New insights into enhancing morphine analgesia

From glia to pharmacokinetics

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ACADEMIC DISSERTATION

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“No matter what else happens, fly the airplane.”
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ABC</td>
<td>Adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>AIF1</td>
<td>Allograft inflammatory factor 1</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>b.i.d.</td>
<td>Bis in die (twice daily)</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood–brain barrier</td>
</tr>
<tr>
<td>Bcrp</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca²⁺/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCD</td>
<td>Chronic compression of the dorsal root ganglion</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injury</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster of differentiation molecule 11b</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CTAP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>CTOP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P 450 family</td>
</tr>
<tr>
<td>DDD</td>
<td>Defined daily dose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPDPE</td>
<td>D-penicillamine(2,5)-enkephalin</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>Half maximal effective dose</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory postsynaptic current</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding regulatory protein</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein coupled receptor kinase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>Intrathecal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Half maximal lethal dose</td>
</tr>
<tr>
<td>LE</td>
<td>Long-Evans (rat strain)</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M3G</td>
<td>Morphine-3-glucuronide</td>
</tr>
<tr>
<td>M6G</td>
<td>Morphine-6-glucuronide</td>
</tr>
<tr>
<td>MAC</td>
<td>Minimum alveolar concentration</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MD2</td>
<td>Lymphocyte antigen 96</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MPE%</td>
<td>Percentage of the maximum possible effect</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>Mrp</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>$N$-methyl-$D$-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>$N$-methyl-$D$-aspartate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Oatp</td>
<td>Organic anion transporter polypeptide</td>
</tr>
<tr>
<td>OIH</td>
<td>Opioid-induced hyperalgesia</td>
</tr>
<tr>
<td>ORL</td>
<td>Opioid receptor-like</td>
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P-gp P-glycoprotein
p.o. Peroral
PAG Periaqueductal gray
PB Parabrachial nucleus
PCA Patient controlled analgesia
PCP Phencyclidine
PDE Phosphodiesterase
PKA Protein kinase A
PKC Protein kinase C
PNS Peripheral nervous system
PTX Pertussis toxin
q.d. *Quaque die*, once daily
RVM Rostroventral medulla
S/N Signal-to-noise ratio
s.c. Subcutaneous
SD Sprague-Dawley (rat strain)
SD Standard deviation
SEM Standard error of the mean
SGC Satellite glial cell
SNL Spinal nerve ligation
TG Trigeminal ganglion
TLR Toll-like receptor
TNF Tumor necrosis factor
UDP Uridine diphosphate
UGT Uridine diphosphate glucuronosyl transferase
ABSTRACT

Opioid analgesics are effective in relieving acute and chronic pain. However, adverse effects and the development of opioid dependence and tolerance may restrict the use of opioids and result in inadequate pain relief. The effects of four structurally and functionally different drugs already on the market, ibudilast, atipamezole, spironolactone, and ketamine, were studied in coadministration with morphine, the prototypical mu-opioid receptor agonist. Experiments were conducted using thermal and mechanical tests of nociception in male Sprague-Dawley rats. Morphine tolerance was produced during four days by subcutaneous or intrathecal delivery of morphine. Drug and metabolite concentrations were measured using high-pressure liquid chromatography-tandem mass spectrometry. The objective of the thesis study was to search for potential drugs to augment morphine antinociception and prevent opioid tolerance.

Ibudilast, a phosphodiesterase and macrophage inhibitory factor inhibitor, had transient sedative effects, but it restored the antinociceptive effect of morphine in morphine-tolerant rats after single and repeated administration. It did not prevent the development of opioid tolerance.

Atipamezole, an alpha-2-adrenoceptor antagonist used for the reversal of sedation in animals during anesthesia, was effective in augmenting intrathecal morphine antinociception in both opioid-naïve and opioid-tolerant animals. These effects were observed at doses lower than those required for the antagonism of alpha-2-adrenoceptors. In subcutaneous administration, low doses of atipamezole did not influence morphine antinociception.

The mineralocorticoid receptor antagonist spironolactone dose-dependently enhanced morphine antinociception. This effect was mediated via the increased access of morphine to the central nervous system by the inhibition of the efflux protein P-glycoprotein. Spironolactone did not inhibit the metabolism of morphine to the pronociceptive metabolite morphine-3-glucuronide, and it did not prevent the development of opioid tolerance.

The effects of ketamine in augmenting opioid analgesia in tolerance are thought to result from a beneficial pharmacodynamic interaction.
When acute ketamine was administered to rats under chronic morphine treatment, the brain concentrations of morphine, ketamine and norketamine were increased compared with the situation where either morphine treatment or acute ketamine were administered alone. The results indicate a potentially beneficial pharmacokinetic interaction between the two drugs.

The results of the thesis study demonstrate that ibudilast and atipamezole modulate nociception at systemic and spinal levels in preclinical models of pain, and they may prove advantageous as an adjuvant to opioid therapy. Spironolactone had a pharmacokinetic interaction with morphine, leading to increased morphine concentrations in the central nervous system. Ketamine, a drug used for the treatment of opioid tolerance in cancer patients, may undergo previously unrecognized beneficial pharmacokinetic interactions with morphine.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (studies I–IV) and some unpublished data.


