Neuroinflammation and pain

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To My Family

Abstract

Background

Persistent pain that remains long after the physiological trigger has been resolved is a disabling condition. A possible mechanism for the transition from acute physiological pain to persistent pain involves low-grade inflammation in the central nervous system, in which inflammatory-activated astrocytes play a significant role.

Aims

The aims of this thesis were to explore novel means for the restoration of inflammatory-activated astrocytes and to investigate whether such experimentally obtained findings, when translated into a clinical setting, are associated with improved pain relief in patients with persistent pain.

Methods

For the experimental studies in cell cultures, Ca^{2+} imaging, Western blot analysis, immunocytochemistry, and enzyme-linked immunosorbent assay (ELISA) were performed. In the clinical study, patients were treated with continuous intrathecal infusions of morphine in combination with naloxone or a placebo.

Results

Inflammatory-activated astrocytes were restored to their normal state and function using a combination of the μ-opioid agonist endomorphin-1, ultralow doses of naloxone, and the antiepileptic drug levetiracetam. For patients with persistent pain who were treated with an ongoing intrathecal morphine infusion, the addition of an ultralow dose of naloxone significantly improved their perceived quality of sleep.

Conclusion

We demonstrated that astrocyte dysfunction, which occurs as a component of low-grade neuroinflammation during prolonged pain states, is experimentally restorable by the combined actions of morphine, naloxone, and levetiracetam. To achieve this response, the choice of an ultralow dose of naloxone seems to be particularly crucial. Additionally, our findings in patients with difficult-to-treat pain show that intrathecal administration of an ultralow dose of naloxone in combination with morphine significantly improves perceived quality of sleep, although concurrent alterations in pain relief were not statistically significant. The concept of targeting inflammatory-activated astrocytes to reduce the development of persistent pain is a promising path that merits further evaluation in clinical settings.

Keywords

Persistent pain, neuroinflammation, astrocytes, morphine, naloxone, intrathecal administration

List of papers

This thesis is based on the following studies, which are referred to in the text by their Roman numerals.

I Forshammar J, Block L, Lundborg C, Biber B, Hansson E (2011) Naloxone and ouabain in ultralow concentrations restore Na^+/K^+-ATPase and cytoskeleton in lipopolysaccharide-treated astrocytes. *J Biol Chem 286:31586–31597.*

II Block L, Forshammar J, Westerlund A, Björklund U, Lundborg C, Biber B, Hansson E (2012) Naloxone in ultralow concentration restores endomorphin-1-evoked Ca^{2+} signaling in lipopolysaccharide pretreated astrocytes. *Neuroscience 205:1–9.*

III Block L, Björklund U, Westerlund A, Jörneberg P, Biber B, Hansson E (2013) A new concept affecting restoration of inflammation-reactive astrocytes. *Neuroscience 250:536–545.*

IV Block L, Lundborg C, Bjersing J, Dahm P, Hansson E, Biber B. Ultralow dose of naloxone as an adjuvant to intrathecal morphine infusion improves perceived quality of sleep in patients with severe, persistent pain. A randomized, double-blind, placebo-controlled study. *Submitted.*

Table of content

Abbrevations and explanations

Analgesia	absence of pain in response to stimulation that would normally be painful
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium ion
Central sensitisation	increased responsiveness of nociceptive neurons in the central nervous system to their normal or sub-threshold afferent input
Chronic pain	same as long-term pain
CNS	central nervous system
Disinhibition	down-regulation of pain-inhibiting descending neurons
ELISA	enzyme-linked immunosorbent assay
EM-1	endomorphin-1
GFAP	glial fibrillary acidic protein
GDNF	glial-derived neurotrophic factor
Hyperalgesia	increased pain from a stimulus that normally provokes pain
Hyperesthesia	increased sensitivity to stimulation
Hypoesthesia	decreased sensitivity to stimulation
IASP	international association for the study of pain
IL	interleukin
IL-1β	interleukin 1-beta
IL-8	interleukin 8
IL-10	interleukin 10
IP_3	inositol (1,4,5) triphosphate
IT	intrathecal

Long-term pain	pain without an apparent biological purpose that has persisted beyond the normal tissue healing period
Long-term potentiation	a wind-up phenomenon, that is maintained for extended time (pain memory)
LPS	lipopolysaccharide
Moderate pain	4–6 on the NRS
Neuronal plasticity	neuronal capacity to change neural pain transmission in response to a strong sensory stimulus, may induce a prolonged duration of pain
Neuropathic pain	pain caused by a lesion or disease of the somatosensory nervous system
NO	nitric oxide
Nociception	the neural process of encoding noxious stimuli
NRS	numeric rating scale
Pain	an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage
Persistent pain	similar to long-term pain, usually describes pain that remains for 3 months or more after an injury
PNS	peripheral nervous system
Severe pain	7–10 on the NRS
SF-36	short form-36
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
VAS	visual analogue scale
Wind up	a repeated, constant, peripheral, painful stimulus that induces an increased nociceptive response in the secondary neurons

Populärvetenskaplig sammanfattning på svenska *Summary in Swedish*

Akut smärta som svar på skada eller potentiell skada är en fysiologisk reaktion, vars syfte är att få oss att undvika skadliga stimuli. Smärta som övergår från akut smärta vid skadetillfället, till en långvarig smärta som kvarstår långt efter att skadan har läkt ut, är ett betydande folkhälsoproblem. Långvarig smärta medför förutom individuellt lidande, även sociala och ekonomiska negativa konsekvenser, för såväl individen som samhället (Breivik et al., 2006, SBU 2006). Långvarig smärta är sådan som kvarstår i 3 månader eller mer efter skadetillfället. Långvarig svår eller medelsvår smärta förekommer hos cirka 20 % av patienter som genomgått planerad kirurgi av blandad karaktär (Johansen et al., 2012). Förekomsten av långvarig smärta efter kirurgi varierar med ingreppets art, och har visats förekomma hos 50 % av de som genomgått en amputation, 30 % av de som genomgått en bröstoperation och hos 10 % av de som genomgått en ljumskbråcksoperation (Aasvang och Kehlet, 2005; Kehlet et al., 2006).

Möjliga bakomliggande mekanismer för övergången från akut till långvarig smärta har i modern litteratur framhållits inkludera persisterande låggradig inflammation i centrala nervsystemet (Vallejo et al., 2010; Tenorio et al., 2013). Vid kirurgi eller trauma aktiveras kroppens inflammatoriska kaskad och då aktiveras även mikroglia och astrocyter som är celler som reglerar och modifierar nervcellernas signalering. Astrocyter och mikroglia underhåller och driver på inflammationen i centrala nervsystemet. Aktiverade astrocyter exciterar smärtsignalerande nervceller. Hos vissa patienter kvarstår astrocyternas aktivering, och därmed en överkänslighet i nervcellerna, under lång tid.

Vår hypotes går ut på att återställa de överaktiva, inflammatoriskt reaktiva astrocyterna och därmed minska den låggradiga inflammationen i nervsystemet. Då bör även de överkänsliga, smärtsignalerande nervcellerna återgå i riktning mot ett normalläge.

De experimentella fynden i arbete I, II och III pekar på att flera olika exogena substanser har en normaliserande effekt på inflammatoriskt aktiverade astrocyter. Av de substanser vi har utvärderat fann vi att astrocyterna kan återställas bäst med en kombination av morfin, ultralåg dos av naloxone och levetiracetam. En kombination av dessa läkemedel i lämpliga doser kan potentiellt vara värdefull för att förebygga kvarstående låggradig inflammation i centrala nervsystemet.

I den kliniska studien (arbete IV) har patienter med långvarig smärta och morfininfusion direkt in i centrala nervsystemet fått en tilläggsbehandling med naloxone i två olika koncentrationer. Studien omfattade 11 patienter och pågick sammanlagt 9 veckor. Tilläggsbehandling med ultralåg dos naloxone var associerad med signifikant förbättrad upplevd sömnkvalitet, dock sågs ingen statistiskt signifikant förbättring av smärtlindring.

Introduction

The clinical problem

Acute pain in response to injury is an important mechanism that serves to protect living beings from harm. Signals conveyed from the site of injury to the brain in states of acute pain promote avoidance of harmful, noxious stimuli. However, persistent pain that is sustained for a long time after an injury has healed serves no useful purpose and is a disabling condition (Merskey and Bogduk, 1994; Macrae, 2001). Persistent pain predominantly starts with acute pain, for example, postoperative pain or trauma (Kehlet et al., 2006). Recent studies concerning persistent postoperative pain confirm that it is a significant clinical problem (Lavand'homme, 2011; Ravindran, 2014; Reddi and Curran, 2014). A study in 2043 patients (Johansen et al., 2012) demonstrated that approximately 20 % of patients who underwent an elective, mixed type of surgery suffered from moderate to severe persistent post-surgical pain, which is defined as pain that remains for 3 months or more after surgery. Moderate to severe pain is represented by a 4–10 on the Numeric Rating Scale (NRS), an eleven-step scale in which 0 represents no pain and 10 represents worst imaginable pain. Severe persistent pain is devastating for individuals suffering from it, and it causes substantial health impairment and significant difficulties -socially, financially, and in work-life (Breivik et al., 2006). Few patients with this condition manage to obtain or keep a job (Patel et al., 2012; Leadley et al., 2012). For society, this is a large burden and results in the use of extensive resources for sick leave, disability retirement, and rehabilitation. The estimated cost for persistent pain, in general, is 80 billion SEK annually in Sweden (SBU, 2006).

Among the different types of long-term pain, neuropathic pain stands out as an especially difficult type of pain to treat (Wallace 2005; Hurley et al., 2013). Neuropathic pain is characterised by damage or dysfunction in the sensory nervous system (Treede et al., 2008, Doth et al., 2010). It is recognised by pain distributed and localised along the affected nerve, and it is always accompanied by a sensory disturbance, such as hyperesthesia or hypoesthesia, in the affected area. The International Association of Pain describes neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system'' (www.iasp-pain.org).

The prevalence of persistent postsurgical pain, varies with the type of surgery, ranging from 50 % for limb amputation, 30 % for breast surgery, and 10 % for hernia repair (Aasvang and Kehlet, 2005, Kehlet at al., 2006). Persistent postsurgical pain is strongly associated with neuropathic pain (Martinez et al., 2012; Dualé et al., 2014). Depending on the type of surgery, neuropathic pain is experienced by 3 % (laparoscopic surgery) to 68 % (breast surgery) of patients with persistent postsurgical pain (Haroutianen et al., 2013).

Underlying mechanisms of persistent pain

Possible mechanisms of the transition from acute physiological pain to persistent pain that remains after the acute injury has healed include inflammation in the peripheral (PNS) and central nervous system (CNS) (Hansson, 2010; Calvo et al., 2012; Ellis and Bennett, 2013). Other possible mechanisms for persistent pain include long-term potentiation, central sensitisation, neuronal plasticity, and disinhibition (Woolf and Salter, 2000; Katz and Seltzer, 2009; Basbaum et al., 2009; Woolf, 2011; Taves et al., 2013). These entities, which all concur with our theory, are not the main focus of this thesis and therefore, will not be further discussed.

It is well known that glial cells are non-excitable neural cells that are active in the development of neuroinflammation (De Leo et al., 2004; Milligan and Watkins, 2009; Skaper et al., 2014). In the last few years, it has become clear that glial cells have important metabolic and immune functions (Scholz and Wolf, 2007; Vallejo et al., 2010; Lyman et al., 2014) and may play an important role in the modulation of synaptic pain transmission (Watkins and Maier, 2003; Suter et al., 2007; Gosselin et al., 2010; Grace et al., 2014). Astrocytes and microglia are glial cells that surround, support, and interact with neurons in the CNS. They respond to inflammatory stimuli and may play an important role in modulating the inflammatory activity in the CNS, observed after a peripheral injury (Hansson and Rönnbäck, 2003; Ren and Dubner, 2010; Skaper et al., 2012). Astrocytes are coupled in networks and communicate with each other and with neurons (Blomstrand et al., 1999; Haydon and Carmignoto, 2006), thereby modulating neuronal activity (Araque et al., 1999). Inflammation causes dysfunction in glial-neuron communication due to inflammatory-induced alterations in astrocyte function that disturb the two-way interaction between astrocytes and neurons. This disturbance results in increased excitability in neurons, and synaptic pain transmission is enhanced and prolonged (Guo et al., 2007; Gao and Ji, 2010, 2010; Chiang, 2012).

