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Acetylcholine in Spinal Pain Modulation

An in vivo *Study in the Rat*

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Abstract
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The spinal cord is an important component in the processing and modulation of painful stimuli. Nerve signals from the periphery are relayed and further conducted to the brain (nociception) in the spinal cord, and the most essential modulation of painful information (antinociception) occurs here. Several neurotransmitters are involved in spinal pain modulation, among them acetylcholine. However, the role of acetylcholine has previously been little investigated.

In the present thesis, the acetylcholine release in the spinal cord was studied *in vivo.* By using spinal microdialysis on anaesthetised rats, the effects on the intraspinal acetylcholine release of various receptor ligands and analgesic agents were examined. This, together with pain behavioural tests and *in vitro* pharmacological assays, was used to evaluate the role of acetylcholine in spinal pain modulation. The four studies in this thesis resulted in the following conclusions:

An increased release of spinal acetylcholine is associated with an elevated pain threshold, while a decreased acetylcholine release is associated with hyperalgesia, as seen after systemic treatment with a muscarinic agonist and an antagonist.

Lidocaine is a potent analgesic when given systemically. It was found to produce an increase of intraspinal acetylcholine after intravenous injection of analgesic doses. This effect was attenuated after muscarinic, and abolished after nicotinic, receptor blockade.

Various a_2-adrenergic ligands, associated with nociceptive or antinociceptive effects, were found to affect intraspinal acetylcholine release via action on nicotinic receptors.

Finally, the involvement of spinal acetylcholine in the analgesic effects of aspirin and paracetamol was examined. It was found that spinal acetylcholine could participate in the analgesic effects of aspirin, but not of paracetamol.

The present thesis provides data that clearly demonstrate a relationship between intraspinal acetylcholine and antinociception, and elucidate interactions between acetylcholine and other mechanisms that mediate antinociception in the spinal cord.

Keywords: Pain, Nociception, Antinociception, Acetylcholine, Muscarinic, Nicotinic, Spinal cord, Microdialysis

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List of papers

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

I Intravenously administered oxotremorine and atropine, in doses known to affect pain threshold, affect the intraspinal release of acetylcholine in rats.
Klas S. P. Abelson and A. Urban Höglund
Pharmacol. Toxicol. 90: 187-192, 2002

II Intravenously administered lidocaine in therapeutic doses increases the intraspinal release of acetylcholine in rats.
Klas S. P. Abelson and A. Urban Höglund
Neurosci. Lett. 317: 93-96, 2002

III The effects of the α_2-adrenergic receptor agonists clonidine and rilmenidine, and antagonists yohimbine and efaroxan, on the spinal cholinergic receptor system in the rat.
Klas S. P. Abelson and A. Urban Höglund
Basic Clin. Pharmacol. Toxicol. 94: 153-160, 2004

IV Spinal cholinergic involvement after treatment with aspirin and paracetamol in rats.
Klas S. P. Abelson, Mahinda Kommalage and A. Urban Höglund
Neurosci. Lett. 368: 116-120, 2004

Contents

Abbreviations

ACh	Acetylcholine
5-HT	5-hydroxytryptamine (serotonin)
ALQ	Anterolateral quadrant
AMPA	α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid
ANOVA	Analysis of variance
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
GABA	γ-amino butyric acid
GLM	General linear model
HPLC	High performance liquid chromatography
IASP	International Association for the Study of Pain
IMER	Immobilised enzyme reactor
mAChR	Muscarinic acetylcholine receptor
mGluR	Metabotrotpic glutamate receptor
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NK-A	Neurokinin A
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NRM	Nucleus raphe magnus
NS	Nociceptive specific
PAG	Periaqueductal gray
SI	Primary somatosensory cortex
SII	Secondary somatosensory cortex
SP	Substance P
WDR	Wide dynamic range

Introduction

Pain

The ability to experience pain is vital for the survival of all mammals. Pain arises when an organism is being injured and serves as a warning signal to make an escape or evasive action possible, and as a signal to avoid strain on and injured body part. Attempts to define the term 'pain' has been made since the days of Aristotle. The difficulty in defining the term has mainly been due to the consideration of pain as a physical or emotional event. In 1979, the International Association for the Study of Pain (IASP) adopted a definition, which in brief reads "An unpleasant and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. (…) Pain is always subjective. (…)" (IASP 1979; Merskey 1991)

Pain is one of the foremost causes of suffering in humans as well as in animals. Several pharmaceutical agents for pain suppression are available, but the existing treatments are not always adequate and often associated with several adverse effects.

In order to develop newer and more efficient pain suppressive agents, the pharmacological and physiological mechanisms that regulate pain transmission and pain modulation, must be understood in detail.

Pain transmission

Nociception

While *pain* is defined as the subjective experience of noxious stimuli, the underlying physiological and pharmacological activities that lead to a painful sensation are denominated *nociception*.

The nociceptive pathways can be described as a three-neuron chain that transmits nociceptive information from the periphery to the cerebral cortex. The *first-order neurons* have their cell bodies in the dorsal root ganglion from where two axons project, one to peripheral tissues and the other to the dorsal horn of the spinal cord. The *second-order neurons* originate from the spinal cord and ascend to the thalamus or other regions of the brainstem.

From the thalamus, the *third-order neurons* project to the cerebrocortex (Cross 1994; Millan 1999).

Peripheral actions

In most peripheral tissues throughout the body such as the skin; muscles; joints; and viscera, the presence of nociceptors has been described. The nociceptors are free, naked nerve-endings that can be directly activated by strong mechanical, thermal or chemical stimuli; or activated after being sensitised during tissue injury, inflammation, ischemia or low pH (Cross 1994; Riedel & Neeck 2001; Willis & Westlund 1997). The sensitisation is mediated by second-messenger systems such as production and release of prostaglandins, bradykinin, serotonin and histamine in the injured area. Receptors for bradykinin, serotonin and histamine are present on the surface of most primary nociceptive afferents, together with opiate, γ-aminobutyric acid (GABA) and capsaicin receptors. (Willis & Westlund 1997).

The nociceptors are associated with the first-order neurons. There are two types of first-order afferent nerve fibres; Aδ- and C-fibres. The Aδ-fibres are myelinated, 2–6 μm in diameter and conduct nerve signals with a velocity of about 30–100 m/s. The C-fibres are unmyelinated and thereby thinner (0.4–1.2 μm) than the Aδ-fibres. The C-fibres are also slower in conducting nerve signals with a velocity of 12–30 m/s (Almeida et al. 2004; Besson & Chaouch 1987).

Stimulation of cutaneous Aδ-fibres results in pricking pain, while C-fibre activation in the skin is related to a dull and burning pain sensation. Aδ- and C-fibres are also present in muscular and articular tissue. Noxious stimuli in muscles give rise to an aching and less localised pain, irrespective of fibre type. In joints, the occurrence of silent nociceptors is common, i.e. nociceptors that cannot be activated under normal conditions, but that are sensitised during inflammation and then respond to noxious stimuli. Different visceral disorders can give rise to a painful sensation, a sensation that is often referred to a cutaneous zone. Both Aδ- and C-fibres have been described in viscera, but much is yet to discover regarding how visceral nociception arises (Almeida et al. 2004; Besson & Chaouch 1987; Mense 1993; Willis & Westlund 1997).

Ascending nociceptive pathways

Spinal cord dorsal horn

The morphological structure and organisation of the spinal cord has been known for long and is well described (Rexed 1952). The superficial dorsal horn of the spinal cord grey matter includes the marginal zone and the substantia gelatinosa, also known as Rexed's laminae I and II (Fürst 1999;

Rexed 1952), and it is mainly here where the afferent Aδ- and C-fibres terminate and where the switch-over to second-order neurons occurs.

In the superficial dorsal horn, a large variety of receptor classes and neurotransmitters can be found. Peripheral noxious stimuli lead to nociceptor activation followed by release of neurotransmitters in the dorsal horn. The most important neurotransmitter classes for nociceptive transmission are excitatory amino acids and neuropeptides.

The nociceptive transmission by excitatory amino acids, such as glutamate and aspartate, is mediated by ionotropic or metabotropic glutamate receptors. The ionotropic receptors can be divided into three subcategories; N-methyl-D-aspartate (NMDA), α-amino-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA), and kainate receptors. The metabotropic glutamate receptors (mGluRs) consist of at least eight subtypes, of which at least two are present in the spinal cord (Coggeshall & Carlton 1997; Fürst 1999; Riedel & Neeck 2001).

Neuropeptides and receptors for neuropeptides are abundant in the dorsal horn and may be involved in many functions, including nociceptive transmission. Substance P (SP), can be considered the most important neuropeptide with respect to its nociceptive mechanisms, but other peptides such as neurokinin-A (NK-A), calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) are also associated with transmission of nociceptive information. SP and NK-A exert their action via neurokinin-receptors, and CGRP via CGRP-receptors. Receptors for CCK are also present in the superficial laminae, and CCK acts nociceptively mainly via an indirect action by inhibiting antinociceptive effects of opioids (Coggeshall & Carlton 1997; Fürst 1999; Millan 1999; Riedel & Neeck 2001).

Nitric oxide (NO) also plays an important role in nociceptive transmission. NO acts as a non-adrenergic, non-cholinergic neurotransmitter and has been proposed to initiate presynaptic glutamate release and thereby enhance nociception (Fürst 1999). This is supported by findings showing that the NO synthase inhibitor L-NAME enhances the antinociceptive effect of oxotremorine (Machelska et al. 1999). There are also studies suggesting antinociceptive effects of NO, which are discussed below.

Ascending tracts

From the spinal cord dorsal horn, the nociceptive information is transmitted to the brain via the second-order neurons. These have their cell bodies in the dorsal horn and their axon terminations in the brain, and are mainly of two types; wide-dynamic-range (WDR) and nociceptive-specific (NS) neurons. The WDR neurons respond to and distinguish between non-noxious and noxious stimuli, while the NS neurons respond solely to noxious stimuli. The second-order neurons reach the brain via several afferent pathways (Almeida et al. 2004; Cross 1994).