* These authors contributed equally to the publication.

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1. INTRODUCTION

The treatment of acute and chronic severe pain remains a major challenge. Opioids, such as morphine and oxycodone, are used as primary analgesics in moderate to severe pain (Hoskin, 2008; Kalso et al., 2004). However, long-term opioid treatment, in particular, has several problems, such as the development of tolerance and dependence with withdrawal symptoms. Tolerance is manifested as a need to increase the opioid dose to reach an equipotent analgesic effect, whereas opioid-induced hyperalgesia is a process of sensitization in which opioids cause hypersensitivity to painful stimuli. Dose escalation of opioids is typically limited by adverse effects such as respiratory depression, nausea, and constipation (McNicol et al., 2003). The adverse effects and tolerance with hyperalgesia may necessitate the discontinuation of opioid treatment and result in inadequate pain control.

The classic models for explaining the development of opioid tolerance and hyperalgesia have mostly focused on cellular and network changes in neurons themselves. The changes caused by repeated administration of opioids and consequent opioid receptor activation may involve adaptive changes in the neurons, such as the internalization of opioid receptors (Zuo, 2005), upregulation of N-methyl-D-aspartate receptor function (Mao et al., 2002; Shimoyama et al., 2005), production of nitric oxide, or down-regulation of glutamate transporters (Pasternak, 2007). Moreover, a counter-regulatory antiopioid system could be involved, including neuromodulators such as cholecystokinin or dynorphin (Xie et al., 2005). The formation of heterodimeric receptor complexes between mu-opioid and other receptors, such as the alpha-2-adrenoceptor, may modulate the function of the mu-opioid–receptor coupled effectors and the efficacy of opioids (Jordan et al., 2003).

Recent studies suggest that opioid administration may also induce tolerance via mechanisms other than those involving neurons. The glial cells of the central nervous system (CNS) have previously been considered as neuroimmune cells that mainly provide support and nutrition for the neurons. New data indicate that activated glial cells may modulate the activity of the nociceptive neurons in the CNS (Ji et al., 2013; Grace et al., 2014) by releasing various proinflammatory substances and actively oppose the analgesic action of opioids on pain-transmission neurons (Wang et al., 2012b). Another line of opioid tolerance research has focused on the pharmacokinetics of opioids,
especially their potentially reduced access to the CNS through the blood–brain barrier (BBB) (Mercer and Coop, 2011). Of the known BBB drug transporters, morphine is an acknowledged substrate of the efflux transporter P-glycoprotein (P-gp) (Letrent et al., 1998; Schinkel et al., 1995; Xie et al., 1999). Upregulation of this transporter by opioids could lead to decreased access of opioids to the brain and compromised analgesia. However, limited knowledge exists concerning other potential opioid-transporting proteins and pharmacokinetic drug–drug interactions between analgesics.

The present investigations were conducted to acquire further information on possible adjuvant drugs that could improve the efficacy of opioids in acute and chronic treatment, with particular emphasis on both pharmacodynamic and pharmacokinetic interactions.
2. REVIEW OF THE LITERATURE

2.1 Physiology of nociception and pain

2.1.1 Primary nociceptors

Somatosensation includes several submodalities, including touch (detection of light mechanical stimuli), proprioception (detection of mechanical stretch of muscles and joints), thermosensation (detection of cold and warmth), and nociception (detection of noxious thermal, mechanical, or chemical stimuli), that evoke pain sensations (Julius, 2013). Noxious stimuli are initially detected by primary afferent sensory nerve fibers that innervate a peripheral target. This information is transmitted to secondary afferent dorsal horn neurons in the spinal cord and further to the brain via ascending neural circuits. The cell bodies of primary afferent sensory nerves, referred to as nociceptors, lie in the dorsal root ganglia (DRG) for the body and the trigeminal ganglion for the face. They have a peripheral and a central axon innervating the target organ and the spinal cord, respectively. The nociceptors only discharge action potentials when they are stimulated to a noxious range, indicating that they are only capable of detecting and responding to potentially injurious stimuli (Todd and Koerber, 2013).

Nociceptors are divided into two major classes: 1) medium-diameter myelinated A-delta fibers that convey acute, fast pain and 2) small-diameter unmyelinated C fibers that convey poorly localized, slow pain. C fibers are usually divided into peptidergic and nonpeptidergic neurons. Peptidergic neurons may contain substance P and calcitonin gene-related peptide (CGRP) and express the nerve growth factor receptor TrkA. The nonpeptidergic population expresses the c-Ret neurotrophin receptor with glial-derived neurotrophic factor as its agonist. They usually lack substance P and CGRP, but express purinergic P2X3 and P2Y1 receptors. The large-diameter A-beta fibers are rapidly conducting primary afferent nerves that do not convey nociceptive information. However, their activity may modulate pain perception by activating inhibitory interneurons in the spinal cord (Todd and Koerber, 2013).
2.1.2 Nociception at the spinal cord level

The dorsal horn of the spinal cord is organized into histologically and functionally distinct laminae. They receive their input by the primary afferent nerve fibers. A-delta fibers generally project to lamina I and also to the deep lamina V, whereas the C fibers project to the superficial laminae I and II, with some terminals in deeper laminae (Fig. 1A). The A-beta fibers, mediating light touch, project to laminae III, IV and V (Basbaum et al., 2009; Todd and Koerber, 2013). In addition to the primary afferent nerves and projection neurons, one-third of the neuron population consists of inhibitory interneurons using gamma-amino butyric acid (GABA) and/or glycine as neurotransmitters. Inhibition at the spinal cord level is an important form of endogenous pain control. For example, in the absence of sensory stimuli, the release of tonic inhibitory neurotransmitter inhibits spontaneous discharges of the spinal dorsal horn neurons (for review, see Todd, 2010).