Pain and neuroinflammation

Inflammation is a physiological response to injury that is designed to remove dangerous stimuli, kill bacteria, remove cell debris, and initiate healing. However, when the inflammatory reaction persists or is exaggerated, it causes undesired negative effects. When an injury occurs in peripheral tissue, pro-inflammatory mediators, such as nitric oxide (NO), bradykinin, tissue factors, and prostaglandins, are released into the bloodstream, and white blood cells are attracted to the injury site (Mölne and Wold, 2007). The endothelium that lines the blood vessels becomes permeable, and leucocytes can migrate from the blood vessels to the injury site (Medzithov, 2008). A peripheral inflammatory process can also induce inflammation in the CNS, known as neuroinflammation (de Vries et al., 1996; Beggs et al., 2010; Echeverry et al., 2011). When an inflammatory response is activated throughout the body,

pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), are released from the inflammatory-activated leucocytes. These cytokines cause the blood-brain barrier (BBB) to become permeable, allowing leucocytes to migrate through and transform into microglia in the CNS (Huber et al., 2001; Moalem et al., 2004; Abbott et al., 2006; Sharma and Johansen, 2007; Terrando et al., 2011). The activated microglia produce more pro-inflammatory cytokines, such as IL-1β and TNF-α. IL-1β in turn activates astrocytes which also release the pro-inflammatory cytokine IL-1 β. This combined response causes a change in the astrocyte network signalling, potentiating neuronal pain transmission (Shao and McCarthy, 1994; Bruce-Keller, 1999; Schäfers and Sorkin, 2008). Furthermore, this reaction is associated with the development of new synapses and dysfunction of existing synapses (De Leo et al., 2006; Chiang et al., 2012; Lyman et al., 2014.)

Neuroinflammation can also be initiated when a local peripheral injury gives rise to inflammatory activation in the CNS, which is conveyed by neurogenic sites of action (Vasudeva et al., 2014). Immediately after an injury to a nerve ending in the periphery, the inflammatory cascade is activated and immunocompetent cells migrate to the site of injury. Macrophages infiltrate the injured nerve and cause an inflammatory reaction in the nerve cell (Scholz and Wolf, 2007). This reaction leads to microglia activation in the CNS and the release of pro-inflammatory cytokines that activate and alter astrocyte function (Vallejo et al., 2010; Calvo and Bennett, 2012; Ji et al., 2013). It has been demonstrated in a rodent model of pain that after a peripheral nerve injury, macrophages invade the end of the injured axon and cause low-grade inflammation (Saade and Jabbur, 2008), along the pain pathway from the periphery to the spinal cord, extending up to the thalamus and further on to the parietal cortex. Once the astrocytes and microglia have become activated, they participate in the development, spread, and potentiation of neuroinflammation (DeLeo et al., 2004; Milligan and Watkins, 2009).

Immune cells are closely associated with the sensory neural system, and neuroinflammation causes pain (Marchand et al., 2005; Scholz and Woolf 2007; Calvo et al., 2012). Neuroinflammation can give rise to neuropathic pain, which is the chronic pain state caused by significant pathological changes in the nervous system (Moalem and Tracey, 2006; Myers et al., 2006; McMahon and Malcangio, 2009; Vallejo et al., 2010; Tenorio et al., 2013).

Astrocytes and their role in inflammation modulation

The CNS consists of neurons and glial cells, which constitute 70 % of the cells in the CNS (Vallejo et al., 2010). The two types of glial cells of interest that are involved in the development of persistent pain are microglia and astrocytes (Grace et al., 2014; Franke and Illes 2014). Additionally, dorsal root ganglion satellite cells, astrocyte-like cells in the PNS, may play a role in the development of neuropathic pain (Ji et al., 2013). However, they will not be further discussed in this thesis. Astrocytes (astro; star, cyte; cell) are the most abundant cell type in the CNS. They are star-shaped cells with long slender processes. Astrocytes are coupled by gap junctions in syncytial networks and occupy a strategic position between the vasculature and the neurons, where they monitor and modify neuronal activity and transmitter release (Giaume and McCarthy, 1996; Araque et al 1999; Abbott et al., 2006; Hansson, 2010). Astrocytes can release a rich variety of neuroactive substances, and they also express receptors for these substances. They surround neural synapses with their end-feet and monitor and modulate synaptic activity (figure 1). One astrocyte can make contact with approximately 100,000 synapses (Bushong et al., 2002; Oberheim et al., 2012), making their impact on synaptic transmission substantial.

Astrocytes communicate within their network by utilising Ca^{2+} waves (Cornell-Bell et al., 1990). Normally, Ca^{2+} waves travel from one cell to the next via gap junctions (Blomstrand et al., 1999). Gap junctions are channels consisting of the

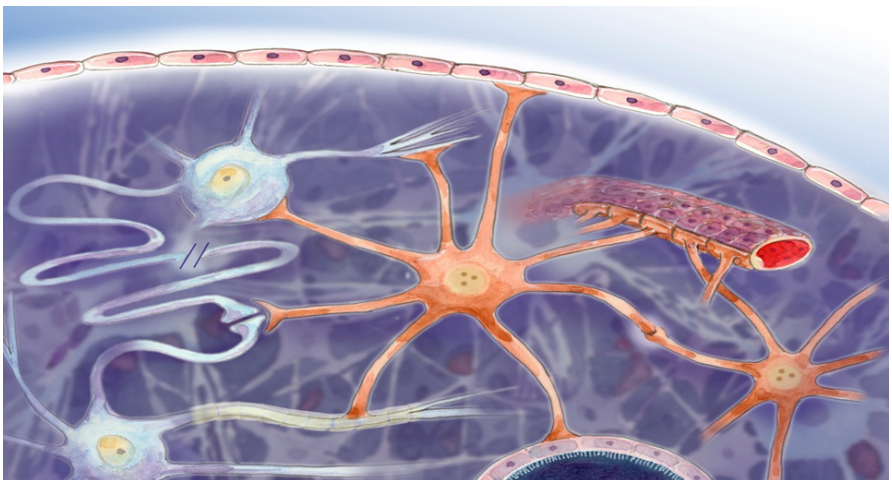

Figure 1. Astrocytes are star-shaped cells coupled by gap junctions in syncytial networks. They are prevalent throughout the CNS, and their end- feet are in contact with other glial cells, neuronal axons, synapses, and cell bodies, as well as the blood-brain barrier, ventricles, and the blood vessels. The drawing was created by Eva Kraft, Gothenburg, Sweden (Hansson, 2006).

protein connexin 43 (Chen et al., 2012). Ca^{2+} waves propagate at a speed of 0.1 mm/s (Hassinger et al., 1996). The Ca^{2+} signalling provides an opportunity for astrocytes to influence synaptic transmission (Chiang, 2012).

One role of astrocytes is to clear the synaptic cleft of glutamate released from neurons, thereby preventing neurotoxicity derived from excessive amounts of glutamate (Danbolt 2001; Kreft et al., 2012). Neuronal activity releases glutamate into the neural synapse. Glutamate is taken up by astrocytes and converted to glutamine by the enzyme glutamine synthetase. Glutamine is released back into the synaptic cleft and is taken up by neurons and metabolised to glutamate, which is again released into the synaptic cleft. This process comprises the glutamate-glutamine cycle (Berl et al., 1961; McKenna 2007, 2013).

Inflammatory-activated astrocytes cannot sufficiently clear the synaptic cleft of glutamate. Glutamate is an excitatory neurotransmitter, and the increased glutamate level in the synaptic cleft renders the neurons more excitable (Hertz and Zielke, 2004).

Microglia

Microglia are resident macrophages in the CNS, and they respond rapidly to injury by proliferating, changing shape, and producing pro-inflammatory cytokines, such as TNF-α, IL-1β, and brain-derived neurotropic factor (BDNF) (Taves et al., 2013). It is well-known that peripheral nerve injury caused by surgery or trauma causes significant activation of microglia in the spinal cord. Within 2 days of an injury there is a marked proliferation of microglia in the spinal cord (Hains and Waxman, 2006; Hains et al., 2010). Thus, microglia are most likely the cells that initiate inflammation in the CNS, leading to further activation and spread of inflammation by the astrocytes (Calvo and Bennett, 2012).

Cellular changes during inflammation

The cellular changes that occur during experimental neuroinflammation render the astrocyte network unable to interact appropriately with neurons, which alter synaptic transmission. When experimental neuroinflammation was induced, lipopolysaccharide (LPS), a potent inflammatory activator composed of endotoxin from gram-negative bacterial cell walls, was utilised.

The inflammatory-induced cellular changes that we focus on in this thesis are listed and explained below and in figure 2.

1. Changes in the receptor expression of Toll-like receptor 4 (TLR4)

TLR4 is an inflammatory receptor that responds to LPS. The expression of TLR4 is increased in astrocytes under neuroinflammatory conditions. Activation of TLR4 not

only leads to Ca^{2+} release, but also to an increase in the release of the pro-inflammatory cytokines TNF-α and IL-1β (Hutchinsson et al., 2011). The activation of TLR4 can be inhibited by ultralow doses of naloxone (Lewis et al., 2012), and interestingly, it can be increased by prolonged morphine administration (Watkins et al., 2009).

2. Changes in Ca^{2+} wave signalling

Astrocytes respond to a variety of substances by releasing intracellular Ca^{2+}. These Ca^{2+} waves can be stimulated by substances released from both neurons and glial cells (Santello et al., 2012; Zorec et al., 2012). Receptors on the surface of astrocytes are coupled to G proteins, and release Ca^{2+} from the endoplasmic reticulum via phospholipase C and inositol-tri-phosphate (IP_3) (Lencesova et al., 2004). These Ca^{2+} waves can propagate from one cell to another via gap junctions. An increase in cytosolic Ca^{2+} leads to the release of gliotransmitters, i.e substances that can influence and modulate synaptic transmission (Blomstrand et al., 1999; Scemes and Giaume, 2006). Prolonged neuroinflammation causes dysfunction of this signalling system (Hansson and Rönnbäck, 2003; Hansson, 2006; 2010; Delbro et al., 2009).

3. Changes in the expression of Na^+/K^+-ATPase

Na^+/K^+-ATPase is fundamental in maintaining cytosolic homeostasis in astrocytes, as well as all other cell types. In astrocytes, this pump indirectly modulates Ca^{2+} signalling; therefore, the activity of this pump is critical for cell function (Liu et al., 2008). Ultralow doses of ouabain and naloxone (10^{-12} M) have the potential to increase the activity of Na^+/K^+-ATPase (Zhang et al., 2008).

4. Changes in cytoskeleton structure

An intact cytoskeleton is required for the propagation of Ca^{2+} waves in astrocytes, and disruption of the cytoskeleton abolishes Ca^{2+} waves by changing the balance between the Ca^{2+}-regulating processes (Cotrina et al., 1998). Neuroinflammation disrupts the cytoskeleton.

5. Amounts of released pro-inflammatory cytokine IL-1β

IL-1β is a pro-inflammatory cytokine that initiates and maintains neuroinflammation. In the CNS, IL-1β is mainly produced by microglia that are active in initiating the inflammatory process, while astrocytes, which also produce significant amounts of IL-1β, are dominant in maintaining neuroinflammation (Watkins et al., 1999, Kiguchi et al., 2012). The increase in IL-1β closes the gap junction, thereby inhibiting the normal propagation of Ca^{2+} waves through the astrocytic network.