The *spinothalamic tract* ascends in the anterolateral quadrant (ALQ) of the spinal cord and terminates in the contralateral thalamus via two projections. In the lateral projection, axons mainly originate in laminae I and V and terminate in the ventral posterior lateral nucleus and the ventral posterior inferior part of the lateral thalamus. In the medial projection, axons originate from deeper parts of the dorsal horn, and from the ventral horn, and terminate in the central lateral locus. The thalamus is considered the most important relay for reception and processing of nociceptive information at the supraspinal level. The lateral part of the thalamus is thought to be involved in the sensory-discriminative component of pain, while the medial part is involved in motivational-affective aspects of pain (Almeida et al. 2004; Cross 1994; Hodge & Apkarian 1990; Willis & Westlund 1997).

The *spinomesencephalic tract* includes projections to different areas in the midbrain. Most axons originate similarly to cells in the spinothalamic tract, i.e. in laminae I and IV–VI, but some have their origin in lamina X or in the ventral horn. The tract terminates in regions such as periaqueductal gray (PAG), nucleus cuneiformis, intercolliculus nucleus, deep layers of the superior colliculus, and anterior and posterior pretectal nuclei. It has been suggested that different components of the tract have different functions. The projections to the PAG seem to contribute to aversive behaviour as well as activation of descending pain modulation, and the deep layers of superior colliculus are likely of importance for orientation (Almeida et al. 2004; Willis & Westlund 1997).

The *spinoreticular tract* originates from deep layers of the dorsal horn, from laminae VI and VII of the ventral horn, ascends through the ALQ and terminates in the reticular formation of the brainstem. One part of the tract terminates in several nuclei in pons and medulla, such as nucleus gigantocellularis, nuclei reticluaris pontis caudalis and oralis, nucleus paragigantocellularis, and nucleus subcoeruleus. Another major termination is in the parabrachial region, including the locus coeruleus and the parabrachial nuclei (Cross 1994; Willis & Westlund 1997). The spinoreticular tract is important for motivational-affective aspects of pain, and for descending modulatory mechanisms (Almeida et al. 2004; Millan 1999).

In addition, several other ascending nociceptive pathways have been described. The spino-limbic tracts consist of *the spinoreticulothalamic, the spinoamygdalar*, and *the spinohypothalamic pathways*. Pathways in the dorsal quadrant, such as *the spinocervicothalamic pathway* and the *postsynaptic dorsal column pathway*, are also present (Almeida et al. 2004; Millan 1999; Willis & Westlund 1997).

Cortical structures

The nociceptive information is transmitted from the thalamus to the cerebral cortex via the third-order neurons. Depending on their origin, the neurons terminate in different parts of the cortex. Neurons from the lateral thalamic

nuclei project to the primary somatosensory cortex (SI), where a conscious localisation and characterisation of the pain occurs. Neurons from the medial nuclei are projected to the anterior cingulate gyrus, which has been suggested to be involved in perception of suffering, and emotional reactions to pain. Several other areas of the cerebral cortex have also been described as important for the processing of nociceptive information and the experience of pain. The secondary somatosensory cortex (SII), regions of the interior and anterior parietal cortex, the insular cortex and the medial prefrontal cortex have all been identified as regions activated by noxious stimuli from cutaneous and intramuscular tissue (Casey 1999; Cross 1994; Davis et al. 1997; Riedel & Neeck 2001; Timmermann et al. 2001).

Basal ganglia

The basal ganglia, including the caudate nucleus, putamen, globus pallidus and substantia nigra, are important regions of the brain regarding motor functions. However, the basal ganglia have also been shown to be important for processing of nociceptive somatosensory information. Several studies have suggested the basal ganglia to be involved in the sensory-discriminative dimension of pain, the affective dimension of pain, the cognitive dimension of pain, modulation of nociceptive information, and sensory gating of nociceptive information to higher motor areas (Barker 1988; Chudler & Dong 1995).

Abnormal pain conditions

The neuroanatomical system presented above describes the mechanisms of the nociceptive system during normal conditions, i.e. when an acute painful sensation arises as a warning signal due to tissue damage. This type of pain is often denominated acute nociceptive pain. However, if the pain sensation for some reason persists and no longer serves as a warning signal, chronic pain may develop. Chronic pain is a major cause of suffering and is difficult to treat. It can be defined as a continuous or intermittent pain or discomfort which has persisted for at least three months (IASP 1986; Smith et al. 2001). Chronic pain may develop as a result of several changes in the nociceptive system. One significant factor for this is central sensitisation at the spinal level, which has been linked to the wind-up phenomenon. Another important factor for chronic pain is neuropathic pain, i.e. damage to the central or peripheral nervous system (Garry et al. 2004; Ossipov et al. 2000; Priest & Hoggart 2002).

Pain modulation

Antinociception

Although the ability to experience pain is vital for the survival of all mammals, it is essential for the organism to be able to control and modulate the pain sensation. The pain control is termed antinociception. The transmission of nociceptive information is rigorously controlled and modulated at most levels in the central nervous system, and the modulation appears to be hierarchically organised in the descending pain modulatory system.

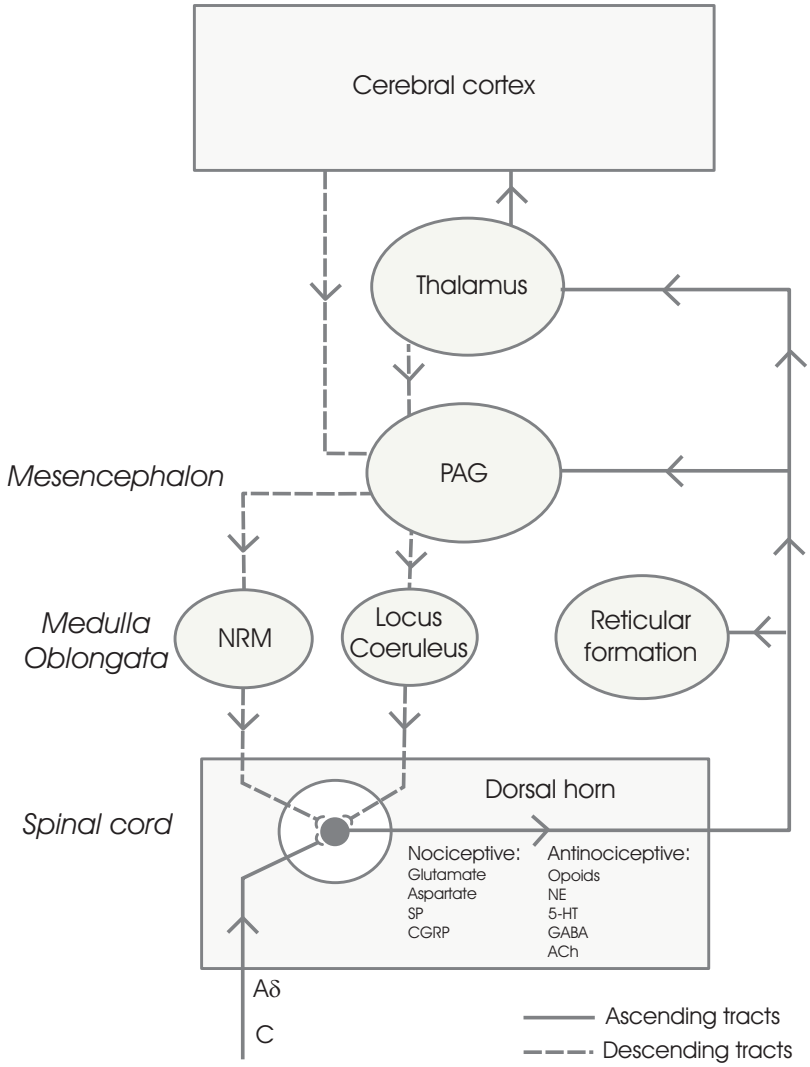

Figure 1. Simplified overview of the nociceptive and antinociceptive pathways.

Descending pain modulatory pathways

From different regions in the brain, such as the frontal and insular cortex, the thalamus, the amygdala and hypothalamus, projections of mainly opioid neurons descend to the PAG in the mesencephalon. The PAG plays an essential role in the modulation of nociceptive information at the supraspinal level, as it serves as a relay station for transmission of antinociceptive information to the lower brainstem. It is likely that antinociception can be generated from all regions of the PAG. However, the ventral portion (dorsal raphe nucleus) has been proposed to be the most effective (Bandler & Shipley 1994; Basbaum & Fields 1984; Harris 1996; Millan 2002).

From the PAG, neurons project to various areas in the reticular formation of the medulla. This includes the medullary nucleus, nucleus raphe magnus, reticularis magnocellularis, nucleus paragigantocellularis and noradrenergic medullary cell groups. In the reticular areas, the antinociception is again relayed and neurons project, directly or via interneurons, to the dorsal horn of the spinal cord. The spinal cord dorsal horn appears to be the level where the strongest antinociception occurs. (Basbaum & Fields 1984; Harris 1996; Millan 2002; Riedel & Neeck 2001).

Opiods can be considered the most important neurotransmitter type in the overall antinociceptive system, but there are several other neurotransmitters of importance in the descending pain modulatory system. In the PAG, GABAergic interneurons are likely involved in the reconnection to the reticular formation. The projections descending from the medulla to the dorsal horn mainly consist of serotonergic and noradrenergic neurons (Basbaum & Fields 1984; Harris 1996; Harris & Westbrook 1995; Millan 2002).

Antinociception at the spinal cord level

The ability to modulate pain at the spinal level has been known for a long time. In 1885, James Leonard Corning showed that cocaine administered into the perispinal space resulted in some degree of anaesthesia in humans (Corning 1885), and in 1899 August Bier tested several anaesthetics in the spinal cords of his assistant, his patients and himself. (Bier 1899).

GABA

In the spinal cord dorsal horn, nociceptive transmission can be inhibited in numerous ways. One of the major inhibitory transmitters is γ-amino butyric acid (GABA). Binding sites for GABA and GABA-containing neurons have been localised in almost all structures in the spinal cord, including interneurons and synaptic terminals. The highest concentration of GABA is in the dorsal horn, especially laminae I–III. There are two main GABA receptor types: the ligand-gated Cl^- channel $GABA_A$, and the GTP-binding protein coupled receptor $GABA_B$. Both types are important in antinociception at the

spinal level. Activation of GABAergic interneurons reduces the release of glutamate, SP and CGRP from primary nociceptive afferents (Fürst 1999; Malcangio & Bowery 1996; Schadrack & Zieglgänsberger 1998).