2.1.3 Nociception and pain in supraspinal structures

Projection neurons located in laminae I and V are responsible for most of the output from the dorsal horn to the supraspinal structures (Basbaum and Jessell, 2000). The spinothalamic tract, which conveys nociceptive messages to the thalamus, is especially relevant for the sensory-discriminative aspects of pain, whereas the spinoreticulothalamic tract conveys nociceptive information to the brainstem and is more relevant in poorly localized nociception. There are also connections to the amygdala via the parabrachial region of the dorsolateral pons. This connection has been associated with information related to the unpleasantness of the nociceptive stimulus. From these loci, information reaches the cortical areas (Fig. 1B). Pain has been suggested to result from the activation of numerous structures, with the somatosensory cortex being largely responsibility for the sensory-discriminative properties of pain and others, such as the anterior cingulate cortex and insular cortex, having responsibility for the emotional aspects (Apkarian et al., 2005). The pain matrix concept involves several interacting neuronal networks, of which the nociceptive matrix (mainly at the posterior insular region), receiving input from the spinothalamic projection, is responsible for cortical nociceptive processing. This information is further transferred to a second-order network and to perception involving parietal, prefrontal, and anterior insular areas (Garcia-Larrea and Peyron, 2013).
According to the International Association for the Study of Pain (IASP), pain is described as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. This implies that pain can only be experienced at the whole-organism level. Animals cannot verbally report pain, but certain recurrent reactions to nociceptive stimuli can be interpreted as nociception. The extrapolation of the analgesic response from vastly differing pain models or the use of measures with unclear relevance is a problem in animal research. Attempts are being made to develop new specific pain measurement methods. For example, a facial expression pain coding system for the mouse has recently been introduced (Matsumiya et al., 2012), a system similar to those used for evaluating pain in nonverbal human populations.
2.1.4 Supraspinal mechanisms of pain modulation

The relationship between the peripheral noxious stimulation and the perceived pain experience depends on many variables, including the behavioral context and other sensory inputs. Modification of the pain response results from the action of CNS networks. Suppression of the nociceptive reflexes and pain sensation is essential in the face of a threat, whereas enhanced pain in tissue inflammation or injury may promote sheltering and recuperation. At present, it is widely acknowledged that the continuous activation of descending pain facilitator networks is a contributor to chronic pain states (Heinricher and Fields, 2013).

The first evidence for supraspinal sites controlling ascending sensory pathways was published in the 1950s, and a specific pain modulatory system theory was introduced by Melzack and Wall (1965) in the Gate control theory of pain. Descending projections to the spinal cord control spinal nociception either directly or through spinal interneurons (Todd, 2010). The discovery of periaqueductal gray (PAG) and its central role in the brain mechanisms of pain processing was a critical step in understanding the modulation of nociception. Simple effects evoked by noxious stimulus at the spinal cord level may be inhibited by the stimulation of PAG or its relay in the rostroventral medulla (RVM). The major output of the RVM is to the spinal cord via neurons including 5-hydroxytryptamine (5-HT), GABA, and several neuropeptides (Skagerberg and Björklund, 1985). PAG receives direct projections from sites involved in supraspinal pain processing, including the cingulate and insular cortex, hypothalamus, and amygdala (Aggleton et al., 1980; Floyd et al., 2000; Rizvi et al., 1996).

The PAG-RVM is crucial for the analgesic actions of opioids and endogenous opioid peptides (see 2.2.3). Interestingly, other substances such as cannabinoids also partly produce their antinociceptive effect by activating the PAG-RVM system. Another important descending modulatory system is the pontomedullary noradrenergic pathway, reciprocally linked with the PAG-RVM system (see 2.3.4).
2.2 Opioids

2.2.1 Indications and prevalence of opioid therapy in Finland

Opioids are the most potent analgesic agents used for pain therapy. They are the gold standard in the treatment of acute postoperative pain and also severe to moderate cancer pain (Caraceni et al., 2012). The usage of opioids in the treatment of non-cancer chronic pain has significantly increased. In Finland, nine different opioids are currently on the market, grouped into weak (codeine and tramadol), intermediate (buprenorphine), and strong (fentanyl, hydromorphone, methadone, morphine, oxycodone, and pethidine) categories based on their pharmacological efficacy. In 1990, the total consumption of opioids was three defined daily doses (DDD) per 1000 inhabitants, increasing to a level of 17 DDD per 1000 inhabitants by 2011, indicating a six-fold increase in opioid consumption during 20 years. This development goes hand in hand with the global development: the use of prescribed opioids increased threefold from 1989 to 2009, and the use of morphine worldwide increased from 6.5 tons in 1989 to 41.8 tons in 2009 (Huxtable et al., 2011). The majority of opioids consumed in Finland are weak opioids, mainly codeine. The consumption of morphine has been stable for the last 10 years (0.27 DDD), whereas the consumption of oxycodone has increased almost threefold to 0.81 DDD. In 2011, strong opioids were prescribed to 25 000 outpatients, and the total number of inhabitants who had used opioids in 2011 was 410 000. Based on the Finnish registries, it is not possible, however, to determine the individual consumption for different diagnoses (Nevantaus et al., 2013; summary in English).