Astrocyte network functions in the healthy cell

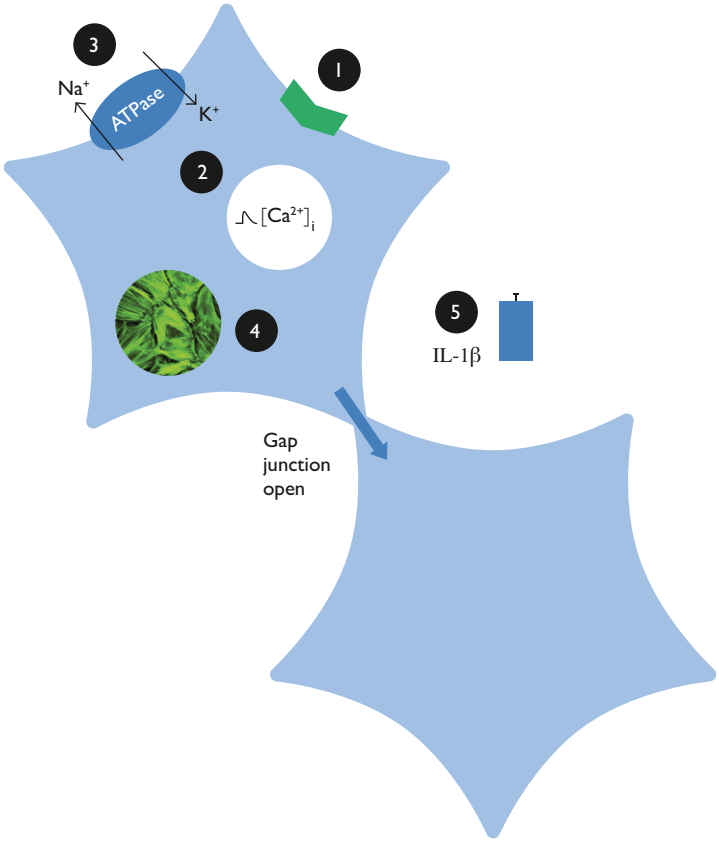

Figure 2a. The astrocyte network functions studied in this thesis are : 1) Expression of the inflammatory receptor TLR4 (green); 2) calcium signalling; 3) expression of Na^+/K^+-ATPase; 4) the actin filament structure; and 5) the release of IL-1β.

Astrocyte network functions during inflammation

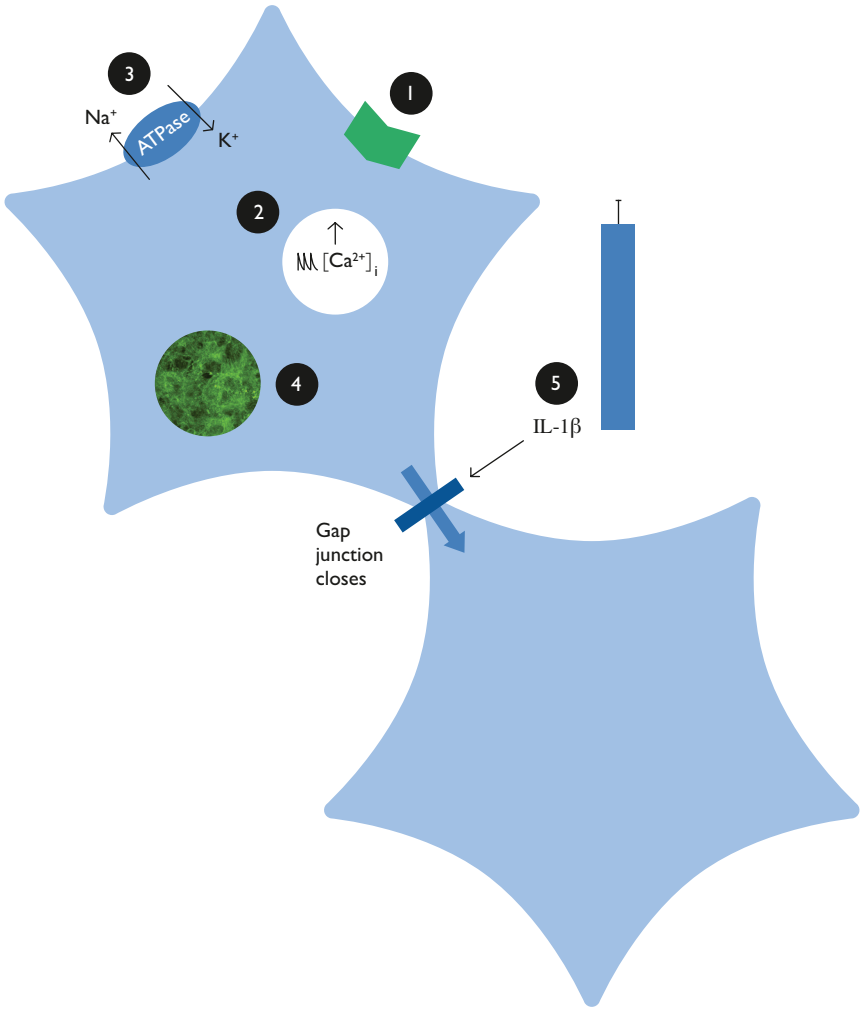

Figure 2b. During experimental neuroinflammation the following changes occur: 1) Expression of the inflammatory receptor TLR4 is increased (green); 2) calcium signalling is disturbed; 3) Na^+/K^+-ATPase is down-regulated; 4) the actin filament structure is altered; and 5) the release of IL-1β is increased, causing gap junction to close.

Pharmacological substances

To restore the cellular changes caused by the experimental neuroinflammation described above, we searched the literature for substances with possible anti-inflammatory properties that are usually used for other purposes in medicine.

Ouabain

Ouabain is a digitalis-derived glycoside, and it is known to inhibit Na^+/K^+-ATPase at higher concentrations and to stimulate it at lower concentrations (Zhang et al., 2008). We used this substance to restore the inflammation-induced decrease in Na^+/K^+-ATPase expression. Ouabain is a toxic substance that has a narrow therapeutic window; therefore, its use in clinical experiments is limited (Valente et al., 2003).

Naloxone

Naloxone is an effective μ-opioid receptor antagonist when used at higher doses (milligrams), and it is widely used in clinical practice to reverse opioid overdoses (Boyer, 2012).

It has also successfully been used intravenously at lower doses (micrograms) to prevent the side effects of morphine treatment in postoperative settings in adults and children (Maxwell et al., 2005). Naloxone in microgram doses also partially blocks the μ-opioid receptor.

At ultralow doses (picograms) the mechanism of naloxone is different. Usually, morphine activates the μ-opioid receptor, which in turn activates the $G_{i/o}$ protein. The complex of μ-opioid receptor and $G_{i/o}$ works by multiple mechanisms to inhibit neural pain impulses, thereby decreasing pain sensations in the brain (Connor and Christie, 1999; Taylor et al., 2013). In states of low-grade neuroinflammation, such as chronic pain states (Huber et al., 2001; Sharma and Johanson, 2007; Hansson, 2010), and even after long-term morphine treatment (Raghavendra et al., 2002; Watkins et al., 2005), the μ-opioid receptor shifts its coupling from the inhibitory $G_{i/o}$ protein to the excitatory G_s protein (Wang et al., 2005; Tsai et al., 2009). This switch causes diminished pain relief and increased morphine tolerance. Naloxone at ultralow concentrations has the ability to block μ-opioid receptor-coupling to the excitatory G_s protein and causes the μ-opioid receptor to couple to the inhibitory $G_{i/o}$ protein (Crain and Shen, 1995; 2000; Block et al., 2012), thereby improving pain relief. Naloxone has been shown to be virtually atoxic; however, it can cause severe withdrawal symptoms when administered to patients with a history of long-term opioid use.

Endomorphin-1

Endomorphin-1 (EM-1) is a peptide and an endogenous μ-opioid receptor agonist (Mizoguchi et al., 2002; Horvath et al., 2003; Fincha et al., 2007). EM-1 is released from nerve endings into the general circulation (Jessop et al., 2000). It has also been found in inflammatory tissue (Masocha et al., 2003), implying that it may interact with immune cells. Astrocytes possess μ-opioid receptors, and endomorphins may play a role in the control of neuroinflammatory activity (Hansson et al., 2008; Lazarus and Okada, 2012). In inflammatory conditions, an up-regulation of endomorphins occurs, and this response has been demonstrated to have functional significance in pain control by producing potent analgesia in states of inflammatory and neuropathic pain in rodents (Horvath and Kekesi, 2006).

Morphine

Morphine (from Morpheus, Roman god of sleep and dreams) has been used for thousands of years due to its analgesic properties (Norn et al., 2005). Morphine is a naturally occurring alkaloid, originally derived from the poppy plant. The main metabolites from morphine are morphine 3-glucuronide and morphine 6-glucuronide. Morphine metabolism occurs primarily in the liver and kidney (Christrup, 1997). Like most opioids, morphine exerts its analgesic effects on the μ-opioid receptors that are prevalent in the periphery, as well as in the spinal cord and brain (Pasternak and Pan, 2013). Opioid receptors are also prevalent on immunocompetent cells, and it is believed that morphine inhibits the inflammatory response, although the exact mechanism for this modulation is unclear (Al-Hashimi et al., 2013). The analgesic effects of morphine are complex and achieved by inhibition of pain-enhancing neurons and the reinforcement of pain-inhibiting neurons (McQuay, 1999; Twycross, 1999; Hojsted and Sjogren, 2007). Common side effects of morphine are nausea and sedation. Some of the side effects of morphine are potentially fatal; the most serious side effect is respiratory depression (Andersen et al., 2003).

Levetiracetam

Levetiracetam is an effective anti-epileptic drug. It has a mechanism of action that is slightly different from that of other antiepileptics which work by blocking Na^+ and Ca^{2+} channels, thereby decreasing neuronal excitability (Sills, 2006; Dooley et al., 2007). Instead, levetiracetam inhibits the neural release of transmitters into the synaptic cleft by binding to a protein that regulates exocytosis (Crowder et al., 1999; Lynch et al., 2004). There are several reports suggesting that levetiracetam has beneficial effects on neuropathic pain (Rowbotham et al., 2003; Frediani, 2004; Price, 2004). In our experimental work, levetiracetam was used for its anti-inflammatory properties. Levetiracetam has been shown, in inflammation-reactive astrocyte

models, to restore functional gap junction coupling (Stienen et al., 2011) by increasing the expression of connexin 43, the predominant gap junction protein, and decreasing the enhanced IL-1β level (Haghikia et al., 2008). Levetiracetam is widely used and generally well tolerated. However, it has some significantly undesirable side-effects, such as psychosis and suicidal behaviour (www.fass.se).

Cytokines as pain markers

Cytokines are produced by immunocompetent cells, such as monocytes, macrophages and endothelial cells. They are locally produced signalling molecules that maintain and regulate inflammatory processes (Mölne and Wold, 2007; Hutchinson et al., 2008). The interleukins IL-1β, and TNF-α stimulate cyclooxygenase, resulting in the release of painful endogenous substances such as prostaglandins, substance P, and bradykinin (Schäfers and Sorkin, 2008; Sommer, 2009). IL-10 has anti-inflammatory properties and reduces activity in C fibres (Lin et al., 2010). BDNF is associated with neuropathic pain and increases in states of neuroinflammation (Ferrini and Konick, 2013; Tsuda et al., 2013). Glial cell line-derived factor (GDNF), induces hyperalgesia when administered peripheral (Malin et al., 2006; Ferrari et al., 2010) but has also been shown to inhibit and reverse neuropathic pain in rats (Boucher et al., 2000).