Opioids

Opioid receptors are important in supraspinal as well as spinal antinociceptive mechanisms. The three major types of opioid receptors are μ-, κ-, and δ-receptors. The μ-receptor is generally considered the most essential in antinociceptive actions, but the κ- and δ-receptors have also been shown to mediate antinociception. In addition, there is a fourth type of opioid receptor; the ε-receptor, which is thought to mediate β-endorphin-induced analgesia, but the existence of such a receptor is still controversial. The endogenous ligands for opioid receptors can be divided in three different families of opioid peptides; endorphins, enkephalins, and dynorphins (Fürst 1999; Kanjhan 1995; Tseng 2001). Opioids can exert their antinociceptive activity through numerous mechanisms. For instance, activation of opioid receptors can inhibit Ca^{2+} channels specifically on primary afferent C-fibres and thereby inhibit their spinal activity. Opioid receptors are also present on interneurons and cell bodies of second-order neurons, where the nociceptive information can be blocked (Ossipov et al. 2004; Taddese et al. 1995). In addition, the opioid system has been found to interact with NMDA receptors, which might contribute to the antinociceptive actions of opioids, but also to development of tolerance to and dependence of opioid agonists. The opioids may act by modulating the NMDA receptor-mediated electrophysiological events or by interacting at an intracellular level (Mao 1999).

Monoamines

The monoamines serotonin and norepinephrine are important neurotransmitters in spinal antinociception. Descending serotonergic and noradrenergic neurons terminate in the spinal cord dorsal horn, where endogenous serotonin (5-HT) and norepinephrine (NE) is released to inhibit nociceptive transmission (Fürst 1999; Yaksh 1979). There are several types of serotonergic and noradrenergic receptor types present in the spinal cord. Serotonergic receptors can be divided into three main classes; 5-HT_1, 5-HT_2 and 5-HT_3. In particular, 5-HT_1 and 5-HT_3 have been shown to play a role in spinal antinociception, although some subtypes seem to also facilitate nociceptive transmission (Ali et al. 1994; Ali et al. 1996; Fürst 1999; Hoyer et al. 1994).

The noradrenergic receptors involved in antinociception are generally α_2-adrenergic receptors, which consist of three subtypes: α_{2a}, α_{2b} and α_{2c}. Stimulation of spinal α_2-adrenergic receptors results in a very potent antinociception, as seen after intrathecal administration of α_2-adrenergic agonists (Eisenach et al. 1989; MacDonald et al. 1997; Reddy et al. 1980; Saunders & Limbird 1999).

Nitric oxide

NO is known to be involved in the transmission of nociceptive information, but several studies have also suggested a role for NO in antinociception. It has been shown to contribute to the antinociceptive actions of morphine as well as of adrenergic and cholinergic agonists, at both spinal and supraspinal levels (Iwamoto & Marion 1994a; Iwamoto & Marion 1994b; Xu & Tseng 1994; Xu et al. 1997)

Acetylcholinergic mechanisms

An involvement of the central cholinergic system in antinociception has been suggested by many studies (see below), but in comparison to other neurotransmitters, very little has been investigated and very little is known about the antinociceptive mechanisms of acetylcholine and its receptors.

Acetylcholinergic receptors

The acetylcholinergic receptors are divided in two main groups: muscarinic (mAChRs) and nicotinic (nAChRs) receptors.

Muscarinic receptors are G-protein coupled receptors with seven transmembrane domains. The muscarinic receptors were defined in 1914 by Sir Henry Dale as receptors activated by muscarine and blocked by atropine (Dale 1914). This definition was accepted until 1980, when pharmacological studies demonstrated that the effects mediated by mAChRs could not be due to merely one receptor type, but that there had to be two subtypes (Caulfield 1993; Hammer et al. 1980). Further pharmacological investigations showed that at least three (Birdsall et al. 1983; Caulfield & Straughan 1983) and later four (Waelbroeck et al. 1990) muscarinic subtypes existed. These pharmacologically defined subtypes were termed M1, M2, M3 and M4. Molecular biology techniques have made cloning of genes, coding for muscarinic receptors, possible, which has demonstrated the presence of five muscarinic receptor subtypes. The cloned subtypes are termed m1, m2, m3, m4 and m5 (Caulfield 1993; Hulme et al. 1990). The different subtypes differ in function with regard to their specific G-protein coupling and second messenger activation. Subtypes M1, M3 and M5 couple to the G_q protein, which activates the inositol polyphosphate generation, which in turn leads to a stimulated effect of the cell. Subtypes M2 and M4, on the other hand, couple to the G_i protein and thereby inhibit cAMP generation, which in turn inhibits the function of the cell (Caulfield 1993; Lambert 1993). Muscarinic receptors have been found in the spinal cord grey matter, including the superficial laminae, in several species such as humans and rats (Gillberg & Aquilonius 1985; Gillberg et al. 1988; Höglund & Baghdoyan 1997).

Nicotinic receptors are pentameric transmembrane proteins belonging to the family of ligand-gated ion-channels. Like the muscarinic receptors, they

were defined by Sir Henry Dale in 1914, as receptors that were activated by nicotine and blocked by curare (Dale 1914). The receptor consists of five subunits arranged symmetrically in the cell membrane to form a central pore. Several types of subunits have been described, which can be divided in two main categories: The α subunits (α1–9) that possess adjacent cysteines for acetylcholine binding, and the non-α subunits (β1–4, γ, δ, and ε) that lack the cysteines. About 15 mammalian subunits have been cloned (Corringer et al. 2000; Le Novere & Changeux 2001; Sargent 1993). Various nicotinic receptor subtypes are present in the spinal cord, also in the superficial laminae. However, the nicotinic receptors appear to exist in lower quantities than muscarinic receptors (Gillberg et al. 1988; Khan et al. 1994; Khan et al. 1997).

Acetylcholine in pain modulation

As mentioned, several studies have been undertaken to investigate the involvement of the acetylcholinergic receptor system in antinociception at both the supraspinal and the spinal level. Systemic as well as intrathecal administration of muscarinic agonists produces potent antinociception in several species (Abram & O'Connor 1995; Gillberg et al. 1989; Gower 1987; Iwamoto & Marion 1993b; Lambert & Appadu 1995; Yaksh et al. 1985; Zhuo & Gebhart 1991). Different pharmacological studies have attempted to determine the spinal muscarinic subtypes relevant for the antinociceptive effect, suggesting involvement of the M1, M2, M3 and M4 subtypes (Bartolini et al. 1992; Duttaroy et al. 2002; Ellis et al. 1999; Lograsso et al. 2002; Naguib & Yaksh 1997).

Neuronal nicotinic receptors are considered a promising target in pain treatment (Flores & Hargreaves 1998). An involvement of nicotinic receptors in antinociception has been known for several decades. In 1932, antinociception of nicotine was reported (Davis et al. 1932), an effect that has been verified by other studies (Iwamoto 1991; Sahley & Berntson 1979). Other nicotinic agonists that produce antinociception after supraspinal or systemic administration are epibatidine (Curzon et al. 1998; Lawand et al. 1999; Qian et al. 1993), A85380 (Curzon et al. 1998) and ABT-594 (Bannon et al. 1998; Bitner et al. 1998). The antinociceptive effects of nicotinic agonists administered into the spinal cord are somewhat controversial, since both nociceptive and antinociceptive effects have been observed (Khan et al. 1998).

The acetylcholinergic receptor system has been found to interact with most other receptor systems in the spinal cord. Muscarinic receptors have been shown to be involved in spinal antinociceptive mechanisms mediated by the GABAergic (Baba et al. 1998; Chen & Pan 2003), opioid (Chen & Pan 2001; Harris et al. 1969; Pert 1975), and adrenergic (Detweiler et al. 1993; Honda et al. 2002; Honda et al. 2003; Klimscha et al. 1997; Pan et al. 1999) receptor systems. Nicotinic receptors are also involved in modulation of nociceptive information by other receptor systems. Interactions with par-

ticularly the serotonergic and adrenergic systems have been demonstrated (Bitner et al. 1998; Cordero-Erausquin & Changeux 2001; Iwamoto & Marion 1993a; Li & Eisenach 2002). In addition, both muscarinic and nicotinic receptors have been suggested to play an important role in the antinociceptive mechanism of NO in the spinal cord (Xu et al. 2000; Xu et al. 1996).

Based on these findings, there should be little doubt that the acetylcholinergic receptor system is an important component in antinociceptive mechanisms. However, the underlying mechanisms responsible for the cholinergic contribution to spinal antinociception are far from fully understood.

One conceivable explanation could be that stimulation of muscarinic or nicotinic receptors results in a release of acetylcholine in the spinal cord, and that acetylcholine in turn inhibits the nociceptive transmission. In 1945, a study showed that subcutaneous injection of the acetylcholine esterase inhibitor neostigmine (prostigmine) significantly increased the antinociceptive effect of morphine in humans (Flodmark & Wramner 1945). In more recent studies, intrathecal administration of neostigmine has revealed that part of the antinociceptive effect of this substance is mediated at the spinal cord level in both humans and animals (Bouaziz et al. 1995; Hood et al. 1995; Hwang et al. 1999). Since neostigmine prevents degradation of acetylcholine in the synaptic cleft, the amount of acetylcholine should be increased. Thus, this strengthens the theory that endogenous acetylcholine is contributing to the inhibition of nociceptive information at the spinal cord level. A few studies have been performed to evaluate this theory (Bouaziz et al. 1996; Eisenach et al. 1996), but much is yet to discover.

Recently, an experimental setup was designed to study the release of acetylcholine in the spinal cord of rats by using microdialysis. This study demonstrated that both muscarinic and nicotinic receptor ligands dose-dependently affected the release of acetylcholine in the dorsal region of the rat spinal cord (Höglund et al. 2000). The microdialyis technique established in this study has made it possible to accurately perform *in vivo* studies of the pharmacological mechanisms of acetylcholine, in relation to pain modulation in the spinal cord.

Aims of the study

General aim

The overall aim of this thesis was to further investigate the role of intraspinal acetylcholine in the mechanisms mediating pain modulation at the spinal cord level of the rat.