2.2.2 Opioid receptors

Opioid receptors belong to the superfamily of guanine nucleotide-binding regulatory protein (G protein) coupled receptors (GPCRs), a family that contains several hundred members in the human genome (Lagerström and Schiöth, 2008). The receptor contains seven transmembrane domains that are connected by short loops. They display an extracellular N-terminal domain and an intracellular C-terminal tail. The opioid receptors were first demonstrated in the 1970s (Pert and Snyder, 1973), and in the 1990s the opioid receptor family, including the classical mu, kappa, and delta receptors, as well as the closely related opioid receptor like (ORL) 1 receptor, were cloned and hence
molecularly identified (Taylor and Dickenson, 1998). Mu, delta and kappa receptors are highly homologous, with greatest variability within the extracellular loops and the N- or C-terminal tails. In 2012, the crystal structure of the kappa receptors was determined with X-ray crystallography (Wu et al., 2012), representing another milestone in opioid research. Pharmacological activities of the prototypal opioid receptor agonists have been tested in mice lacking the mu-, delta- or kappa-opioid receptor genes. The results suggest that the mu-opioid receptor is responsible for the analgesic activities of morphine (Kieffer and Gavériaux-Ruff, 2002) (Fig. 2).

Mu-opioid receptors are coupled to pertussis toxin-sensitive $G_\alpha$ proteins $G_o$ and $G_i$ to inhibit the cyclic adenosine monophosphate (cAMP) pathway by inhibiting adenylyl cyclase activity (Connor and Christie, 1999; Laugwitz et al., 1993). By initiating the release of the $G_{\beta\gamma}$ dimer from $G_i$ or $G_o$, opioids reduce the opening of voltage-dependent calcium channels (Saegusa et al., 2000) or activate inwardly rectifying potassium channels (Ikeda et al., 2000), phenomena leading to decreased neuronal excitability and analgesia. Opioids also may activate mitogen-activated protein kinase (MAPK) cascades, affecting the transcriptome of the cell.

![Figure 2. Opioid receptor-mediated effects. The main pharmacological activities of the opioid receptor agonist prototypes have been assessed in mu-, delta-, and kappa knockout mice. Modified from Kieffer and Gavériaux-Ruff (2002). DPDPE, D-penicillamine(2,5)-enkephalin.](image-url)
2.2.3 Mechanisms of opioid-induced analgesia

The actions of opioids in mediating analgesia after systemic administration present their actions in both the brain and spinal cord.

Within the spinal cord, opioid receptors are predominantly located in laminae I and II of the dorsal horn, with smaller quantities in deeper layers. The contribution of mu, delta, and kappa receptors to opioid binding in the rat spinal cord is roughly 70%, 24% and 6%, respectively (Besse et al., 1990; Rahman et al., 1998). Over 70% of the receptors are located on the central terminals of small-diameter (mostly C and alpha-delta) primary afferent neurons, implying that the main mechanism of spinal opioid analgesia lies in the activation of presynaptic opioid receptors, decreasing the release of excitatory transmitters and nociceptive transmission. Another confirmation of the presynaptic action is provided by the observation that the release of primary afferent transmitters such as substance P and CGRP, contained in small afferents, is reduced after opioid administration (Go and Yaksh, 1987). Taken together, the capacity of spinal opioids to reduce the release of excitatory neurotransmitters and to decrease neuron excitability in the dorsal horn is considered to mediate the powerful effects of opioids on nociception at the spinal cord level. In humans, there is extensive literature indicating the clinical efficacy of spinal opioids (Yaksh, 1997).

As opioid receptors are synthesized in the DRG cell bodies, they are also transported peripherally to the axons. The peripheral application of exogenous opioid agonists has only been antinociceptive in inflammatory states (Machelska et al., 1999).

Microinjections of opioids to specific brain sites have demonstrated that opioid agonists, in a manner consistent with their activity on the mu-opioid receptor, block nociceptive behavior. The supraspinal sites where morphine microinjections have shown efficacy are the insular cortex, amygdala, hypothalamus, PAG, and RVM. Interest in the PAG-RVM system was raised when it was discovered that opioid analgesia is also partly mediated via direct post-synaptic hyperpolarization of a neuron subpopulation in this area (Pan et al., 1990; Yaksh et al., 1988). On the other hand, RVM may also contribute to hyperalgesia and allodynia in inflammatory and neuropathic pain (Porreca et al., 2002) or prolonged opioid administration (Vanderah et al., 2001). Additionally, especially in humans, opioids also have supraspinal mood-altering effects that modulate the experience of pain. In addition to relief of distress and increased
tolerability of pain, patients may also experience euphoria (Yaksh and Wallace, 2011).

