Hökfelt T. PD134308, a selective antagonist of cholecystokinin type B receptor, enhances the analgesic effect of morphine and synergistically interacts with intrathecal galanin to depress spinal nociceptive reflexes. *Proc. Natl. Acad. Sci. USA.*, 1990, 87: 7105-7109.

Wiesenfeld-Hallin Z, Xu XJ. The role of cholecystokinin in nociception, neuropathic pain and opiate tolerance. *Regul. Pept,* 1996, 65: 23-28.

Wiesenfeld-Hallin Z, de Arauja Lucas G, Alster P, Xu XJ, and Hökfelt T. Cholecystokinin/opioid interactions. *Brain Res.,* 1999, 848: 78-89.

Wiesenfeld-Hallin Z, Xu XJ. Neuropeptides in neuropathic and inflammatory pain with special emphasis on cholecystokinin and galanin. *Eur J Pharmacol.* 2001, 429:49-59

Willis WD Westlund KN. Neuroanatomy of the pain system and of the pathways that modulate pain. *J. Clin. Neurophysiol.,* 1997, 14:2-31.

Winter CA, Risley EA, Carrgeenan-induced.edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc Soc Exp Biol Med* 1962, 111: 544-547

White DM. Mechanism of prostaglandin E2-induced substance P release from cultured sensory neurons. *Neuroscience.* 1996, 70:561-565.

Woolf CJ, Doubell TP. The pathophysiology of chronic pain--increased sensitivity to low threshold A beta-fibre inputs. *Curr Opin Neurobiol.* 1994, 4:525-34

Wu J, Fang L, Lin Q, Willis WD. Nitric oxide synthase in spinal cord central sensitization following intradermal injection of capsaicin. Pain. 2001, 94:47-58.

Xu X-J, Puke MJC, Verge VMK, Wiesenfeld-Hallin Z, Hughes J, Hökfelt T. () Up-regulation of cholecystokinin in primary sensory neurons is associated with morphine insensitivity in experimental neuropathic pain in the rat. *Neurosci. Lett.* 1993, 152:129-132.

Xu XJ, Hao JX, Seiger A, Hughes J, Hokfelt T, Wiesenfeld-Hallin Z.Chronic pain-related behaviors in spinally injured rats: evidence for functional alterations of the endogenous cholecystokinin and opioid systems. *Pain.* 1994, 56:271-277

Yaksh TL, Jessell TM, Gamse R, Mudge AW, Leeman SE. Intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo. *Nature*, 1980, 286: 155-157.

Yaksh TL, Noueihed R.The physiology and pharmacology of spinal opiates. Annu Rev Pharmacol Toxicol. 1985, 25:433-462.

Yaksh TL. Opioid receptor systems and the endorphins: a review of their spinal organization. J Neurosurg. 1987, 67:157-176

Yaksh TL. Substance P release from knee joint afferent terminals: modulation by opioids.Brain Res 1988;458:319-324.

Yaksh TL, Malmberg AB. Central pharmacology of nociceptive transmission. In: P.D. Wall and R. Melzack (Eds.), *Textbook of Pain*, Churchill Livingstone, Edinburgh, 1994, pp. 165-200.

Yashpal K, Henry JL. Endorphins mediate overshoot of substance P-induced facilitation of a spinal nociceptive reflex. *Can J Physiol Pharmacol.* 1983 61:303-307

Yashpal K, Radhakrishnan V, Coderre TJ, Henry JL.CP-96,345, but not its stereoisomer, CP-96,344, blocks the nociceptive responses to intrathecally administered substance P and to noxious thermal and chemical stimuli in the rat. *Neuroscience.* 1993, 52:1039-1047

You ZB, Herrera-Marschitz M, Brodin E, Meana JJ, Morini P, Hökfelt T, Silveira R, Goiny M, Ungerstedt U. On the origin of striatal cholecystokinin release: studies with in vivo microdialysis. *J. Neurochem.* 1994, 62:76-85.

Zadina JE, Hackler L, Ge LJ, and Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature.* 1997, 386:499-502.

Zhang X., Dagerlind Å, Elde RP, Castel M-N, Broberger C, Wiesenfeld-Hallin Z, and Hökfelt T. Marked increase in cholecystokinin B receptor messenger RNA levels in rat dorsal root ganglia after peripheral axotomy. *Neuroscience.* 1993; 57:337-233.

Zhang X, Bao L, Arvidsson U, Elde R, and Hökfelt T. Localization and regulation of the delta-opioid receptor in dorsal root ganglia and spinal cord of the rat and monkey: evidence for association with the membrane of large dense-core vesicles. *Neuroscience*, 1998, 82:1225-1242.

Zhou Y, Sun YH, Zhang ZW, and Han JS. Increased release of immunoreactive cholecystokinin octapeptide by morphine and potentiation of mu-opioid analgesia by CCKB receptor antagonist L-365.260 in rat spinal cord. *Eur. J. Pharmacol.* 1993, 234:147-154.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain,* 1983, 16:109-110.