Specific aims

The specific aims of the thesis were:

- to test the hypothesis that spinal acetylcholine contributes to the regulation of pain transmission at the spinal cord level, by investigating the relationship between spinal release of acetylcholine and the thermal pain threshold after treatment with a muscarinic receptor agonist and an antagonist.

- to study how systemic administration of lidocaine, in analgesic doses, affects the intraspinal release of acetylcholine, and to investigate the involvement of muscarinic and nicotinic receptors in this effect.

- to study the interactions between the acetylcholinergic and α_2-adrenergic receptor systems at the spinal level, and to determine the role of muscarinic and nicotinic receptors in these interactions.

- to study the possible role of spinal acetylcholine in the modulation of non-inflammatory pain conditions by aspirin and paracetamol.

Materials and Methods

Animals

All animal experiments presented in this thesis were approved by the Animal Ethics Committee in Uppsala, Sweden. In total, 244 adult, male outbred Sprague-Dawley rats (B&K, Sollentuna, Sweden) were included in the different studies. The animals were housed in groups of 4-5 rats in Makrolon cages, size IV (59 cm × 38 cm × 20 cm). They had free access to food (R36, Ewos, Vadstena, Sweden) and tap water at all times. The animals were kept in rooms with a temperature of 20 ± 2 ^{o}C and a relative humidity of about 50%. The air was changed approximately 15 times per hour. Diurnal rhythm was regulated with a 12-h light:12-h dark cycle with lights on 6.00 a.m.–6.00 p.m. The animals were acclimatised after delivery for one week before they were used.

Intraspinal microdialysis (I–IV)

Anaesthesia

In the first two studies, anaesthesia was induced with Brietal (methohexital sodium), 40 mg/kg intraperitoneally. During anaesthesia, the animals were intubated and connected to a Harvard ventilator (Harvard Apparatus Inc., South Natic, Massachusetts, USA). Brietal anaesthesia was subsequently replaced with isoflurane in 100% oxygen. During study II, the use of Brietal® for induction of anaesthesia was omitted and anaesthesia was instead induced by 4.5% isoflurane. The latter induction method was used in studies III and IV. The reason for changing induction method was the suspicion that methohexital could affect acetylcholinergic receptors and thereby the results of the experiments. Although we could see no effect on the relative changes in acetylcholine release after methohexital induction compared to isoflurane induction (study II), we decided to continue using isoflurane for induction, as gas anaesthesia is less stressful than injectable anaesthesia for the animals (O'Brien et al. 1995). Anaesthesia was maintained with isoflurane 2.5 – 3% during surgery and isoflurane 1.3% during sampling of dialysate. Body core temperature was maintained at 37.5^{o}C and end-tidal pCO_2 was kept at 4.0 kPa.

Surgical preparation

A U-shaped spinal microdialysis probe (Marsil Enterprises, San Diego, CA, USA), was placed in the spinal cord for sampling of acetylcholine, and for administration of substances. For insertion of the microdialysis probe, the rat's head was fixed, and a midline incision was made at the back of the scull. The neck muscles were dissected to expose the cisterna magna. The dura and pia mater were cut open and the spinal microdialysis probe was placed along the dorsal part of the spinal cord so that the tip of the probe was approximately at the C5 level. For intravenous administration of substances (studies I and II), a polyethylene catheter was inserted in the femoral vein. After completion of surgery, the rats rested for 40 minutes before sampling was carried out. The experimental setup is shown in figure 2.

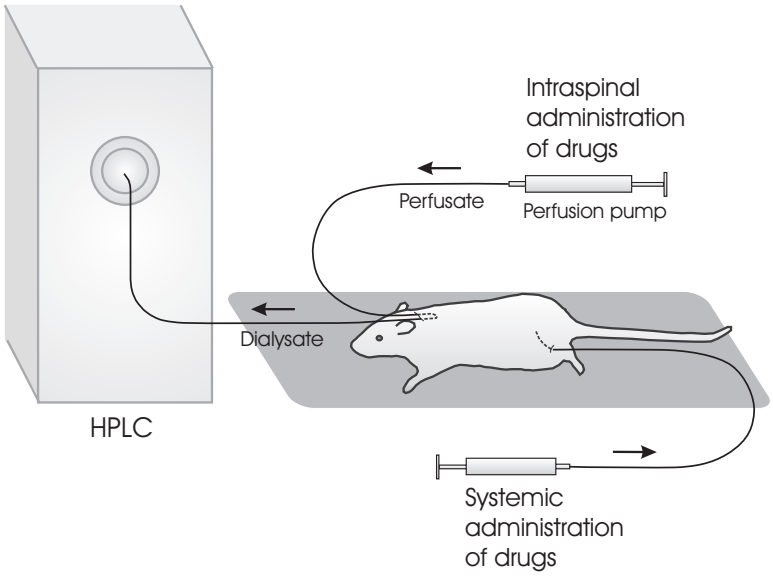

Figure 2. Experimental setup of the intraspinal microdialysis.

Microdialysis

The dialysis probe consisted of a dialysis membrane, constructed by a hollow fibre with an outer diameter of 300 μm and with a cut-off at a molecular weight of 11 kDa. The dialysis membrane was bent to form a U-shaped loop with a length of 12 mm.

The probe was perfused with Ringer's solution (147 mM NaCl, 2.4 mM $CaCl_2$ and 4.0 mM KCl), containing 10 μM of the acetylcholine esterase inhibitor neostigmine to prevent degradation of acetylcholine, and thereby make detection possible. The use of neostigmine has been found not to influ-

ence drug-receptor pharmacology in several studies (Billard et al. 1995; Damsma et al. 1987; Damsma et al. 1988; Höglund et al. 2000; Roth et al. 1996). In studies I and II, the perfusion rate was 3 µl/min, since dialysate was collected in tubes in samples of 30 µl, of which 20 µl were injected into the HPLC. In studies III and IV, dialysate was injected online into the HPLC-loop, and the perfusion rate was set at 2.5 µl/min.

The probe recovery was determined *in-vitro* before and after each experiment, to ensure that the recovery was stable throughout the whole experiment, and that detected changes in acetylcholine release actually reflected the situation in the tissue and were not confounded by damage to the probe during the experiment.

Acetylcholine analysis (I–IV)

High Performance Liquid Chromatography (HPLC) with electrochemical detection (Antech, Leyden, The Netherlands) was used to analyse acetylcholine in the dialysate. The dialysate samples were loaded into a 20 µl loop and thereafter transported into the HPLC columns (Acetylcholine/Choline analytical and IMER columns, Bioanalytical Science Inc.). A phosphate buffer (50 mM dibasic Na_2HPO_4, pH 9.0, flow rate at 1 ml/min) was used as mobile phase. Substances in the dialysate were separated on the analytical column, and further transported into the immobilised enzyme reactor (IMER) column, containing acetylcholine esterase and choline oxidase. In the enzymatic column, acetylcholine was degenerated to choline, which in turn was oxidised by the choline oxidase. The latter reaction produced hydrogen peroxide, which was detected by a platinum electrode with a voltage of 500 mV. This enabled detection of acetylcholine after 4.25 minutes, followed by choline after 5.5 min.

A standard calibration curve ranging from 1 pmol to 20 pmol (corresponding to 50 nM–1 µM) acetylcholine and choline was established before each experiment from two samples of each concentration. During experiments, each dialysate sample was collected during 10 min. After the 40-min resting period, five samples were analysed to calculate the baseline release of acetylcholine. After a stable baseline was established, drugs were administered either intraspinally via the microdialysis probe (I – IV) or systemically by intravenous (I and II) or subcutaneous (IV) injections, and the relative changes of acetylcholine release compared to baseline levels were measured. The term ‘release’ is used throughout this thesis, when describing basal levels as well as changed levels of acetylcholine. This is the term commonly used when describing the amount of a neurotransmitter at a certain time point in the tissue.

Pain behavioural tests (I)

Thermal pain threshold in the tail was investigated using the tail-flick latency test. The investigation was undertaken to evaluate the effects on pain threshold of systemically administered oxotremorine and atropine, in doses that were found to affect the intraspinal acetylcholine release.

All animals were handled daily one week before the tail-flick tests started, in order to condition the animals to the experimental procedure. Tail-flick latency was measured using an IITC model 33 analgesia meter (IITC Inc., Woodland Hills, CA, USA) with a sensitivity setting of 10, beam at 8, and a cut-off time set at 12 sec. Baseline tail-flick latency was obtained by systemic administration of saline. Drugs were injected systemically and tail-flick latencies were measured and compared to baseline.

Receptor binding studies (III)

To study the binding properties of various ligands to spinal muscarinic and nicotinic receptors, competition binding assays were performed on homogenised spinal cord tissue. Rats were decapitated and the spinal cord was removed by flushing the spinal canal with high pressure using a 12.5 ml syringe containing ice-cold saline. Spinal cords were homogenised in 50 × volumes of ice cold 50 mM phosphate buffer (50 mM dibasic Na_2HPO_4, 50 mM monobasic $NaH_2PO_4 \bullet H_2O$ and 1 mM $MgCl_2$). The homogenate was centrifuged 48.000 × g for 10 min. The pellet was resuspended in fresh buffer, then homogenised and centrifuged as above. The second pellet was resuspended in distilled water to degrade the cell membranes and bring the receptors free in suspension. The suspension was homogenised and centrifuged and the final pellet was resuspended in fresh buffer.

The competition assay mixture consisted of 3.75 mg tissue homogenate (corresponding to approximately 200 μg protein) in a final incubation volume of 1 ml. The homogenate was incubated with 260 pM [N-methyl-^{3}H]scopolamine for muscarinic receptor binding and 500 pM [^{3}H]epibatidine for nicotinic receptor binding, respectively, and twelve concentrations of a competitive ligand ranging from 1 nM to 1 mM. Each concentration was done in duplicates. Protein quantity was determined by Bio-Rad DC protein assay. The assay buffer was the same as the homogenisation buffer. Competition binding assays were initiated by the addition of the tissue suspension to the mixture of radioactive ligand and competitive ligand followed by rapid mixing. Incubation was carried out in room temperature for four hours. The assays were terminated by dilution of 3 ml ice-cold buffer followed immediately by rapid filtration under vacuum through Whatman GF/C filter papers, pre-soaked in 0.1% polyethyleneimine. The filters were then rinsed twice with 3 ml ice-cold buffer, placed in counting vials and mixed vigorously

with 5 ml scintillation fluid (Zinsser Analytic). The vials were counted in a Phillips liquid scintillation counter. Specific binding was determined as the difference between total binding and binding in the presence of 1 μM atropine for the muscarinic assay and 40 μM nicotine for the nicotinic assay respectively.