2.2.4 Morphine

Morphine \([5α,6α]-7,8\text{-didehydro}-4,5\text{-epoxy}-17\text{-methylmorphinan}-3,6\text{-diol}\) (Fig. 3) is the main active substance in opium. As it is difficult to synthesize, it is still extracted from poppy straw or obtained from opium. It has become the gold standard of opioids against which all other opioids are compared. Its main effect is mediated by binding to and activating the mu-opioid receptors in the CNS. Morphine is also the prototypical agonist of the mu receptor (Yaksh and Wallace, 2011).

![Figure 3. Structural formula of morphine.](image)

After peroral administration, the absorption of morphine from the gastrointestinal tract is modest with a bioavailability of approximately 25%. Hence, its first-pass metabolism in the liver and gut wall is significant. In parenteral intravenous, intramuscular, and subcutaneous (i.v., i.m., and s.c., respectively) administration, the onset of the effects is more rapid and the effect of the same dose is larger. Morphine is also available as suppositories with a rather good absorption through the rectal mucosa. About one-third of morphine in plasma is bound to proteins after a therapeutic dose. The analgesic plasma concentration range in patients suffering from cancer is very large, 22–364 ng/mL (Neumann et al., 1982).

Morphine is glucuronidated to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) by uridine diphosphate (UDP)-glucuronosyl
transferase 2B7 (UGT2B7) in humans (Coffman et al., 1997), and mostly to M3G by Ugt2b1 in rodents (Coffman et al., 1996; King et al., 1997; Pritchard et al., 1994). A small amount is metabolized to normorphine via the cytochrome P-450 (CYP) system, especially CYPs 3A4 and 2C8 (Projean et al., 2003). In humans, after oral morphine administration, the areas under curve (AUC) of M6G and M3G exceed that of the parent drug by factors of 9:1 and 50:1, respectively (Osborne et al., 1990). Morphine is mainly excreted via the kidney in the 3-glucuronide form; very little morphine is excreted unchanged. M6G is also excreted by the kidney, and in kidney failure it may accumulate, probably explaining the increased and prolonged effect of morphine in this patient group (Owen et al., 1983). Some enterohepatic circulation of morphine and its glucuronides occurs, which can be seen as traces of morphine in the feces for some days after the end of therapy (Walsh and Levine, 1975).

In rodents, the disposition of morphine in the CNS is modulated by P-gp (Letrent et al., 1999; Schinkel et al., 1995; Xie et al., 1999), an efflux protein at the blood–brain barrier (BBB). Thus, P-gp inhibition could lead to increased access of morphine to the CNS (see 2.3.5).

2.2.5 Adverse effects of morphine

Opioid therapy may be compromised by adverse effects (Table 1 and Fig. 4), of which most are mediated through the opioid receptors. The severity of these adverse effects on the patient may range from mild to severe: constipation is common but it may generally be alleviated with pharmacological methods, whereas respiratory depression is the most common cause of death associated with opioid toxicity (Pattinson, 2008). The risk of respiratory depression in pain treatment is lower than in opioid abuse. It has been suggested that the respiratory center also receives nociceptive input, and pain thus acts as a physiological antagonist for opioid-induced respiratory depression (Hanks and Twycross, 1984). Acute potentially dangerous adverse effects of opioids may be antagonized with naloxone, the prototypical opioid receptor antagonist, given intravenously or subcutaneously. The bioavailability of naloxone is poor, leading to minimal systemic concentrations after peroral administration. Interestingly, this effect may be advantageous in the treatment of constipation, in which the target of opioid receptor antagonism lies in the enteric nervous system of the gut. Indeed, oral naloxone and subcutaneous methylnaltrexone are currently in use to reverse opioid-induced constipation without compromising analgesia (Leppert, 2010).
Table 1. Possible adverse effects of opioids in chronic pain treatment (Lawlor and Bruera, 1998; Schug et al., 1992).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropsychiatric</td>
<td>Delirium, sedation, mood changes, anxiety, sleep disturbance</td>
</tr>
<tr>
<td>Neurological</td>
<td>Myoclonus, miosis, hyperalgesia, allodynia, seizures</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Dry mouth, nausea and vomiting, constipation, biliary spasm</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Respiratory depression, pulmonary edema, cough suppression</td>
</tr>
<tr>
<td>Urological</td>
<td>Micturitional disturbance</td>
</tr>
<tr>
<td>Dermatological</td>
<td>Pruritus, sweating</td>
</tr>
<tr>
<td>Endocrine</td>
<td>Hormonal changes, immune changes</td>
</tr>
<tr>
<td>Pharmacological</td>
<td>Tolerance, dependence</td>
</tr>
</tbody>
</table>