The project

Patients with persistent pain after surgery or trauma often have a neuropathic pain component. When established, this pain is very difficult to treat, and in many cases, conventional analgesics provide insufficient pain relief. To better treat these patients, new or novel uses of existing therapeutics must be considered. Neuroinflammation is one of the underlying mechanisms of persistent neuropathic pain. Microglia and astrocytes are known to initiate, maintain, and spread neuroinflammation. The inflammatory-activated microglia produce pro-inflammatory cytokines, consisting predominantly of IL-1β. Astrocytes are activated by the pro-inflammatory cytokines released from microglia, and because astrocytes are coupled in networks, they are well suited to spread and maintain this inflammation. In an effort to determine the cellular changes that are associated with neuroinflammation, astrocyte cultures, and more specifically, their communication in networks in normal and inflammatory states, were studied experimentally. Our aim in these experimental studies was to identify substances that had the ability to restore astrocyte network Ca^{2+} signalling, which had been disturbed by neuroinflammation, to its normal state. The results from our experimental studies showed that three substances used in combination had this potential effect. Hoping to improve pain relief, we then investigated the effect of these substances on patients with long-term pain. In a pilot study in eleven patients

suffering from persistent pain, we used two of the substances to investigate whether they could improve pain relief in these patients. The rationale for not using all three agents at once is that we wanted the option to investigate the effect of each agent per se in patients with persistent pain.

Specific background of papers I, II, III and IV

I. Astrocyte Ca^{2+} waves can be evoked by transmitters released from neurons and glial cells (Scemes and Giaume, 2006). The influx of Ca^{2+} across the plasma membrane is driven by the Na^+ electrochemical gradient across the plasma membrane and the Na^+ pump, Na^+/K^+-ATPase, which indirectly modulates Ca^{2+} signalling (Liu et al., 2008). Inflammatory stimuli disturb the Ca^{2+} homeostasis in astrocyte networks, possibly by interfering with the activity of Na^+/K^+-ATPase (Hansson, 2006; 2010). Ouabain is known for its ability to modulate Na^+/K^+-ATPase; specifically at high concentrations, it inhibits Na^+/K^+-ATPase increasing intracellular Ca^{2+}. Interestingly, at low concentrations (nanomolar and picomolar), ouabain stimulates Na^+/K^+-ATPase activity (Zhang et al., 2008).

II. It has been reported that μ-opioid receptors in healthy cells exert their effect by coupling to the inhibitory second messenger $G_{i/o}$ protein (Connor and Christie, 1999). A switch in G protein coupling from $G_{i/o}$ to the excitatory G_s protein has been observed during inflammation and following chronic administration of opioids in rats (Wang et al., 2005). An increased coupling to the G_s protein by the opioid receptor is associated with tolerance to and a diminished anti-nociceptive effect of morphine (Crain and Shen, 1995). Naloxone at ultra-low doses restores the $G_{i/o}$ protein coupling by blocking the G_s protein, thereby also restoring the anti-nociceptive effect of morphine (Tsai et al., 2009).

III. Under conditions that lead to neuroinflammation, several receptors are influenced and their expression is changed. These changes in the glial cells can lead to pathogenic chronic neuroinflammation. Subsequently, the neurons change their excitability and signalling properties. Levetiracetam, an effective anti-epileptic drug, has been shown, in inflammation-reactive astrocyte models, to restore functional gap junction coupling (Stienen et al., 2010) by increasing the expression of connexin 43, the predominant gap junction protein, and decreasing the enhanced IL-1β level (Haghikia et al., 2008), thereby restoring cellular functions altered by inflammation.

IV. Most pain conditions can be treated to a satisfactory degree using conventional therapies. However, in certain cases, pain remains that is so severe that alternative options have to be considered. Pain treatment with indwelling spinal catheters and implantable pumps for non-malignant pain syndromes has been shown to yield effective results (Nitescu et al., 1991;1998) and to be safe (Dahm et al., 1998; Willis and Doleys, 1999; Lundborg et al., 1999). Even with this invasive method, acceptable pain relief is still not achieved in some patients; therefore the identification and development of additional therapies to improve pain relief are essential. This study focused on the potential beneficial effects of supplementing intrathecal opioids with concurrent and similarly administered naloxone.

Aims

General aims

The goals of this study were to explore the nature of astrocyte signalling in normal and inflammatory states, investigate cellular changes induced by inflammation, identify a drug combination that can restore these changes in vitro, and evaluate the effects of such a drug combination when administered to patients with long-term pain.

Specific aims

I. To *experimentally* evaluate how inflammatory stimuli alter cellular function with respect to expression of the inflammatory receptor TLR4, intercellular communication through Ca^{2+} signalling in astrocyte networks, expression of Na^{+}/K^{+}-ATPase, organisation of actin filaments, and release of the pro-inflammatory cytokine IL-1β. Furthermore, we aimed to evaluate whether the postulated inflammatory changes can be attenuated by the proposed anti-inflammatory substances naloxone and ouabain.

II. To *experimentally* evaluate the responses in astrocytes to the endogenous μ-opioid agonist EM-1 with respect to intercellular communication through Ca^{2+} signalling. Additionally, we aimed to study possible changes in intercellular communication caused by inflammatory stimuli, to determine whether postulated changes in communication can be restored with ultralow concentrations of the assessed anti-inflammatory substance naloxone, and to evaluate possible mechanisms of this process.

III. To *experimentally* evaluate whether the combination of EM-1, naloxone, and levetiracetam can counteract the inflammatory induced disturbances in 1) astrocyte Ca^{2+} signalling, 2) expression of Na^{+}/K^{+}-ATPase activity, 3) organisation of actin filaments, and 4) astrocyte release of IL-1β.

IV. To *clinically* investigate whether pain relief can be achieved in patients with severe persistent pain by delivering continuous intrathecal morphine and adjuvant nanogram doses of naloxone, administered by the same route.

Experimental methods and materials

Cell cultures (Papers I, II and III)

To obtain the cell cultures used in papers I, II and III, astrocytes from the cerebral cortices of newborn rats were co-cultured with microvascular endothelial cells from adult rat brains (Hansson et al., 2008; Delbro et al., 2009). The astrocytes were first cultured for 6 days and then co-cultured with endothelial cells for 9–11 days. As endothelial cells are not in direct physical contact with the astrocytes in vitro, the interaction between them was induced by the shared medium. Co-cultured astrocytes are affected by substances that are released from endothelial cells (Huber et al., 2001; Abbott et al., 2006; Willis and Davis, 2008) and are morphologically differentiated by long, slender processes. Co-cultured astrocytes exhibit greater Ca^{2+} responses and cytokine release than mono-cultured astrocytes. The μ-opioid receptor, as well as TLR4 (Forshammar et al., 2011; Stokes et al., 2013), are better expressed in co-cultured astrocytes (Hansson et al., 2008; Byrne et al., 2012) compared to mono-cultured astrocytes. Cell cultures are studied in vitro, and the results achieved in this setting are difficult to translate to an in vivo setting. The results obtained using this in vitro model should therefore be interpreted with particular caution. It must also be noted that the cells used for the experiments discussed in this thesis were derived from living animals, and the condition of an animal, such as stress or disease state may also influence the results.

The inflammatory reaction in cell cultures was produced using LPS, an endotoxin from the cell wall of gram-negative bacteria. LPS is widely used to initiate a fast and powerful inflammatory reaction (Zielasek and Hartung 1996; Nakamura, 2002; Tarassishin et al., 2014).

Calcium imaging (Papers I, II and III)

Calcium imaging consists of incubating astrocytes with the fluorophore probe Fura-2/AM for 30 minutes prior to the experiments. The probe, which is an ester, is cleaved by an intracellular esterase, becomes negatively charged, and binds to the positively charged Ca^{2+} ions when they are released from the endoplasmic reticulum. The probe is a Ca^{2+} dye that has an excitation spectrum that shifts from 380 nm in its unbound state to 340 nm when it becomes bound to Ca^{2+} (Grynkiewicz et al., 1985). This method can be used to determine alterations in intracellular Ca^{2+} concentration (Cornell-Bell et al., 1990). The cells are then stimulated with an agent, such as LPS (paper I), EM-1 (paper II), or glutamate (paper III) (Forshammar et al., 2011;

Block et al., 2012; Block et al., 2013), for 30 s and viewed with an inverted epifluorescence microscope with excitatory light that alternates between 340 and 380 nm. The emitted light was captured by a camera and analysed according to intensity, reflecting the intracellular Ca^{2+} levels (figure 3). The amplitude was calculated as the maximum increase in the ratio of light emitted at 340/380 nm. The amplitude, number of peaks, and the total area under the curve were analysed to provide measures of the intensity of the Ca^{2+} response. Ca^{2+} releases were measured before and after the cells were induced to be inflammatory-reactive by incubation with LPS. The incubation-time was between 30 minutes and 24 hours. To counteract the inflammatory reaction caused by LPS, cells were pre-treated for 3.5 minutes with naloxone, ouabain, EM-1 and levetiracetam, separately and in combination, as described in papers I, II and III. This method allows for the measurement of released Ca^{2+} from astrocyte intracellular stores as a response to a variety of agents (Paredes et al., 2008; Li et al., 2014). The experiments can be repeated, but the result may vary between different sets of cultivated cells. It is a time-consuming method that demands highly technological and computerised equipment.

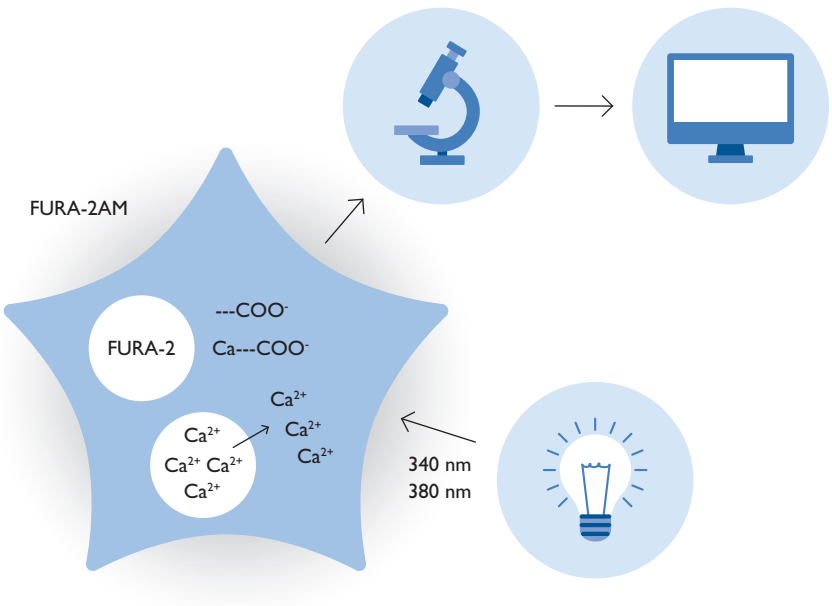

Figure 3. Astrocytes are incubated with Fura-2/AM, which is cleaved by esterases inside the cell and becomes negatively charged. The positive Ca^{2+} ions bind to the Fura-2 and are exposed to light with alternating wavelength and the Ca^{2+} release can be visualised and measured.

Protein determination and Western blot analysis (Papers I and III)

Protein determination was performed in accordance with the manufacturer's instructions, based on the method by Lowry et al. (Lowry et al., 1951), with some modification by Persson et al. (Persson et al., 2005). Astrocytes were lysed, and an equal amount of protein was delivered to each lane of the electrophoresis gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane for Western blotting. The membrane was probed with primary antibodies, and unspecific binding was inhibited. The antibodies were detected by adding conjugated secondary antibodies and visualised by chemiluminescence. The resulting bands were quantified by densitometry.

Immunocytochemistry and fluorescent probes (Papers I and III)

Immunocytochemistry was used in paper I to visualise the expression of TLR4 and in paper III to visualise microglia in the astrocyte cultures. Briefly, cells were incubated with a primary antibody directed against the target. Thereafter, cells were incubated with a secondary colour-conjugated antibody that targets the primary antibody. Finally, the cells were mounted on a microscope slide in fluorescent medium and viewed under a microscope.