Statistical analysis

Microdialysis data

The intraspinal microdialysis data were analysed using analysis of variance (ANOVA) and general linear model (GLM) using SPSS versions 10.0–11.5.

To compare relative changes of acetylcholine release at each time point against baseline release, ANOVA with Dunnett's post-hoc test was performed. To calculate the differences between groups of animals treated with different concentrations or doses of substances, the data was analysed with ANOVA with Tukey's post-hoc test, and/or GLM with repeated measures. P values < 0.05 were considered significant.

Tail-flick tests

Changes in tail-flick latencies after treatment with atropine were compared to baseline using ANOVA with Dunnett's post-hoc test. After treatment with oxotremorine, however, the tail-flick latency reached the cut-off time of 10 sec., why the non-parametric Wilcoxon Signed Ranks Test was used to analyse the differences against baseline.

Competition binding assays

Competition binding data were analysed in GraphPad Prism version 3.0. IC_{50} values and k_i values of each substance were determined by non-linear regression. Calculation of k_i values for the nicotinic receptor binding was performed with the two-site binding of [^{3}H]epibatidine to nicotinic receptors taken into consideration. To determine whether the curves were best fit to a one-site binding or two-site binding, the two types of curves were compared using the F test.

Drugs and chemicals

The anaesthetic drug Brietal® (methohexital sodium) was purchased from Eli Lilly, Indianapolis, Indiana, USA, and Forene® (isoflurane) was purchased from Abbot Scandinavia, Kista, Sweden. The chemicals NaCl, $CaCl_2$ and

KCl for Ringer's solution as well as the Na_2HPO_4 for the mobile phase were obtained from Kebo Lab, Spånga, Sweden (later VWR International AB, Stockholm Sweden) and neostigmine bromide was purchased from Sigma-Aldrich, Stockholm, Sweden. Acetylcholine chloride and choline chloride for preparation of standard calibration curves were also obtained from Sigma-Aldrich. All chemicals used for the competition binding buffer were purchased from Kebo Lab (later VWR). The various drugs and chemicals used for investigating acetylcholinergic mechanisms in the different experiments are presented in table 1.

Table 1. *Drugs and chemicals used in studies I–IV*

Drug	Study	Main properties	Manufacturer
Oxotremorine	I	Muscarinic receptor agonist	Sigma-Aldrich, Stockholm, Sweden
Atropine	I, II	Muscarinic receptor antagonist	Sigma-Aldrich, Stockholm, Sweden
Lidocaine	II	Local anaesthetic, Na^+-channel blocker	Sigma-Aldrich, Stockholm, Sweden
Mecamylamine	II, III	Nicotinic receptor antagonist (non-specific, non competitive)	Sigma-Aldrich, Stockholm, Sweden
Clonidine	III	α_2-adrenergic receptor agonist; Imidazoline I_1 receptor ligand	Sigma-Aldrich, Stockholm, Sweden
Yohimbine	III	α_2-adrenergic receptor antagonist	Sigma-Aldrich, Stockholm, Sweden
Rilmenidine	III	α_2-adrenergic receptor agonist; Imidazoline I_1 receptor ligand	Tocris, Bio-Nuclear, Bromma, Sweden
Efaroxan	III	α_2-adrenergic receptor antagonist; Imidazoline I_1 receptor ligand	Tocris, Bio-Nuclear, Bromma, Sweden
[N-methyl-^{3}H]scopolamine	III	Muscarinic receptor antagonist (tritium labelled)	Amersham Pharmacia Biotech, Uppsala Sweden
[^{3}H]epibatidine	III	Nicotinic receptor agonist (tritium labelled)	Amersham Pharmacia Biotech, Uppsala Sweden
Acetylsalicylic acid	IV	Analgesic, antipyretic and anti-inflammatory drug	Sigma-Aldrich, Stockholm, Sweden
Paracetamol (acetaminophen)	IV	Analgesic and antipyretic drug	Sigma-Aldrich, Stockholm, Sweden

Results

Study I

Effects of muscarinic receptor ligands on intraspinal acetylcholine release and pain threshold

In this study, a possible relation between intraspinal acetylcholine release and pain threshold was investigated. The study was designed to test the hypothesis that systemically administered oxotremorine, in doses that increase pain threshold, also increases the intraspinal acetylcholine release, while systemic administration of hyperalgesic doses of atropine decreases the release of acetylcholine.

It was found that intravenous administration of 30 µg/kg oxotremorine resulted in a 20% increase of intraspinal acetylcholine release for 10 min after injection. Oxotremorine in doses of 100–300 µg/kg increased the acetylcholine release 27% and 73% respectively, and in this case, the increase persisted for 20 min after injection (figure 3).

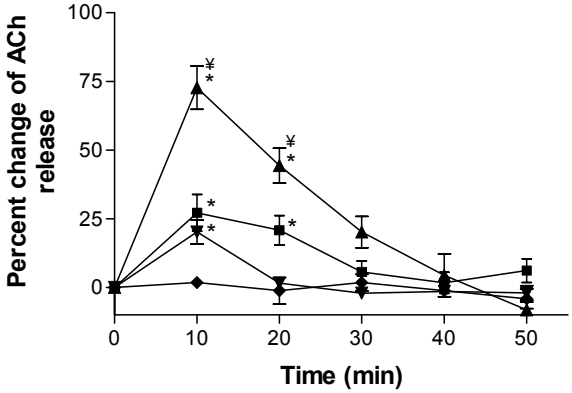

Figure 3. The effects on intraspinal acetylcholine release of intravenously injected oxotremorine in doses of 10 (W), 30 (R), 100 (V) and 300 (Q) µg/kg. The effect is expressed as percent change from baseline.

Systemic administration of oxotremorine in doses of 100 and 300 µg/kg significantly increased tail-flick latency 20 min after injection (figure 4).

During microdialysis, typical clinical signs of muscarinic receptor activation, such as increased salivation and decrease of body temperature, were observed after administration of oxotremorine. The increase of salivation was observed only after 100 and 300 µg/kg. During the tail-flick tests, further signs of muscarinic receptor activation were observed. Extensive salivation was observed after injection of 100 µg/kg, and increased salivation, lacrimation and defecation was observed after 300 µg/kg.

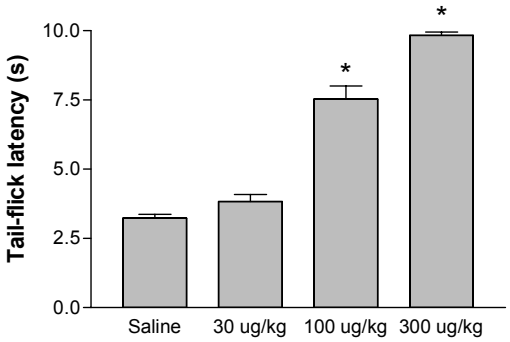

Figure 4. The effects on tail-flick latency of systemically administered oxotremorine and saline control.

Intravenous administration of 5000 µg/kg atropine during microdialysis was found to cause a persistent decrease of the intraspinal acetylcholine release. The decrease was eventually stabilised at approximately 30% (figure 5). The dose of 5000 µg/kg was also found to significantly decrease tail-flick latency 15 min after intraperitoneal injection.

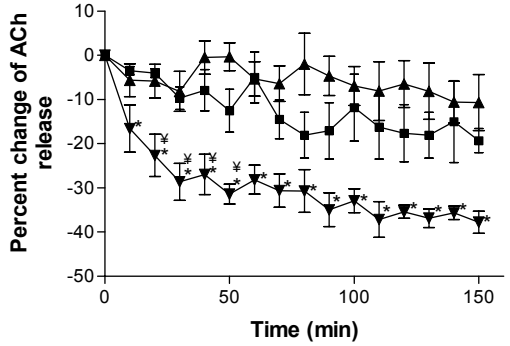

Figure 5. The effects on intraspinal acetylcholine release of intravenously injected atropine in doses of 0.1 (ⱽ), 10 (ᵠ) and 5000 (ᴿ) µg/kg. The effect is expressed as percent change from baseline.

Intraspinal administration of atropine in concentrations of 0.1, 1, and 10 nM resulted in a persistent significant decrease of intraspinal acetylcholine release compared to baseline. The maximum decrease was stabilised at about -30%. None of the routes of administration of atropine caused any detectable changes in body temperature.

The main conclusion that can be drawn from this study is that changes in intraspinal acetylcholine release contribute to the regulation of pain threshold at the spinal cord level. An increase of acetylcholine of approximately 27% increases pain threshold, while a 30% decrease of acetylcholine decreases pain threshold.

Study II

Effects of lidocaine on intraspinal acetylcholine release

The aims of this study were first; to test the hypothesis that systemic administration of the local anaesthetic lidocaine, in analgesic doses, affects the intraspinal acetylcholine release, and second; to investigate the importance of muscarinic and nicotinic receptors for this effect.

Intravenous administration of lidocaine in doses of 10 and 30 mg/kg significantly increased the intraspinal release of acetylcholine. The lower dose produced an increase of 117% at 20 min after injection. The increase declined over time but lasted for another 30 minutes. The higher dose resulted in an increase that lasted throughout the whole experiment, with a maximum effect of 137% at 20 min after injection.

Both muscarinic receptor blockade by 100 μM intraspinal atropine, and nicotinic receptor blockade by 50 μM intraspinal mecamylamine, significantly attenuated the effect of intravenous lidocaine (figure 6).