Opioids affect two major hormonal systems: the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonadal axis. Morphine causes a strong decrease in plasma cortisol levels in adults (Banki and Arato, 1987) and laboratory animals (Bartolome and Kuhn, 1983; Collu et al., 1976; Rolandi et al., 1983). In the gonadal axis, typical changes in hormonal secretion are a decrease in luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and estrogen levels, whereas an increase in prolactin levels may be observed (Malaivijitnond and Varavudhi, 1998). Effects of opioids on the immune system may be mediated through their direct effects on immune cells, as opioid-related receptors on immune cells have been discovered (Makman, 1994), or indirectly via changes in neuroendocrine system. Opioids appear to alter both innate and adaptive immune systems (Risdahl et al., 1998; Roy and Loh, 1996). Results from in vivo and in vitro studies suggest that prolonged opioid exposure is likely to suppress immune function in the periphery (Roy et al., 2006; Sacerdote et al., 1997); for a review, see (Ninković and Roy, 2013). Interestingly, microglial cells, immune cells of the CNS, have recently gained considerable attention in the study of pain (see 2.3.3).
2.3 Modulation of opioid-induced antinociception

2.3.1 Opioid tolerance and opioid-induced hyperalgesia

Opioid tolerance is a pharmacological phenomenon that develops during chronic, repeated use of opioids and leads to the need to increase the opioid dose to reach an equipotent analgesic effect, i.e. the dose-response curve is shifted to the right. Two types of tolerance, associative and non-associative tolerance, can be distinguished and they appear to involve different neurotransmitter mechanisms (Grisel et al., 1996; Mitchell et al., 2000). Associative tolerance involves psychological factors, and they may be clinically connected to non-pharmacological aspects of patient care, for example admission to the hospital ward. Non-associative tolerance occurs at the cellular or organism level and involves numerous intracellular changes, as well as changes in the function of wider neuronal networks (Grisel et al., 1996). A prevailing theory is that opioid tolerance is a complex process that involves multiple regulatory mechanisms occurring at the level of both neurons and neuronal networks. Many features of opioid tolerance may be seen as a natural attempt of neuronal homeostasis to...
reach equilibrium, i.e. involving regulatory or opponent processes to overcome prolonged activation of opioid receptors. Desensitization, on the other hand, is a process referring to the acute loss of mu-opioid receptor and effector coupling that occurs within seconds to minutes after the initiation of opioid exposure. Both tolerance and desensitization may be used interchangeably to describe the loss of receptor activity after chronic treatment. (Williams et al., 2013) Tolerance, however, does not necessarily specify the cellular or molecular mechanisms that are responsible for the phenomenon.

Multiple opioid receptor-related phenomena may contribute to cellular tolerance. Phosphorylation of the mu-opioid receptor is a widely accepted theory of desensitization. Many kinases, e.g. G protein receptor kinase (GRK), c-Jun N-terminal kinase (JNK), protein kinases A and C (PKA and PKC), Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII), and MAPK, may be involved in desensitization and receptor endocytosis (Koch and Höllt, 2008). The endocytosis pathway does not necessarily lead to receptor degradation, but it may also lead to dephosphorylation of the receptor and its replacement on the surface of the cell. However, morphine is only a weak stimulator of mu-opioid receptor phosphorylation (Zhang et al., 1998). Based on these data, it has been speculated that morphine tolerance may not be a result of receptor desensitization but rather the lack of desensitization, which may lead to prolonged receptor signaling (Whistler and Zastrow, 1998; Williams et al., 2013). Downregulation of functional mu-opioid receptors is not considered relevant for morphine tolerance, as functional binding studies have implied that the loss of over 80% of functional mu-opioid receptors is needed to observe a rightward shift in the half maximal effective concentration (EC\(_{50}\)) of morphine (Stafford et al., 2001; Williams et al., 2013).