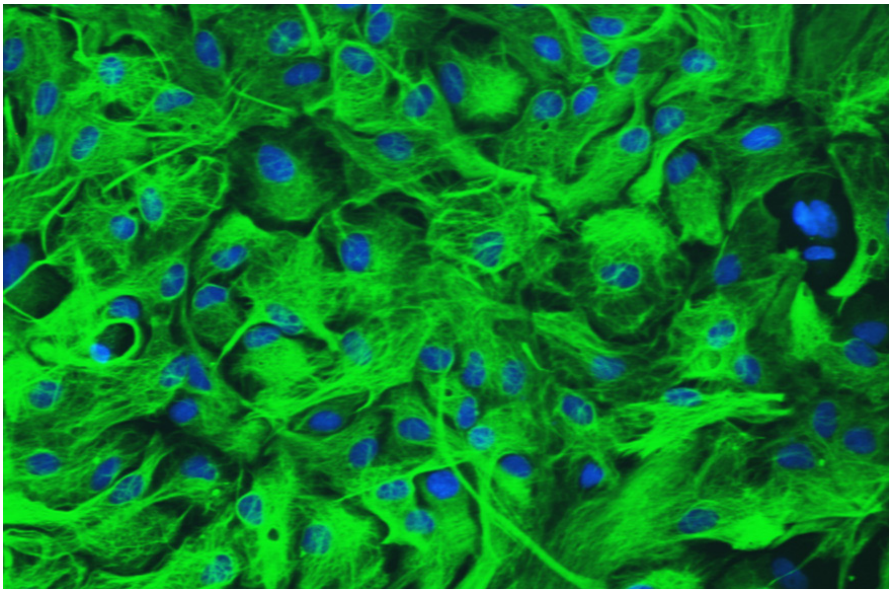

Figure 4. Immunocytochemistry was performed on cultured astrocytes. Alexa 488 targets glial fibrillary acidic protein (GFAP); therefore cell bodies are seen as green. Hoechst 33258 targets DNA; therefore cell nuclei are seen as blue.

Actin visualisation

Actin visualisation is a form of immunohistochemistry, and the techniques are basically the same as those described in the immunocytochemistry section. The organisation of the astrocyte cytoskeleton was evaluated by staining actin filaments with an Alexa 488-conjugated phalloidin probe in papers I and III, followed by observation with a fluorescence microscope (Wulf et al., 1979).

Viability assay

Cell viability assays were conducted to verify that increased Ca^{2+} release from astrocytes during LPS stimulation was not caused by cell death. This is a semiquantitative method that detects rather explicit changes in cell viability. Details can be found in Paper I.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used in experimental studies to detect the levels of IL-1β in papers I, II and III, and to detect IL-1β, TNF-α, IL-8, IL-10, GDNF, and BDNF in paper IV. Cytokines were quantified using commercial high-sensitivity ELISA kits according to the manufacturer's instructions.

Clinical methods and patients, paper IV

Intrathecal pain treatment

Pain treatment using indwelling spinal catheters and implantable pumps for non-malignant pain syndromes has been shown to produce effective results and to be safe (Nitescu et al., 1991, 1998; Dahm et al., 1998; Willis and Doleys, 1999; Lundborg et al., 1999, 2009; Atli et al., 2010; Raphael et al., 2013). A recent study which used the short form-36 (SF-36) to measure health status, also showed that this type of treatment improved all quality of life parameters, with the exception of working capacity (Lara et al. 2011). A catheter with its tip placed at a high lumbar level is inserted into the intrathecal space. The catheter is then tunnelled subcutaneously to the anterior side of the body and connected to a subcutaneous pump (Synchro Med or Iso Med, Medtronic Corp. Minneapolis, USA). The pump is placed in the right or left fossa. The pump, coupled to the intrathecal catheter, continuously delivers a fixed rate of morphine (Smith et al., 2002), and the patient cannot change the set infused rate.

There are some differences between the pumps used; for example the Synchro Med pump is battery-driven and has to be changed every 5 years. In this pump, the rate of drug delivery can be changed via telemetry with a wireless remote control. All the Synchro Med pumps are filled with morphine 10 mg/ml. The other pump used in the study; the Iso Med pump, is gas-driven and does not need to be replaced as long as it is working properly. The Iso Med pump delivers a fixed rate of morphine. To change the amount of drug administered to a patient, the concentration of the drug needs to be altered.

This method offers long-term pain reduction to 2/3 of patients with refractory non-malignant pain (Nitescu et al., 1998). It can be used for many years; however it has some limitations. First, the installation of the catheter and the pump carry a risk of infection, the most severe of which is meningitis (Dahm et al., 1998). Furthermore, it is an invasive procedure and, for the Synchro Med pump system, the procedure of installing the pump has to be repeated every 5 years when the pump battery expires. Long-term treatment with an intrathecal opioid is associated with somewhat different side effects than those associated with classical systemic opioid therapy. Respiratory depression or sedation is rarely observed in long-term intrathecal opioid treatment. Nausea, urinary retention and oedema are known side effects of the intrathecal opioid route, affecting 5–8 % of treated patients (Atli et al., 2010). More common side effects include endocrine disturbances, such as hypogonadism (90 %), cortisol deficiency (15 %), growth hormone deficiency (15 %), and disturbed

lipid metabolism (Chaney, 1995; Abs et al., 2000). Intrathecal morphine infusion can cause granulomas and has the potential to cause neurological damage at the tip of the catheter (De Andres et al., 2010; Ver Donck et al., 2013).

Patients and study design in paper IV

Study IV included male and female outpatients ≥ 18 years of age who had been receiving long-term (≥ 2 years) continuous intrathecal morphine for severe pain and were still being treated. Twelve patients provided informed written consent to participate in the study.

The 12 patients, consisting of 8 women and 4 men aged 39–70 years (median 52 years), used intrathecal systems that had been installed for 2–13 years (median 7 years). Pain intensity scores during daily activities, such as walking or doing light housework ranged from 3–10 (median 5) on the NRS at the beginning of the study. Nine patients claimed that their pain was due to failed back surgery. Two patients reported multiple abdominal surgeries, and one patient reported trauma as the cause of their chronic pain. All of the study patients had undergone multiple surgeries, and they all had some neurological deficits, such as hyperesthesia, numbness, tingling sensations, and buzzing. The type of pain experienced was a combination of neuropathic and nociceptive pain. One patient worked full time, and one worked part time. Two patients were retired, and 8 patients had disability retirement. No opioids, besides the intrathecal morphine, were allowed. Opioid doses were not changed during the course of the study. Exclusion criteria included diseases that complicated assessments of pain status and functional capacity, inability to provide informed consent for the study, and pain <3 on the NRS during daily activities at the time of screening. The patients used morphine intrathecally at dosage ranging from of 0.6–4 mg/24 h.

The study consisted of three consecutive 3-weeks study periods during which patients in randomised order, in addition to their respective steady-state morphine medication, received additional intrathecal medications including placebo, low dose of naloxone (400 ng/24 h) and ultralow dose of naloxone (40 ng/24 h). According to the study design, patients experiencing severe self-reported worsening pain would receive oral oxycodone as needed.

At the start of the initial 3-week study period, patients were randomised in a non-stratified manner into three parallel groups that received treatment in different orders. Blinding was maintained throughout all treatment periods by strict protocols at the pain clinic. Patients stated the pain intensity in interviews before the study and after each treatment. We specifically asked about pain during activity. Following study completion, patients returned to their pre-study intrathecal morphine regimen.

Assessment of pain and quality of life

A self-reported NRS was used to assess pain in activity after each treatment. According to Cepeda et al. (Cepeda et al., 2003) a 1.3 scale-step change in the NRS in acute pain is experienced as a meaningful change to the patient. For long-term pain, a reduction of 30 % is considered moderate pain relief according to (Dworkin et al., 2008). Similarly, in the present study, a 2-step change in the NRS scale in either direction was considered to indicate pain improvement or worsening.

The NRS is a well-validated scale that has been used in several human trials concerning pain during recent years. It has a demonstrated statistically significant correlation with the Visual Analogue Scale (VAS) (Paice and Cohen, 1997; Breivik et al., 2000). It is also recommended by the Change Pain Advisory Board for use in clinical trials (Müller-Schwefe et al., 2011).

To assess changes in quality of life, the Swedish standard version of the SF-36 was used. (Sullivan et al., 1995; Sullivan and Karlsson, 1998; Persson et al., 1998). The version used in this study was altered such that instead of evaluating the previous 4 weeks, the previous 3 weeks were evaluated, which corresponds better to the study protocol. This tool has the advantage of being well recognised, widely used, and validated.

The assessment of pain status and quality of life is challenging, as both entities are subjective experiences unique to each patient. Long-term pain is even more complex to measure, as it is influenced more by other emotional and psychological factors. Several instruments for the measurement of pain and quality of life exist; however none are optimal (Breivik et al., 2008). The rationale for choosing the NRS and SF-36 is that patients in this study were well acquainted with both of these methods from previous trials and clinical investigations.

Statistics

Papers I, II and III: Differences between the different treatments were identified using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

Paper IV: To compare NRS scores, we used a non-parametric, paired Wilcoxon signed rank test. Treatment with different dosages of naloxone (40 ng/24 h and 400 ng/24 h) were compared with placebo treatment. Comparisons between the two naloxone treatments were also performed. Scores after the placebo treatment were compared with scores before the study. Scores from the SF-36 were also assessed using the Wilcoxon signed rank test. To analyse the perceived quality of sleep, Fischer's exact test was used to compare the number of patients who reported improved sleep with the number of patients who reported same or worsening sleep. A Mann-Whitney U test was used to assess differences in the levels of cytokines.

Main results

Paper I

In paper I, using a co-cultured astrocyte model, we showed that after a long incubation (24 h) with LPS, the inflammatory receptor TLR4 is up-regulated, LPS-evoked Ca^{2+} signalling is disturbed, Na^{+}/K^{+}-ATPase is down-regulated, and the actin filaments are disorganised. In the low-dose range, naloxone demonstrates the ability to limit some of these these LPS-induced alterations. Ultralow concentrations of naloxone restore the actin filaments and prevent LPS-induced down-regulation of Na^{+}/K^{+}-ATPase (figures 5 and 6). Ultralow concentrations of naloxone gave more consistent results than higher concentrations. Ouabain, but not naloxone at ultralow concentrations attenuated the release of IL-1β from astrocytes.

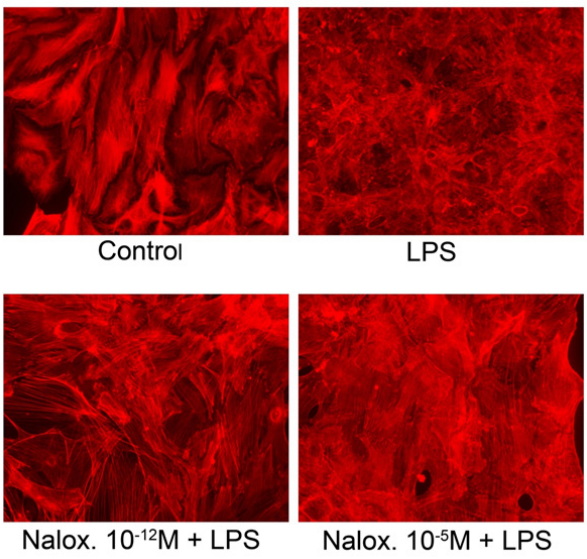

Figure 5. Naloxone at an ultralow concentration (10^{-12} M) restores inflammatory-disturbed actin filaments in astrocytes.