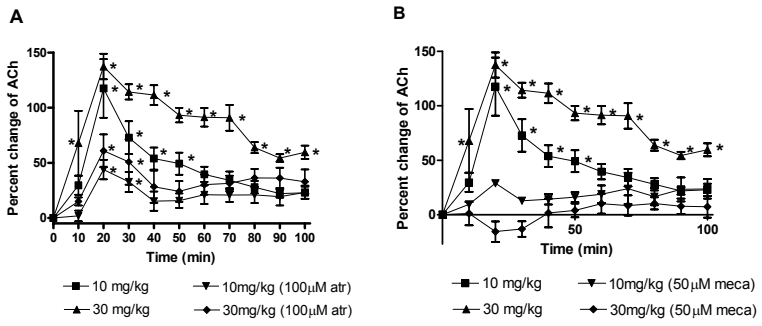

Figure 6. The effects of 10 and 30 mg/kg intravenously injected lidocaine on spinal acetylcholine release, in comparison with the effect during **A)** muscarinic receptor blockade and **B)** nicotinic receptor blockade.

These data suggest that the central analgesic effect of systemic lidocaine is acting through the same mechanisms as muscarinic agonists, by increasing the intraspinal release of acetylcholine. The action of lidocaine is evidently involving both spinal muscarinic and spinal nicotinic receptors.

Study III

Interactions between the α_2-adrenergic and acetylcholinergic receptor systems

This study was designed to thoroughly investigate the effects of various α_2-adrenergic agonists and antagonists on intraspinal acetylcholine release *in-vivo*, and their binding properties to muscarinic and nicotinic receptors *in-vitro*.

***In vivo* experiments**

The α_2-adrenergic receptor agonists clonidine and rilmenidine, and antagonists yohimbine and efaroxan, all affected the intraspinal release of acetylcholine when administered intraspinally (figure 7).

Clonidine 30 μM–1 mM and rilmenidine 10 μM–1 mM, both increased the acetylcholine release in a dose-dependent manner. The effect of rilmenidine appeared to be more potent than that of clonidine. The calculated maximum effect of clonidine was 327% increase, while the maximum effect of rilmenidine was calculated to 703%.

Yohimbine and efaroxan affected intraspinal acetylcholine release differently. Yohimbine in a range of 10 μM–1 mM decreased the intraspinal acetylcholine release in a dose-dependent manner with a calculated maximum of -70%. Efaroxan, on the other hand, affected the acetylcholine release differently depending on concentration. Lower concentrations (10–100 nM) decreased, while higher concentrations (100 μM–10 mM) increased the spinal acetylcholine release. The increase was at highest after 1 mM efaroxan. After 10 mM efaroxan, the increase declined over time. Since efaroxan affected the acetylcholine release in different manners, no dose-response curve could be established.

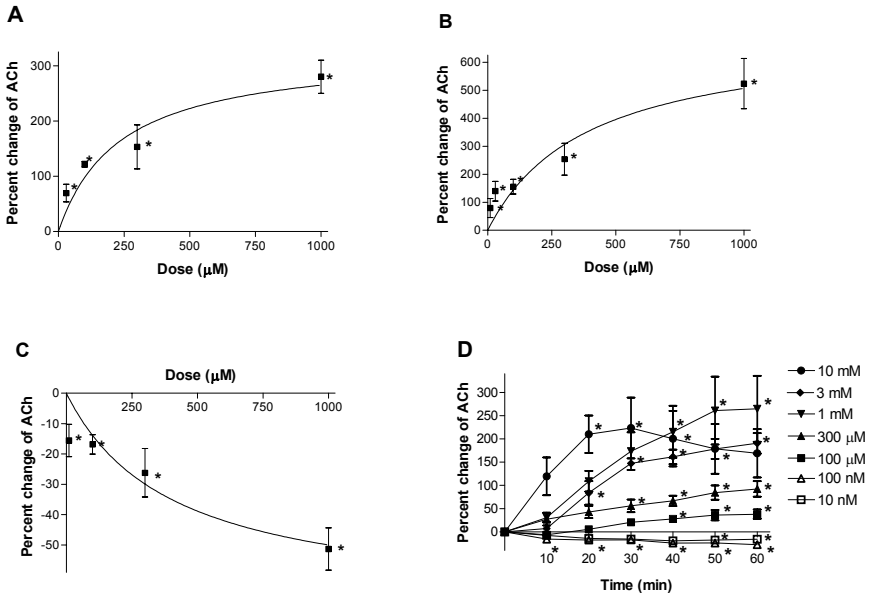

Figure 7. The effects on intraspinal acetylcholine release after intraspinal administration of **A)** clonidine, **B)** rilmenidine, **C)** yohimbine and **D)** efaroxan. **A**–**C** are shown as dose-response curves while **D** is shown as a time-response curve concentration by concentration.

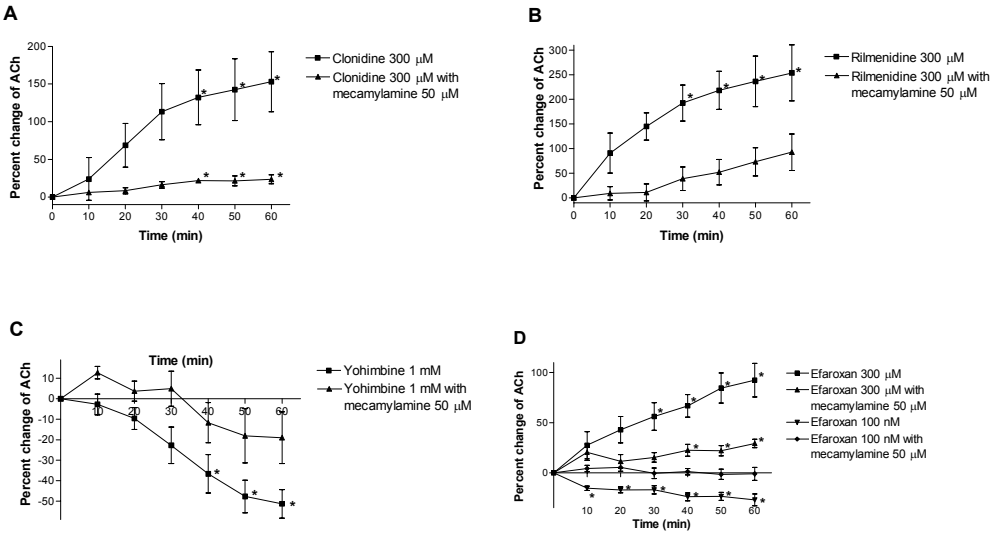

Figure 8. The effects on intraspinal acetylcholine release after intraspinal administration of **A)** clonidine, **B)** rilmenidine, **C)** yohimbine and **D)** efaroxan, with or without nicotinic receptor blockade.

The α_2-adrenergic ligands were also administered intraspinally during nicotinic receptor blockade, i.e. in presence of 50 μM mecamylamine. The observed effects on intraspinal acetylcholine release were compared with the effects in absence of mecamylamine. It was found that nicotinic receptor blockade significantly attenuated the effects of all ligands (figure 8).

***In vitro* experiments**

Competition binding assays demonstrated that none of the investigated α_2-adrenergic ligands possessed binding capacity for spinal muscarinic receptors. It was found, however, that all ligands were able to bind to spinal nicotinic receptors. The statistical analysis suggested curves best fit to one-site binding for clonidine and yohimbine, and two-site binding for rilmenidine and efaroxan. All binding data are presented in table 2.

Table 2. *Competition binding experiments for α_2-adrenergic ligands to nicotinic receptors*

Competitor	B_{max} (fmol/mg)	High affinity sites IC_{50} values (nM)	k_i values (nM)	Low affinity sites IC_{50} values (nM)	k_i values (nM)
Clonidine	85.2 ± 28.2	–	–	2590 ± 1.3	1084 ± 1.3
Rilmenidine	57.5 ± 0.8	106 ± 2.5	9.64 ± 2.4	3553 ± 1.2	1422 ± 1.3
Yohimbine	86.3 ± 31.6	–	–	5226 ± 1.3	2188 ± 1.3
Efaroxan	54.8 ± 0.8	193 ± 2.2	23.8 ± 2.2	8571 ± 1.6	3589 ± 1.6

Nicotinic receptor binding of clonidine, rilmenidine, yohimbine and efaroxan in competition with [3H]epibatidine in rat spinal cord. Number of experiments (n) = 3 for each competitor. Each experiment was performed in duplicate. B_{max} represents the maximum binding of the radioligand.

The data in this study demonstrate that all the investigated α_2-adrenergic ligands interact with the acetylcholinergic receptor system in the spinal cord and affect the release of acetylcholine. The facts that nicotinic receptor blockade inhibits the actions, and that all ligands bind to nicotinic receptors; strongly indicate that the interactions are mediated by spinal nicotinic receptors.

Study IV

Effects of peripheral analgesics on intraspinal acetylcholine release

The aim of this study was to investigate how systemic and intraspinal administration of the analgesics aspirin and paracetamol affect the intraspinal release of acetylcholine.

It was found that subcutaneous aspirin in doses of 100 and 300 mg/kg resulted in a small and short-lasting increase of spinal acetylcholine release. Subcutaneous paracetamol (300mg/kg), on the other hand, clearly decreased the acetylcholine release throughout the whole experiment (figure 9). Intraspinally administered aspirin or paracetamol had no effect on the acetylcholine release.

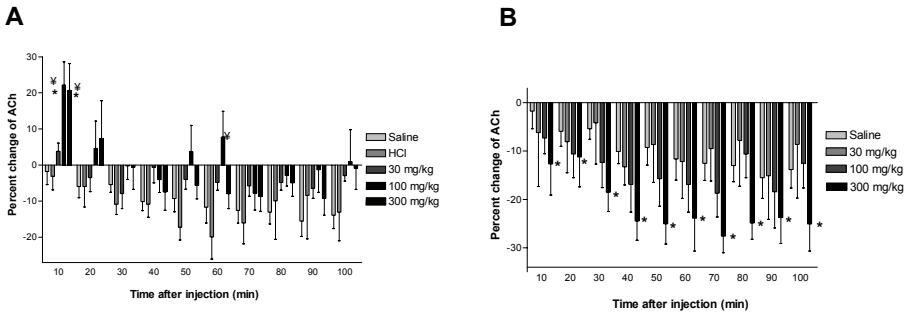

Figure 9. The effect of subcutaneously administered **A)** acetylsalicylic acid and **B)** paracetamol on the intraspinal release of acetylcholine.