The mu-opioid receptor is coupled to cellular effectors such as adenylyl cyclase and Ca\(^{2+}\) and K\(^{+}\) channels, affecting the release of neurotransmitters. Repeated opioid administration initiates counterregulatory processes in these systems, of which the best described is the superactivation of adenylyl cyclase and increased intracellular cAMP concentrations. This, in turn, induces PKA activation and an increased inward cation current through Ca\(^{2+}\) channels (Bailey and Connor, 2005; Collier, 1980). Even though different opioids share the same binding site (presumably the mu-opioid receptor) to produce their physiological effects, there may be significant differences in the activation of different G proteins and intracellular downstream responses. This is called functional selectivity or biased agonism; for review, see Raehal et al. (2011).
Opioid tolerance not only has receptor- and effector-level effects, but also includes system-level counteradaptation. The observed tolerance may result from not only pharmacological tolerance, but also enhanced excitability of the opioid-regulated pathways. Indeed, opioids may initiate a pronociceptive process also known as opioid-induced hyperalgesia (OIH). While tolerance at simplest is characterized by a progressive decrease in the half maximal effective dose ($ED_{50}$) that can be overcome by increasing the drug dose, OIH is a process of sensitization in which opioids cause hypersensitivity to pain (Fishbain et al., 2012). The relative contributions of these two processes to the observed overall decrease in the opioid analgesic effect has not been established, and these processes may have mutual intercellular characteristics and contribute in parallel to the decrease the clinical efficacy. The common mechanisms may involve activation of bulbospinal pathways that increase the secondary neuron excitability of the spinal dorsal horn via a counter-regulatory antiopioid system, including neuromodulators such as cholecystokinin or dynorphin (Gardell et al., 2002; Xie et al., 2005). Glutamatergic cellular mechanisms that lead to upregulation of $N$-methyl-$D$-aspartate (NMDA) receptor function may also contribute to OIH (Mao et al., 1994). OIH has been described in rodents (Célèrier et al., 2000; Li et al., 2001a; Mao et al., 1994; Vanderah et al., 2001, Minville et al., 2010) and pain-free human volunteers receiving opioid infusions (Koppert et al., 2003; Luginbühl et al., 2003; Tröster et al., 2006, Vinik and Kissin, 1998); for a meta-analysis, see Fishbain et al. (2009). However, data in many other settings are contradictory (Fishbain et al., 2009), and there are no clinical data on the prevalence of OIH (Lee et al., 2011). During opioid maintenance therapy in addicts, a modality-specific increased pain sensitivity has been observed in studies with small populations (Compton et al., 2001; 2012; Prosser et al., 2008; Doverty et al., 2001). In postoperative studies, OIH has mainly been studied after opioid-based anesthesia (Chia et al., 1999; Cooper et al., 1997; Guignard et al., 2000; Katz et al., 1996; Lahtinen et al., 2008; Schmidt et al., 2007; Schraag et al., 1999; Shin et al., 2010). In a recent meta-analysis, high intraoperative doses of remifentanil, a potent opioid with a short elimination half-life, were associated with a small increase in postoperative pain (Fletcher and Martinez, 2014). OIH may also be a clinical problem in chronic pain (Hay et al., 2009; Chu et al., 2006; Hooten et al., 2010). In practice, the discontinuation of a large dose of morphine and substitution with another opioid (opioid rotation) has been a route to reverse opioid tolerance, implicating
morphine or its accumulating metabolite morphine-3-glucuronide in the phenomenon (Andersen et al., 2003; Sjøgren et al., 1994; Smith, 2000).

Pharmacokinetic factors, such as decreased absorption of oral preparations, accelerated drug elimination in the liver and kidney, or changes in the BBB drug transport function may lead to decreased opioid access to the CNS (see 2.3.5). It is also important to distinguish the marked differences between the actions of M3G and M6G, the primary metabolites of morphine in humans. Upon morphine administration, both metabolites are observable in the brain, even though they have limited permeability through the BBB (Bickel et al., 1996). Systemically administered M6G is approximately twice as potent as morphine in humans (Osborne et al., 1990) and also in animal models (Paul et al., 1989). During prolonged oral administration in humans, the plasma concentration of M6G generally exceeds that of morphine. In rats, however, M6G is not formed at detectable concentrations (Coughtrie et al., 1989). M3G, the other primary metabolite of morphine, has the opposite actions. It has very little affinity for the opioid receptor, but may contribute to the harmful excitatory effects of morphine and OIH (Andersen et al., 2003; Sjøgren et al., 1994; Smith, 2000).

On an individual level, there are marked differences in the variation of the response to a given opioid. The genetics involved in opioid pharmacodynamics or pharmacokinetics may play a role in the response to opioid drugs, but their influence on this variation is complex. Personalized medicine is a rising field of medicine that aims at the customization of medical treatment to personally suit the needs of an individual patient. The majority of genetic studies have so far focused on single genes or single nucleotide polymorphisms, and research into the involvement of gene–gene interactions in the opioid response is only just starting; for a review, see Branford et al. (2012). Environmental factors that cause changes in chromosomes without altering the deoxyribonucleic acid (DNA) sequence by altering gene transcription are called epigenetics. Chronic opioid exposure may stimulate DNA methylation at the mu-opioid receptor coding OPRM1 gene. This finding did not induce increased opioid dosing requirements, but was associated with increased chronic pain. The mechanism is still unresolved, but may provide a new interesting approach to research on OIH (Doehring et al., 2013).

In conclusion, the need for opioid dose elevation during chronic administration in a clinical setting may be a sum of the pharmacologic opioid tolerance, non-associative tolerance, OIH, alterations in pharmacokinetics, and disease progression. Genetic factors also have to be considered.
2.3.2 Role of the glutamatergic system in opioid tolerance and opioid-induced hyperalgesia

Adaptation to opioid use can also result from the upregulation of glutamate and other pathways in the brain, which can exert an opioid-opposing effect and thereby reduce the effects of opioid drugs by altering downstream pathways, regardless of mu-opioid receptor activation. Extensive research has centered on the excitatory amino acid system, in particular the NMDA receptors. These are ionotropic glutamate receptors that are crucial for excitatory synaptic transmission. They are involved in numerous cognitive processes such as learning and synaptic plasticity. In the resting state, the receptor is blocked by Mg$^{2+}$. Activated NMDA receptors are permeable to Ca$^{2+}$, Na$^+$, and K$^+$ (Frohlich and Van Horn, 2014).

Acute pain is mainly signaled by the release of glutamate from the central terminals of primary afferents, which after binding to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors causes transient excitatory postsynaptic currents (EPSCs) in second-order dorsal horn neurons. The sum of these EPSCs may lead to an action potential and transmission to high-order neurons. During the constant barrage of action potentials from the periphery, the increased neurotransmitter release and postsynaptic depolarization will be long enough to activate NMDA receptors (Frohlich and Van Horn, 2014).