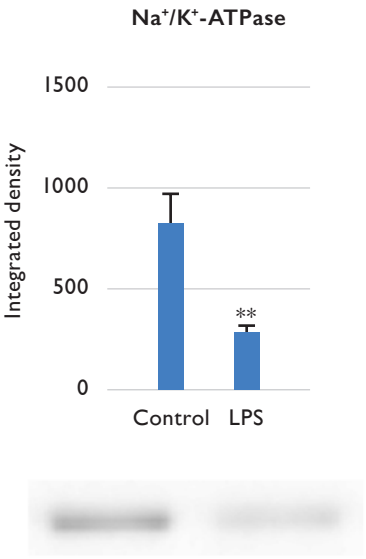

Figure 6a. Incubation with LPS down-regulates the Na^+/K^+-ATPase.

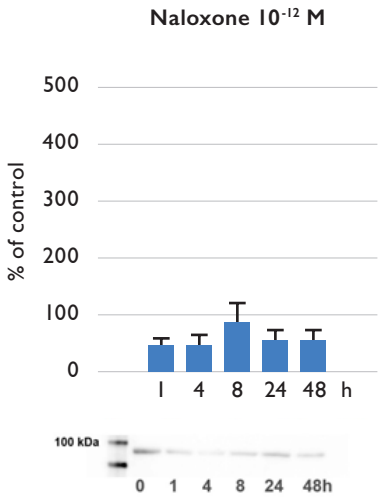

Figure 6b. Expression of Na^+/K^+-ATPase is maintained when astrocytes are pre-treated with an ultralow dose of naloxone (10^{-12} M).

Paper II

The main finding of this study was that the endogenous opioid agonist EM-1 induces intracellular Ca^{2+} release in co-cultured astrocytes at doses ranging from 10^{-15} M to 10^{-4} M. Ca^{2+} release was attenuated when the cells were exposed to the inflammatory inducer LPS for a short time (4 h), but it increased after a longer incubation period (24 h). The intracellular Ca^{2+} release, attenuated by LPS (4 h), was restored by treatment with an ultralow concentration of naloxone (10^{-12} M) (figure 7). This is most likely a result of switching the target of μ-opioid receptor stimulation from the inhibitory $G_{i/o}$ protein to the stimulating G_s protein. Picomolar concentrations of naloxone block the G_s protein, and the stimulatory effect then switches the target of the μ-opioid receptor back to the $G_{i/o}$ protein. Naloxone appears to have a homeostatic effect, resulting in restoration of the EM-1–evoked astrocyte Ca^{2+} signalling.

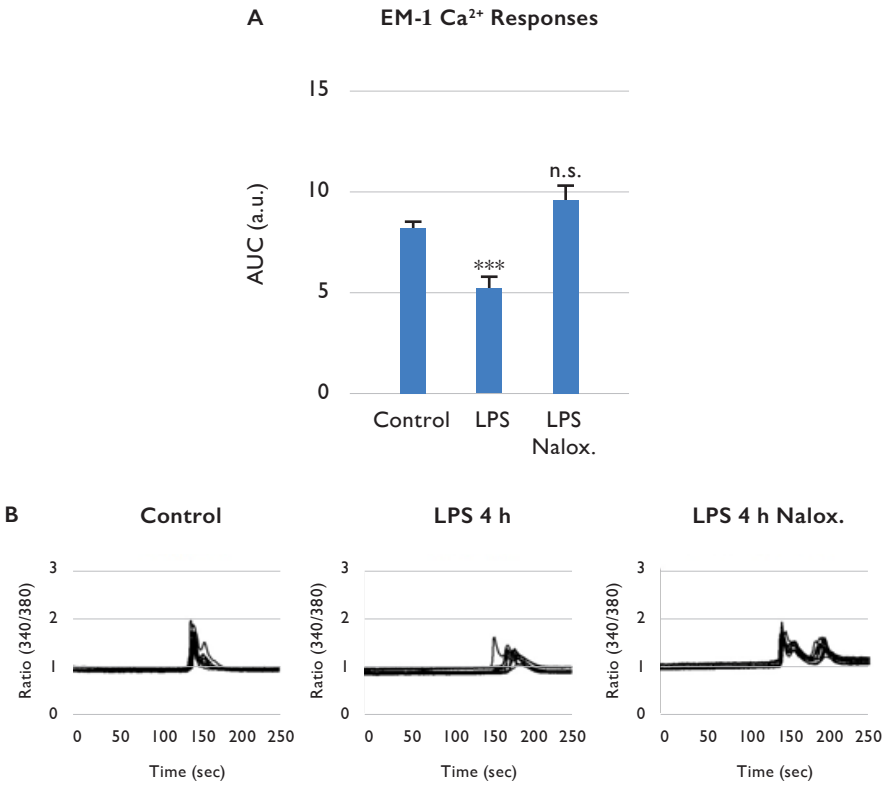

Figure 7. The intensity of Ca^{2+} release (in arbitrary units (a.u)) when EM-1 is used as a stimulating agent is reduced after incubation with LPS for 4 h, but is restored when naloxone (10^{-12} M) is added.

Paper III

In paper III, the antiepileptic drug levetiracetam was added to the naloxone and EM-1 treatment regimen, to determine whether the cellular changes induced by LPS could be completely restored. In this paper, the expression of Na^+/K^+-ATPase, Ca^{2+} signalling, actin filament organisation, and the release of IL-1β were studied. The inflammatory-induced cellular changes were fully restored. The combination of ultralow concentrations of naloxone, EM-1, and levetiracetam stimulated Na^+/K^+-ATPase activity and restored the actin filaments and intercellular Ca^{2+} signalling. IL-1β release was also attenuated (figure 8).

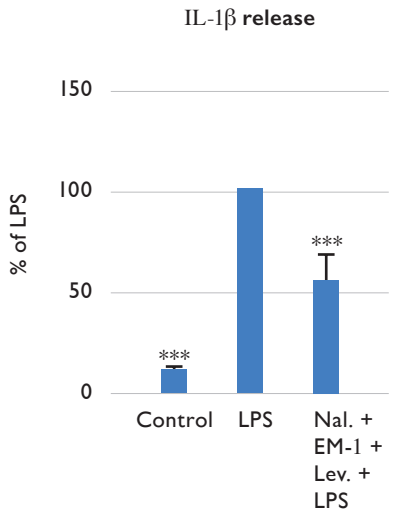

Figure 8. Release of IL-1β from inflammatory activated astrocytes is reduced by treatment with a combination of naloxone, EM-1, and levetiracetam.

Paper IV

This study addressed the novel hypothesis that a combination of intrathecally administered morphine and naloxone at low doses will improve pain relief in patients with persistent and difficult-to-treat pain. The two dosages of naloxone used were 40 ng/24 h and 400 ng/24 h. Neither of these interventions was associated with statistically significant changes in pain status, as assessed by the NRS (figure 9).

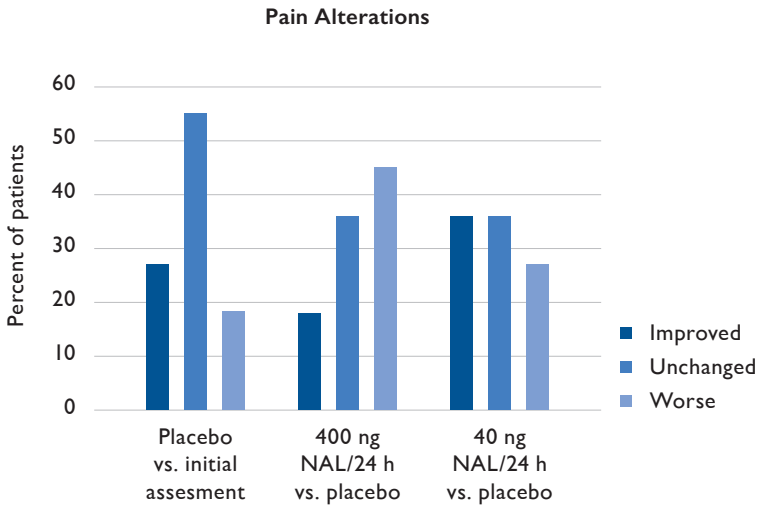

Figure 9. Effects of interventions on pain (percent of subjects), expressed as either "Improved", "Unchanged"or "Worse". NAL= naloxone. N=11.

A change of 2 steps or more in either direction on the NRS was considered as a change in pain intensity. However, adjuvant naloxone 40 ng/24 h significantly improved the perceived quality of sleep compared with placebo (figure 10), which was an unexpected finding.

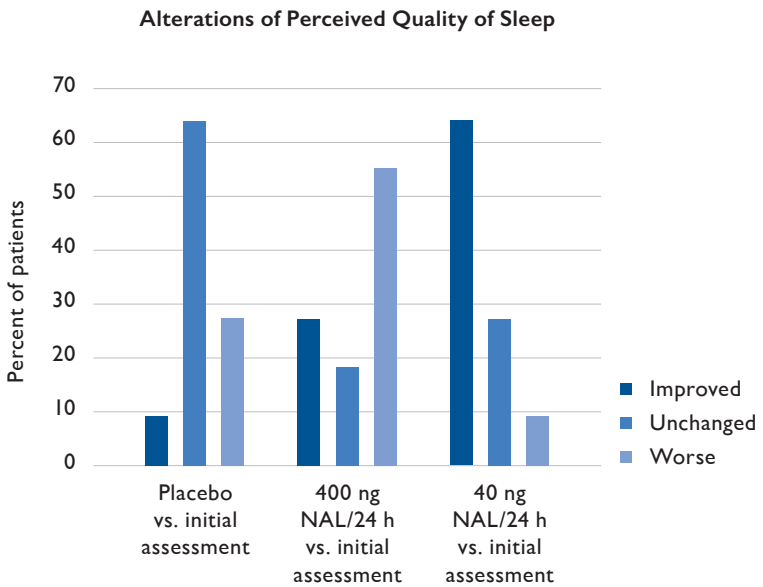

Figure 10. Effects of interventions on perceived quality of sleep (percent of subjects), expressed as either "Improved", "Unchanged", or "Worse". NAL= naloxone. p indicates a comparison between placebo and NAL 40 ng/24 h. N=11.

Discussion

Normal state

In a healthy normal state, astrocytes respond to neural activity by increasing the cytosolic Ca^{2+} level. In response to this increase in Ca^{2+}, astrocytes release neurotransmitters, such as glutamate and ATP, that modulate synaptic transmission (Santello and Volterra, 2009). Proper function of this bidirectional communication is essential for normal pain transmission (De Leo et al., 2006; Ren and Dubner, 2008; Gao and Ji, 2010); therefore, the control of cytoplasmic Ca^{2+} levels is important. In the normal state, the release of Ca^{2+} is initiated by a variety of stimuli, such as the endogenous neural release of EM-1. EM-1 stimulates the μ-opioid receptor, which activates the second messenger protein $G_{i/o}$. Ca^{2+} is released from the endoplasmic reticulum, via IP_3, into the cytoplasm (Di Castro et al., 2011). The Ca^{2+} wave propagates from one cell to another via diffusion of Ca^{2+} or IP_3 through gap junctions (Hassinger et al., 1996; Berridge, 2007).

Na^+/K^+-ATPase is a protein pump that maintains the electrochemical gradient across the plasma membrane, thereby regulating the flow of ions across the plasma membrane. These actions indirectly affects intracellular Ca^{2+} signalling, as it regulates the flow of Ca^{2+} ions across the plasma membrane (Liu et al., 2008). EM-1 and morphine have been shown to stimulate Na^+/K^+-ATPase activity in vitro and in vivo (Masocha et al., 2003). Interestingly, when Na^+/K^+-ATPase is inhibited, the antinociceptive effect of morphine is antagonised (Horvath et al., 2003).