In conclusion, these data demonstrate that peripheral analgesics such as aspirin and paracetamol act through other mechanisms than merely cyclooxygenase inhibition, in order to produce analgesia. These other mechanisms may involve the acetylcholinergic receptor system.

Discussion

The spinal acetylcholinergic system has long been suggested to contribute to the modulation of pain transmission at the spinal cord level, although little has been known about this system in comparison to other pain inhibitory neurotransmitters. The studies presented in this thesis have all aimed to further investigate the function of acetylcholine as a pain modulator in the spinal cord. The microdialysis technique used has been a very efficient tool, since it has made it possible to study the spinal acetylcholine release in a very accurate manner *in vivo*. The use of the spinal microdialysis technique, alone as well as in combination with other methods, has contributed to a further understanding of the role of acetylcholine in spinal pain modulation.

The results from each study will be discussed in detail below. To summarise, the data presented in this thesis clearly demonstrate that an increased release of acetylcholine in the spinal cord contributes to the modulation of pain transmission. It is also clear that the intraspinal release of acetylcholine is regulated by several other substances involved in pain modulation, and that muscarinic as well as nicotinic receptors are of importance in these mechanisms.

Acetylcholine and pain threshold

Since muscarinic receptor agonists have antinociceptive effects after systemic as well as intrathecal administration (Abram & O'Connor 1995; Gillberg et al. 1989; Gower 1987; Iwamoto & Marion 1993b; Lambert & Appadu 1995; Yaksh et al. 1985; Zhuo & Gebhart 1991), and since muscarinic agonists increase the release of acetylcholine after intraspinal administration (Höglund et al. 2000), it was assumable that an increased release of intraspinal acetylcholine is involved in mediating the antinociceptive effect. Data demonstrating a nociceptive effect after administration of a muscarinic antagonist (Ghelardini et al. 1990) gave further strength to this idea. However, prior to the present study (I), no attempt had been made to confirm a relationship between the increased acetylcholine release and the increase in pain threshold. The effect of systemic treatment with muscarinic agonists on the intraspinal acetylcholine release had not been shown either. Thus, a hypothesis that an increase in spinal acetylcholine release would produce antino-

ciception, and that a decrease in acetylcholine release would produce hyperalgesia, was tested.

The results demonstrate that systemic administration of oxotremorine in doses of 100 and 300 μg/kg produced a potent antinociceptive effect in thermal pain, as seen in the tail-flick test. This effect of oxotremorine corresponded well to previous findings (Gower 1987; Machelska et al. 1999). The antinociceptive doses of oxotremorine were also found to significantly increase the intraspinal release of acetylcholine during microdialysis. Thus, these findings, taken together with earlier reports, indicate a relationship between the intraspinal acetylcholine release and the increase in pain threshold, and strengthen our hypothesis. The decrease in intraspinal acetylcholine release after systemic atropine 5000 μg/kg also supports our hypothesis, as this dose caused a decrease in thermal pain threshold.

The tail-flick test requires a cut-off time in order to avoid tissue damage in the animal. Treatment with oxotremorine entailed that the tail-flick latency reached the cut-off level in a few animals after 100 μg/kg, and in almost all animals after 300 μg/kg. This means that the tail-flick test is not a fully gradable response, which unfortunately makes it difficult to correlate the increase of intraspinal acetylcholine with the increase in pain threshold. However, the results demonstrate that a 27% increase of intraspinal acetylcholine release is sufficient to obtain a potent antinociceptive effect, while a decrease of acetylcholine of 30% was associated with hyperalgesia. Part of this effect is likely mediated by muscarinic receptors at the spinal level, since both oxotremorine (Höglund et al. 2000) and atropine affected the acetylcholine release after intraspinal administration.

Acetylcholine and centrally acting lidocaine

Lidocaine is commonly used as a local anaesthetic, acting through a fast-sodium-channel blocking mechanism. However, lidocaine also has a pain suppressing effect on both neuropathic and non-neuropathic pain when given systemically in humans and animals (Attal et al. 2000; Bartolini et al. 1987; Dirks et al. 2000; Ferrante et al. 1996; Rigon & Takahashi 1996). Several studies have implied an involvement of central nervous receptor mechanisms, such as α_2-adrenergic (Kawamata et al. 1997; Uchihashi et al. 2000) and opioid (Fraser et al. 1992; Saito et al. 1998), in systemic antinociception of lidocaine. In addition, the acetylcholinergic system has been suggested to participate in the central antinociception of lidocaine. Lidocaine has been shown to compete with a muscarinic receptor agonist in binding to muscarinic receptors *in vitro* (Taylor et al. 1980), and muscarinic antagonists are able to block the lidocaine-induced antinociceptive effect *in vivo* (Bartolini et al. 1987). Lidocaine has also been shown to bind to nicotinic receptors in

a non-competitive manner (Arias 1999; Ryan & Baenziger 1999). Because of these mechanisms, it was hypothesised that systemic lidocaine produces antinociception in the same manner as muscarinic agonists, by increasing the intraspinal release of acetylcholine. In study II, this hypothesis was tested, and the involvement of muscarinic and nicotinic receptors was investigated.

Doses of lidocaine that produce central antinociception in mice, rats and humans (Bartolini et al. 1987; Ferrante et al. 1996), clearly enhanced the release of acetylcholine in the spinal cord when given systemically. This finding gives further strength to the theory that an increased release of acetylcholine is associated with an increased pain threshold.

To further evaluate the mechanisms of lidocaine, the acetylcholine increasing effect was studied during muscarinic and nicotinic receptor blockade. The effect of lidocaine was significantly attenuated in the presence of a muscarinic and nicotinic antagonist, respectively. Thus, both muscarinic and nicotinic receptors are evidently essential for the regulation of acetylcholine release, and accordingly for the central antinociception, mediated by lidocaine. The study provides no evidence for whether the effect of lidocaine is mediated by a direct or indirect action on acetylcholinergic receptors. However, since lidocaine is known to possess binding properties for muscarinic as well as nicotinic receptors, a direct action cannot be excluded.

It is noteworthy that the shape of curves in figure 6 differs after muscarinic blockade compared to nicotinic blockade. After muscarinic receptor blockade, the time-effect curve follows the same pattern as after injection with lidocaine only, which indicates that lidocaine is acting in a competitive manner directly on muscarinic receptors. In the case of nicotinic receptor blockade, the acetylcholine increase is completely abolished, and the curve shape is different after 10 mg/kg compared to 30 mg/kg lidocaine. This indicates that nicotinic receptors are important for mediating the effect of lidocaine, but also that lidocaine, depending on dose, affects nicotinic receptor subtypes with different sensitivity to lidocaine.

Acetylcholine in α_2-adrenergic mechanisms

Previous studies have suggested that both muscarinic and nicotinic acetylcholinergic receptors play an important role in antinociception mediated by α_2-adrenergic receptor activation (Honda et al. 2002; Iwamoto & Marion 1993a; Pan et al. 1999; Xu et al. 2000). Based on this, a study (III) was designed to thoroughly investigate the effect of various α_2-adrenergic receptor ligands, which are associated with effects on the antinociceptive system, on the spinal acetylcholinergic receptor system *in vivo* and *in vitro*.

It was found that the α_2-adrenergic agonists clonidine and rilmenidine increased, while the antagonist yohimbine decreased, the acetylcholine release, when administered intraspinally. This agrees with the hypothesis, since clonidine produces spinal antinociception (Buerkle & Yaksh 1998; Detweiler et al. 1993; Naguib & Yaksh 1994; Womer & Shannon 2000), and since intraspinal yohimbine is able to block the antinociceptive effects by other substances at the spinal level (Monroe et al. 1995; Naguib & Yaksh 1994). Rilmenidine has not yet been associated with antinociceptive effects, but due to its chemical and physiological resemblance to clonidine (Head et al. 1998; Reis 1996; Szabo et al. 1999), it was considered important to study this substance as well. The effect of efaroxan was not in agreement with the hypothesis. It was expected that efaroxan, like yohimbine, would decrease the acetylcholine release, since it is an α_2-adrenergic antagonist and since it has been shown to block the antinociceptive effects of α_2-adrenergic agonists (Shannon & Lutz 2000). Instead, efaroxan was found to increase acetylcholine release at higher concentrations, and to decrease the release at lower concentrations.

The potency of the tested ligands, with respect to the effect on acetylcholine release, is remarkable. The maximum increase produced by clonidine was estimated to 327%, and by rilmenidine to 703%. In study I, it was suggested that a 27% increase of acetylcholine should be enough to produce antinociception after oxotremorine treatment. Oxotremorine has been shown to increase acetylcholine release with a maximal observed effect of 160% (Höglund et al. 2000) when administered intraspinally. In addition, yohimbine was very efficient in decreasing the acetylcholine release, with a maximal calculated effect of –70%, which is extraordinary compared to the maximal observed effect of –30% after treatment with systemic and intraspinal atropine. Thus, the α_2-adrenergic system has a powerful influence on the spinal acetylcholinergic system, which explains part of the potent antinociceptive effect of for instance clonidine. The unexpected effects of efaroxan are difficult to explain, but interestingly, the effect of high concentrations of efaroxan resembles the effect of nicotine in similar concentrations (Kommalage & Höglund 2003). Therefore, it cannot be excluded that efaroxan in high concentrations is increasing acetylcholine release by acting on nicotinic receptors.

Both muscarinic and nicotinic receptors have been suggested to be involved in α_2-adrenergic antinociception. Therefore, the importance of these receptors for the α_2-mediated increased release of acetylcholine was investigated. The results demonstrate that nicotinic receptor blockade by mecamylamine attenuated the effect of all ligands, which shows that spinal nicotinic receptors are essential for the effect of α_2-adrenergic ligands on the intraspinal acetylcholine release.

The *in vitro* experiments were undertaken to investigate the binding properties of the α_2-adrenergic ligands to muscarinic and nicotinic receptors. The idea of studying the binding properties arose as an attempt to test whether the effect of the ligands was mediated by direct or indirect action on acetylcholinergic receptors. None of the ligands possessed any affinity for muscarinic receptors. Thus, it is evident from the binding data that any involvement of muscarinic receptors in α_2-mediated spinal antinociception is mediated via an increased release of acetylcholine evoked by an indirect, and not direct, action on muscarinic receptors. On the other hand, all ligands were able to compete with epibatidine in binding to spinal nicotinic receptors. With respect to this, interactions between α_2-adrenergic ligands (agonists or antagonists) and nicotinic receptors in spinal antinociception could be mediated by a direct as well as by an indirect action on nicotinic receptors.