There is an association between Na^+/K^+-ATPase, the actin filaments that constitute the cytoskeleton, and the endoplasmic reticulum by the adaptor protein ankyrin B (Liu et al., 2008). The Na^+/K^+-ATPase is connected to the actin filaments by the protein ankyrin B, and, the actin filaments are connected to the endoplasmic reticulum by the same protein. If the actin filaments are disrupted, Ca^{2+} release is disturbed. An intact actin filament is essential for normal Ca^{2+} signalling (Cotrina et al., 1998; Sergeeva et al., 2000).

The inflammatory receptor TLR4 is minimally activated in the normal state; therefore, release of the pro-inflammatory cytokine IL-1β is minimal (figure 11 a).

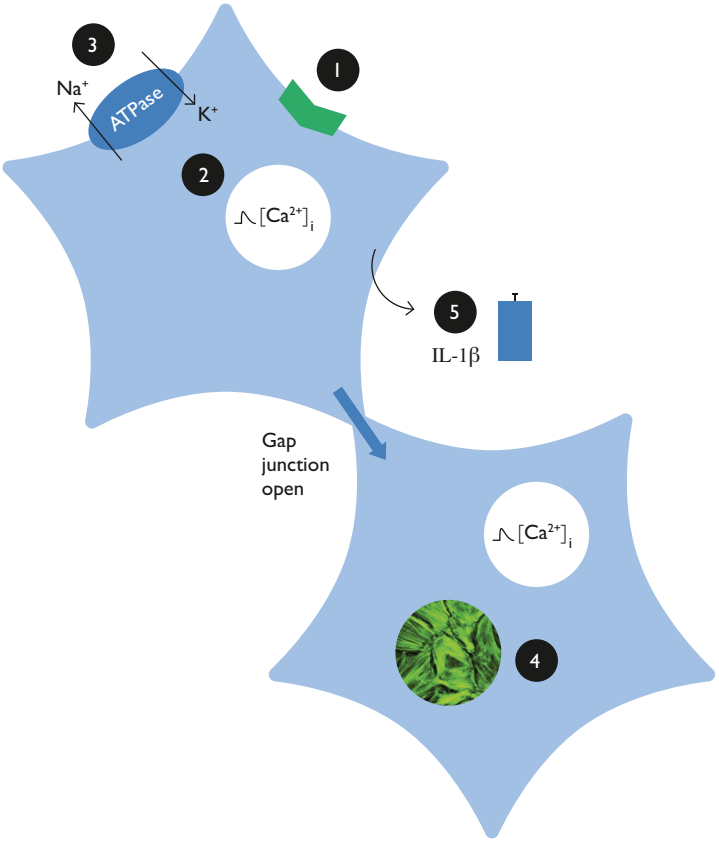

Figure 11a. In the normal state; 1) TLR4 is down-regulated; 2) Ca^{2+} waves are controlled; 3) Na^{+}/K^{+}-ATPase is working optimally; 4) actin filaments are well-organised; 5) IL-1β release is minimal, and gap junctions are open.

Inflammation

In the inflammatory state, TLR4 receptor activity is increased, which leads to an increased production of the pro-inflammatory cytokine IL-1β. This increase in production is induced via an up-regulation of gene expression that regulates immune responses (Hutchinsson et al., 2008). IL-1β affects the gap junction channel protein connexin 43 and closes the gap junctions. In the inflammatory state, Na^+/K^+-ATPase is down-regulated, and the actin filaments are disorganised (Namekata et al 2008; Forshammar et al., 2011). The increase in pro-inflammatory cytokines leads to an increased production of adenosine-tri-phosphate (ATP). These changes disturb the normal Ca^{2+} signalling (Guthrie et al., 1999), which can result in Ca^{2+} oscillations. Gap junctions have a lower capacity for intercellular Ca^{2+} waves and the propagation from cell to cell is attenuated (Meme et al., 2004). The increased extracellular release of ATP acts on purinergic receptors (P2X7) on adjacent astrocytes, which stimulates the release of intracellular Ca^{2+} in adjacent cells (Cotrina et al., 1998 and 2000; Haydon and Carmignoto, 2006). This stimulation leads to a poorly controlled extracellular propagation of the Ca^{2+} waves, and the increased intracellular Ca^{2+} release behaves in an oscillatory manner (Chen et al., 2012).

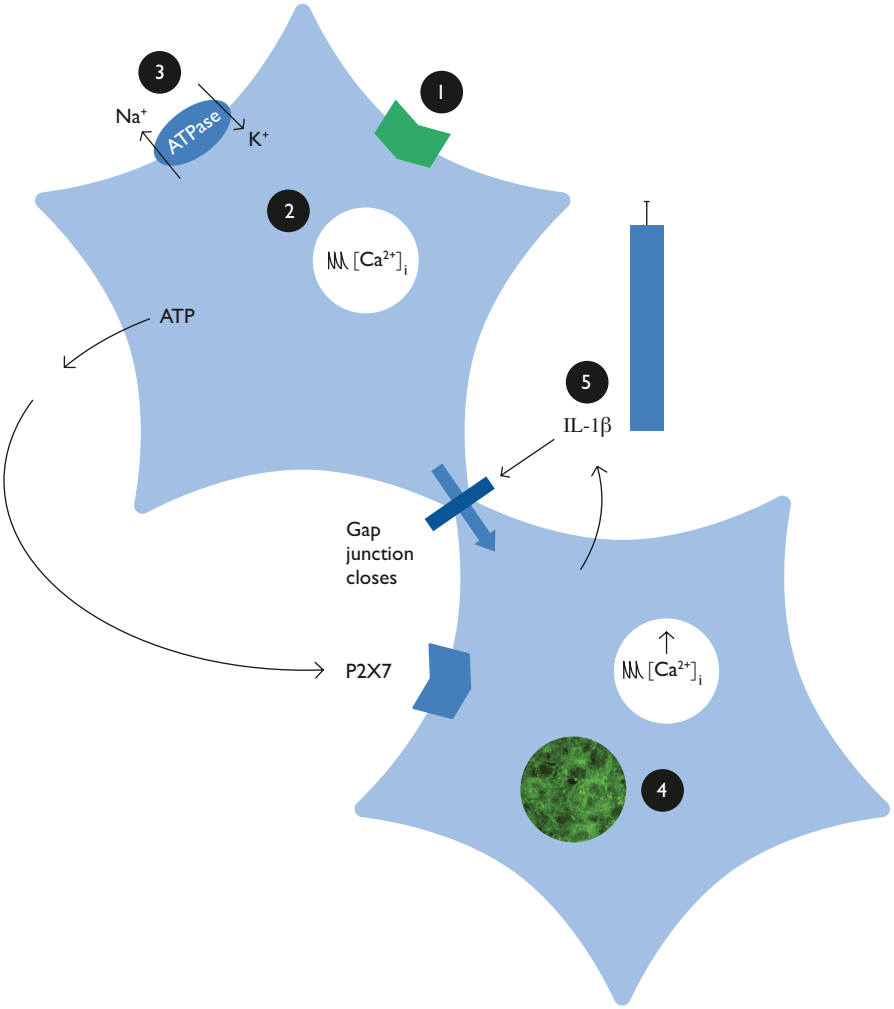

Figure 11b. During the inflammatory state 1) TLR4 is up-regulated; 2) Ca^{2+} waves are oscillating; 3) Na^{+}/K^{+}-ATPase is down-regulated; 4) actin filaments are disrupted; 5) IL-1β release is increased, and gap junctions close. Furthermore, there is an increased extracellular release of ATP which causes Ca^{2+} release in adjacent astrocytes via the P2X7 receptor.

Restoration

To restore the inflammation-induced changes, we first used a combination of EM-1 and naloxone. EM-1 and naloxone stimulate Na^+/K^+-ATPase, as well as the μ-opioid receptor. During inflammation, the μ-opioid receptor switches its normal activation target from the $G_{i/o}$ protein to the G_s protein. Naloxone at ultralow doses blocks G_s and forces $G_{i/o}$ activation, which promotes normal EM-1-induced Ca^{2+} release. Second, the addition of levetiracetam in combination with EM-1 and ultralow doses of naloxone restores the actin filaments, as well as attenuates the release of IL-1β and unblocks the gap junctions, allowing restoration of Ca^{2+} signalling.

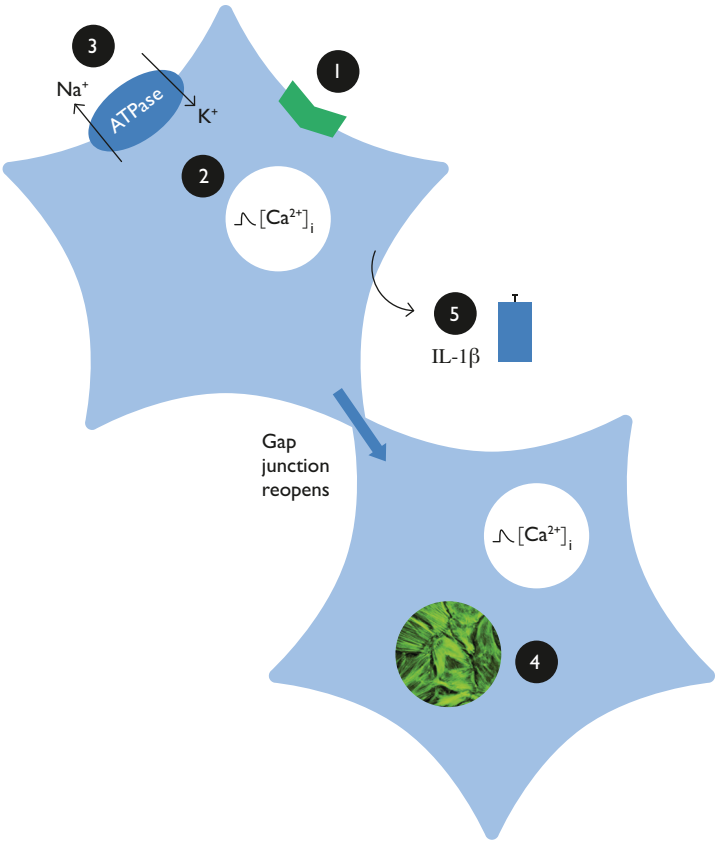

Figure 11c. Treatment with the combination EM-1, naloxone and levetiracetam restore 1) TLR4 expression, 2) Ca^{2+} release, 3) Na^+/K^+-ATPase expression, 4) actin filament organisation, and 5) release of IL-1β. Furthermore the combination unblocks gap junctions and promotes the intercellular Ca^{2+} waves to propagate through the gap junctions.

The experimental findings in this study demonstrate that a combination of EM-1 and ultralow doses of naloxone can attenuate inflammatory-induced cellular changes, and restore intercellular Ca^{2+} signalling. With the addition of levetiracetam, the beneficial results were even more pronounced. It is likely that this type of action influences the intercellular communication between astrocytes and also exerts effects on synaptic pain transmission in neurons. Still, there is a large difference between results produced in a laboratory from cellular cultures and outcomes in a clinical setting; the results from the experimental studies should therefore be interpreted with particular caution. Notwithstanding, in this thesis, specific findings from the clinical study (paper IV) relate well to the experimental and more conceptual findings (papers I and II).

Clinical applications

There are a few research reports showing that analgesia can be improved with an ultralow dose of naloxone in addition to morphine in patients (Hamann and Sloan, 2007; Hamann et al., 2008), and several reports a reduction in the need for opioids with an additional low dose of naloxone (Cepeda et al., 2004; Maxwell et al., 2005; Movafegh et al., 2012).

In study IV, we exclusively recruited study patients who had experienced severe pain for a long period of time while undergoing continuous intrathecal morphine administration. This protocol yielded a relatively homogenous study group and allowed unique assessments of pain and pain-associated symptoms during the interventions. Another noteworthy feature of the study subjects is that, despite the quite complex treatment, they were still highly affected by pain, which impaired their quality of life (figure 12).