It is difficult to correlate the binding properties *in vitro* with the effect on acetylcholine release *in vivo*. The administered concentrations of the ligands are not equal to the concentrations actually present in the spinal cord during the microdialysis experiments. In addition, the affinity for nicotinic receptors *in vivo* is likely not the same as the affinity *in vitro*, since the *in vitro* experiments describe the situation in isolated tissue, with a different buffering and without the regulatory mechanisms present *in vivo*. However, the concentrations administered *in vivo* were higher than those that should affect exclusively α_2-adrenergic receptors, why the administered ligands must affect other receptor types than merely α_2-adrenergic.

Taken together; nicotinic receptor blockade attenuated the effect of the ligands, the α_2-adrenergic ligands possessed binding affinity to nicotinic receptors, and nicotinic ligands have been shown to affect the acetylcholine release via spinal nicotinic receptors (Höglund et al. 2000; Kommalage & Höglund 2003) in a similar manner to the α_2-adrenergic ligands. These facts indicate that the α_2-adrenergic ligands might exert their acetylcholine increasing effect via a direct action on nicotinic receptors. This idea may appear somewhat controversial, as it suggests other interactions between the α_2-adrenergic and acetylcholinergic receptor systems than what has been previously described. It implies a possible physiological role of receptor stimulation by non-specific concentrations, which is something that becomes necessary to consider when studying pharmacological events *in vivo.*

Acetylcholine and peripheral analgesics

Peripheral analgesics such as aspirin and paracetamol are exerting their antinociceptive action mainly via an inhibition of cyclooxygenases (COX), either at the peripheral site of inflammation, which is the case for aspirin (Smith et al. 2000), or in the central nervous system which is the case for

paracetamol (Bujalska 2003). In addition to the COX inhibitory effect, several studies have suggested a role for aspirin and paracetamol in other, non-inflammatory pain conditions, such as thermal, visceral and mechanical pain in rats and mice (Björkman 1995; Miranda et al. 2001). The mechanisms underlying this non-inflammatory pain modulation are not fully understood, but it has been suggested that central antinociceptive receptor mechanisms such as the adrenergic (Pinardi et al. 2002), serotonergic (Courade et al. 2001), opioid (Pini et al. 1997) and cholinergic (Miranda et al. 2002; Pinardi et al. 2003) systems are involved.

If the cholinergic system is involved, it is reasonable to assume that the spinal acetylcholine release is contributing to the antinociception, similarly to the effects of other receptor systems and of lidocaine. A study by Pinardi et al (2003) showed that systemic atropine could reverse antinociception by paracetamol, and suggested that this effect would be mediated by an increased release of acetylcholine in the spinal cord, but provided no data to support this idea. Study IV was designed to further evaluate the role of spinal acetylcholine in non-inflammatory pain modulation by aspirin and paracetamol.

The results show that systemic aspirin increases, while systemic paracetamol decreases the spinal acetylcholine release, and that intraspinal administration of the drugs had no effect. The latter indicates that the effects observed are not mediated by spinal muscarinic or nicotinic receptors, which is the case for other substances studied in this thesis. However, aspirin as well as paracetamol were able to affect the spinal acetylcholine release. Hence, an involvement of spinal acetylcholine in the antinociceptive effects of these drugs cannot be excluded. The systemically administered doses were high but relevant, as doses within the range used in study IV have been used for pain studies in both mice and rats (Choi et al. 2001; Pinardi et al. 2003).

Aspirin increased acetylcholine release with approximately 20%. This is a small increase compared to the effects of for instance intraspinally administered clonidine and rilmenidine (study III), but not far from the requisite 27% increase shown in study I. It is therefore likely that aspirin in the doses tested, affect descending mechanisms at the supraspinal level, which in turn increases the release in the spinal cord.

Paracetamol, on the other hand, had an opposite effect to what was expected. The antinociceptive effect on non-inflammatory pain of paracetamol is therefore not mediated by spinal acetylcholine. In fact, the reduction of acetylcholine should rather produce hyperalgesia. Since this was not the case, it is evident that the cholinergic involvement as demonstrated by others (Pinardi et al. 2003) is mediated by a system that does not include acetylcholinergic receptors at the spinal level. How this system works remains to be discovered.

Function of acetylcholine in spinal pain modulation

The findings presented in this thesis have contributed to a further understanding of the relationship between spinal acetylcholine and antinociception. The findings have also elucidated interactions between acetylcholine and other substances that mediate antinociception in the spinal cord. Based on the results presented, it appears that acetylcholine, together with muscarinic and nicotinic receptors, plays a key role in the complicated network that mediates antinociception at the spinal cord level. The studies do not demonstrate the precise function of the acetylcholine released and the precise mechanisms of the activation or inhibition of muscarinic and nicotinic receptors. In the *in vivo* studies, the microdialysis probe was placed in the dorsal region of the spinal cord, in order to measure acetylcholine release as close to the superficial laminae of the dorsal horn as possible. However, the loop shaped probe used in the experiments does not allow an exact placement that can be repeated with great accuracy from one animal to another. In addition, substances administered, and acetylcholine itself, are probably diffusing in the tissue. Therefore, it cannot be excluded that the changes of acetylcholine release are reflecting events in the whole diameter of the cord, and this must be taken into consideration. Thus, precisely how acetylcholine is being released, and precisely what acetylcholine actually does once released remains to be found out. The present results, however, along with previous knowledge, permit some reflections about possible mechanisms.

Release of acetylcholine

The release of spinal acetylcholine can originate from supraspinal sites. Stimulation of nicotinic and muscarinic receptors in the nucleus raphe magnus activates descending noradrenergic and serotonergic, but also cholinergic, neurons that terminate in the spinal cord (Iwamoto & Marion 1993a). In addition, nicotinic antinociception induced at several supraspinal regions can be inhibited at the spinal cord level (Meyer et al. 2000; Rogers & Iwamoto 1993). However, it is evident that the acetylcholine release also can originate from the spinal level, since direct stimulation or inhibition by intraspinal administration of different agents affects the release, as seen in the results in this thesis. It is likely that acetylcholine is released after stimulation of receptors present on acetylcholine containing interneurons in the spinal cord. This is consistent with the known presence of intrinsic cholinergic neurons that likely mediate cholinergic innervations in the spinal cord (Borges & Iversen 1986). Another idea that supports this theory is the fact that spinal acetylcholine release is not regulated by pre-synaptic autoreceptors (Höglund et al. 2000), together with the observation seen in all experiments presented herein, namely that the released acetylcholine is not inducing further release of itself. Basal, and continuously stimulated, release can be sta-

bilised, and a stimulated release is transient after a bolus treatment. Acetylcholine release from the interneurons is probably tonically regulated by other transmitters in the spinal cord, among them norepinephrine and serotonin from descending pathways which has been indicated by other studies (Bouaziz et al. 1996; Eisenach 1999). However, the origin of acetylcholine is not necessarily neuronal. Many neurotranmitters in microdialysates may have a glial origin (Stiller et al. 2003), and acetylcholine should be no exception. Muscarinic as well as nicotinic receptors have been found present on astrocytes from the rat spinal cord (Hösli & Hösli 1988; Hösli et al. 2001).

Action of acetylcholine

The general role of the acetylcholinergic receptor system in the spinal cord is reasonably that an increased release of acetylcholine results in a decreased firing from nociceptive fibres, thereby inhibiting the synaptic transmission of nociceptive information to the brain. This suggestion is supported by studies that have shown that cholinergic agonists applied at the spinal level can reduce the release of both substance P (Smith et al. 1989) and glutamate (Li & Zhuo 2001). There are several plausible explanations for how this inhibition could occur. Muscarinic or nicotinic receptors could be present on both first-order neurons and second-order neurons. When the acetylcholine release increases, it is possible that acetylcholine binds to the receptors on the nociceptive neurons, followed by a reduction of the activity in these nerve cells. Muscarinic M2 and M4 subtypes are known G_i coupled receptors that should reduce the nerve activity when stimulated. In addition, nicotinic receptors can also reduce the cell activity when stimulated, depending on subtype. This has been shown in a study demonstrating that different nicotinic antagonists affect the acetylcholine release differently depending on subtype specificity (Kommalage & Höglund 2003). Other explanations could be that the released acetylcholine binds to receptors present on other interneurons that in turn release inhibitory transmitters or further stimulate descending neurons, which eventually leads to a decreased nociceptive transmission.

The studies presented in this thesis do not provide evidence for the suggested biological activity of acetylcholine in the spinal cord. However, the findings presented add important insights into the spinal acetylcholinergic mechanisms, which is essential in order to fully define and characterise the role of acetylcholine in spinal pain modulation.

Conclusions

The studies presented in this thesis provide new and interesting information regarding the role of acetylcholine and interactions between the acetylcholinergic receptor system and other systems in spinal pain modulation. The main conclusions drawn from the results are summarised as follows:

- There is a distinct relationship between changes in the tonic release of intraspinal acetylcholine and the pain threshold. An approximately 27% increase of acetylcholine release is associated with an elevated pain threshold, while an approximately 30% decrease of intraspinal acetylcholine lowers the pain threshold.

- Systemic antinociceptive effects of the local anaesthetic lidocaine are acting through similar mechanisms as muscarinic agonists by increasing the intraspinal release of acetylcholine. The acetylcholine increase by lidocaine is mediated by muscarinic as well as nicotinic receptors.

- The interactions between α_2-adrenergic ligands and the acetylcholinergic system involve an affected release of intraspinal acetylcholine release, which likely contributes to the antinociceptive or nociceptive effects of these ligands.

- The α_2-adrenergic agonists and antagonists affect the acetylcholine release via mechanisms involving spinal nicotinic receptors. Part of this mechanism could be mediated by a direct action on nicotinic receptors.

- Acetylcholine release is increased after systemic treatment with aspirin, which suggests a possible involvement of acetylcholine in the suppression of non-inflammatory pain conditions of aspirin. The similar antinociceptive effect of paracetamol, on the other hand, is not mediated by acetylcholine.

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