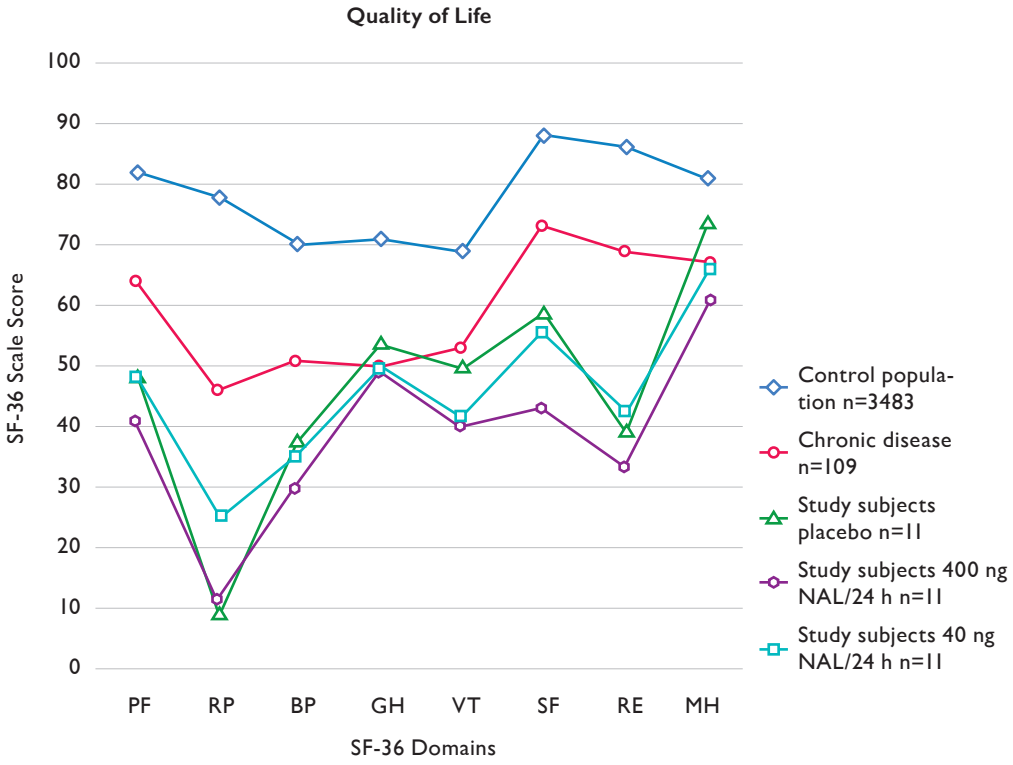

Figure 12. Composite illustrations of SF-36 scores derived from an age-matched healthy control population (blue), an age-matched population with chronic disease/handicap (red), and the present study cohort of patients during treatment with either placebo (green), naloxone 400 ng/24 h (purple) or naloxone 40 ng/24 h (turquoise).

Note the comparatively low quality of life in the study group irrespective of the intervention. The SF-36 domains (x-axis) refer to Physical Function (PF), Role-Physical (RP), Body Pain (BP), General Health (GH), Vitality (VT), Social Function (SF), Role-Emotional (RE) and Mental Health (MH). Adapted with permission from the Institute of Health and Care Sciences (Sullivan et al., 2002).

Additionally, one of the benefits of the design of this study was that compliance was nominally 100 %, as patients themselves could not alter the rate of intrathecal drug administration.

The findings of this study do not statistically support the concept that intrathecal naloxone administered as an adjuvant to intrathecal morphine, improves pain relief. Notwithstanding, three study patients, who did not improve with placebo experienced marked pain relief with either dose of adjuvant intrathecal naloxone.

Placebo treatment has significant effects in patients with severe chronic pain, as has been demonstrated in several studies (Turner et al., 1994; Kahn et al., 2003, Finniss et al., 2010). Placebo effects were also prominent in the present study, with 27 % of patients reporting improved pain relief when intervention with placebo was performed.

One unexpected finding was that seven patients experienced an improved perceived quality of sleep with adjuvant naloxone 40 ng/24 h. This result was significantly better than that achieved with placebo. The possibility of such a significant improvement in perceived quality of sleep with this treatment was not specifically considered when we designed the study, but this appeared to be an important finding. Additionally, levels of activity were increased with naloxone treatment, but not significantly. All study subjects who reported an increased level of activity also reported an improved perceived quality of sleep. This observation is novel and requires confirmation and further exploration.

The underlying mechanisms of the clinical findings in this study are complex. The μ-opioid receptor responsible for the action of morphine normally stimulates the inhibitory $G_{i/o}$ protein, but in states of low-grade inflammation, such as chronic pain states, this coupling decreases and the coupling of the μ-opioid receptor to the excitatory G_s protein increases (Crain and Shen, 1998; 2000; Wang et al., 2008; Wang and Burns 2009). It has been shown in cellular cultures that ultralow doses of naloxone can inhibit the G_s protein, and the μ-opioid receptor coupling to the $G_{i/o}$ protein subsequently increases (Shen and Crain, 1997; Wang et al., 2005; Tsai et al., 2009).

The SF-36 data revealed that the study patients generally had a low quality of life. It has previously been shown that patients with chronic non-malignant pain have an even lower quality of life than patients with malignant pain (Fredheim et al., 2008). In our study, there were no significant differences between placebo and adjuvant naloxone 40 ng/24 h treatments regarding quality of life, which was not surprising, as the intervention was administered over a relatively short time period.

There are some limitations in this study. One limitation that was particularly relevant, was that, as a result of the inclusion criteria, the patients' pain histories were generally long-standing while the duration of the study was relatively short. We acknowledge that this characteristic has implications for the generality of our findings. However, we argue that the inclusion criteria served to focus the study on patients with well documented and severe pain. Additionally, we suggest that a longer study protocol could, through spontaneous individual variations of pain status, have endangered the interpretation of study findings. We also acknowledge that assessments of the different dimensions of sleep are complex. In the interest of simplicity, we chose a robust technique when gauging alterations in perceived sleep quality, i.e., a three-choice ordinal query. Additionally, to maintain blinding throughout the study, we had to accept a non-stratified randomisation format. Finally, more females than males were recruited into the study; women also expressed more intense pain and larger changes in pain with both active treatment and with placebo. However, this is in accordance with a finding that was described previously (Rosseland and Stubhaug, 2004).

Final conclusions

I. The findings in paper I show that experimentally induced inflammation causes the following changes in astrocyte function: 1) the expression of the inflammatory receptor TLR4 is increased; 2) Ca^{2+} wave signalling is disturbed; 3) the expression of Na^{+}/K^{+}-ATPase is decreased; 4) the actin filaments are disorganised; and 5) the release of IL-1β is increased. Naloxone and ouabain at ultralow doses can partially attenuate some of these changes.

II. The endogenous μ-opioid agonist EM-1 can induce Ca^{2+} release in astrocytes. Experimentally induced inflammation alters Ca^{2+} signalling. The disturbed signalling can be restored by pre-treating the astrocytes with ultralow doses of naloxone. The underlying mechanism involves the blocking of excitatory second messenger protein G_s by ultralow concentrations of naloxone. Subsequently, the action of the inhibitory second messenger protein $G_{i/o}$ is enhanced.

III. The combination of EM-1, naloxone, and levetiracetam successfully counteracted the inflammatory-induced cellular changes caused by LPS related to 1) Ca^{2+} signalling, 2) Na^{+}/K^{+}-ATPase, 3) actin filament organisation and 4) IL-1β release. This restoration is essential for intercellular astrocyte communication and for the modulation of synaptic pain transmission.

IV. In the clinical study in this thesis, two intrathecally administered agents were combined, i.e., morphine and naloxone, and the latter was administered at two different dosages (40 ng/24 h and 400 ng/24 h). With this regimen, the addition of naloxone at the ultralow dosage of 40 ng/24 h was associated with a significantly improved perceived quality of sleep, although concurrent alterations of pain levels were not statistically significant.

Translational research

This thesis encompasses research fields that are both experimental and clinical in an effort to bring experimental and clinical research closer together, working from both ends to achieve a greater common knowledge base. To identify and define a clinical problem, work to assess this problem in the laboratory and then apply the experimentally achieved results in a clinical setting is a logical and productive method of solving medical questions. Translational research enables the possibility of understanding the mechanisms associated with different issues. Additionally, preclinical research needs to be closely coordinated with daily medical clinical challenges to more accurately target the issues that need to be addressed. This type of research also has limitations, as it is difficult to validate experimentally achieved results in a clinical setting. In vitro results may not be applicable to in vivo situations and experimentally achieved results must be confirmed in vivo to gain significant meaning.

Future perspectives

Surgery and trauma causes inflammation at the site of injury. Nerve injury causes low-grade inflammation in the central and peripheral nervous systems. Neuroinflammation that persists when the injury heals may be an important component of establishing persistent postsurgical neuropathic pain. Restoring inflammatory-activated astrocytes, thereby inhibiting enhanced pain transmission in neurons, is a very interesting method of preventing persistent postsurgical neuropathic pain. By targeting astrocytes instead of neurons, a new arena for development of pharmacological agents is opened, and larger studies in this area are needed. One may also consider the possibility that adding an anti-epileptic drug to the type of regimen described in study IV of this thesis may represent a potential future application to pain therapy.

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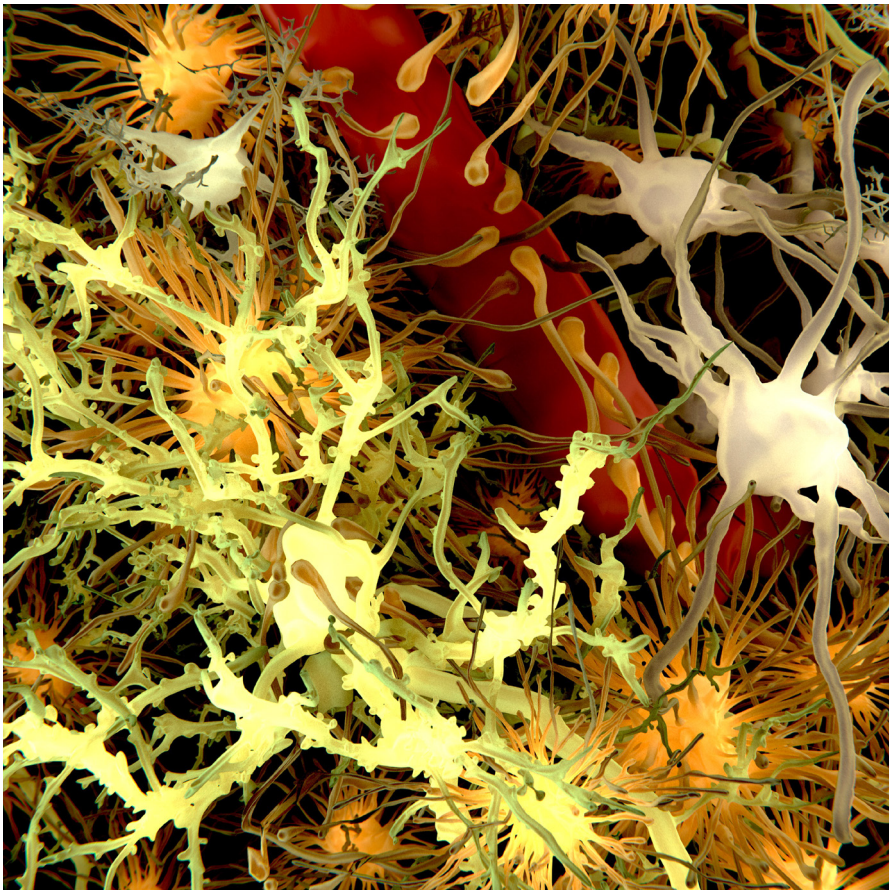

It is complicated. The main cells of the brain: astrocytes-orange; neurons-light yellow/greenish; oligodendrocytes-grey; and microglia-white. Also pictured is a blood vessel-red. Picture used with permission from Shutterstock.com, Juan Gartner.