Functional NMDA receptors (Fig. 5) are heteromeric complexes consisting of a NR1 subunit and modulatory subunits of the NR2 (NR2A–D) family (Frohlich and Van Horn, 2014). NMDA receptors are involved in synaptic long-term modulation (long-term potentiation) and glutamate neurotoxicity when overactivated. Systemic administration of the NMDA receptor antagonists MK-801 or ketamine has been shown to reverse opioid-induced mechanical or thermal hyperalgesia and opioid tolerance after acute or chronic opioid administration in numerous studies (Li et al., 2001b; Manning et al., 1996; Mao et al., 2002; Trujillo and Akil, 1991; 1994; for a review, see Angst and Clark (2006). In many of the studies, a role for the intracellular effector PKC has been suggested to lie behind the induction of OIH, as PKC may upregulate NMDA receptors, and the development of OIH was not observed in PKC-gamma knockout mice (Célérier et al., 2004; Zeitz et al., 2001). Interestingly, increased nociception and hyperalgesia after opioid infusion was also observed in opioid receptor triple knockout mice lacking all three genes encoding opioid receptors (Juni et al., 2007), demonstrating a opioid receptor-independent route of hyperalgesia development. Furthermore, the development of hyperalgesia could be reversed by the NMDA
antagonist MK-801 in control (wild-type) mice, but not in opioid receptor knockout mice, indicating that other mechanisms also contribute to the development of hyperalgesia (see 2.3.3). In another triple knockout study using acute fentanyl injections, the development of hyperalgesia could be reversed by an MK-801 co-injection (Waxman 2009).

Figure 5. Schematic representation of the N-methyl-D-aspartate receptor complex. PCP, phencyclidine. Adapted from Frohlich and Van Horn (2014).

Glial cells may also influence the clearance of neurotransmitters, especially glutamate, from the synaptic cleft. Chronic morphine induced downregulation of the astrocyte glutamate transporters glial glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST) (Mao et al., 2002; Sung et al., 2003), leading to increased glutamate and synaptic transmission, morphine tolerance, and thermal hyperalgesia. Glia also incorporate NMDA receptors that have a different subunit configuration compared to neurons, making them less permeable to Ca$^{2+}$. The function and significance of glial NMDA receptors has not yet been established; for a review, see Dzamba et al. (2013).

Finally, excess glutamate may also play a role in functional changes and the expression of drug transporters at the BBB. Glutamate was shown to upregulate P-gp expression in microvessel endothelial cells of the rat brain (Zhu and Liu, 2004), a phenomenon that was attenuated by the NMDA receptor antagonist MK-801, implying an NMDA receptor-mediated mechanism behind the finding. A simplified representation of the glutamatergic system in opioid tolerance is presented in Figure 6.
Figure 6. A simplified representation of the glutamatergic system in opioid tolerance at the spinal cord level.
1. Morphine administration in acute pain.
2. Chronic pain, opioid tolerance and opioid-induced hyperalgesia.
EAAT, excitatory amino acid transporter; Glu, glutamate; Mo, morphine; MOR, μ-opioid receptor; NMDAR, N-methyl-D-aspartate receptor.
↑, increased concentration or activity; ↓, decreased concentration or activity.
2.3.3 Glial cells and opioid antinociception

Recent years have seen the introduction of the neuroimmune interface as a new concept in modulating CNS neurotransmission. Neuronal excitability can be notably enhanced by the classical neuronal transmitters, but also by immune mediators released from glial cells in the CNS as well as infiltrating cells such as T cells and macrophages. In several painful conditions discussed below, glial cells function in enhancing pain sensitivity via neuroglial interactions. Opioid treatment may also cause glial activation, which may attenuate the analgesic potency of opioids.

Glial cells of the CNS consist of three distinct types of cells: astrocytes, microglia, and oligodendrocytes. In the peripheral nervous system (PNS), glial cells are found as satellite glial cells (SGCs) in the dorsal root ganglia (DRGs) and trigeminal ganglia (TGs), and as Schwann cells enclosing the peripheral nerves (Gao and Ji, 2010; Hutchinson et al., 2011).

The most numerous glia in the CNS are called astrocytes. They have an essential role in the regulation of the chemical content of the extracellular space. Astrocytes form a complex network, an envelope, over the synaptic junctions in the brain to restrict the spread of neurotransmitters. They also communicate intercellularly via $\text{Ca}^{2+}$ signaling. Astrocytes are typically immunostained with the detection of glial fibrillary acidic protein (GFAP), although this only stains the major branches of the cells. Astrocytes are a part of the “tripartite synapse” theory, in which glial cells have a crucial role as active modulators of synaptic transmission. Glia may react to neuronal activity and either enhance or suppress neuronal activity and the synaptic strength with chemical transmitters (Gao and Ji, 2010; Hutchinson et al., 2011; Ji et al., 2013).

Oligodendroglial cells provide layers of membrane that insulate the neuronal axons. One oligodendroglial cell can provide myelin for several axons (Hutchinson et al., 2011).

Microglial cells are responsible for the primary immune response in the brain. These cells originate from bone marrow-derived monocytes that migrate to the CNS during the prenatal period. Their distribution in the CNS is heterogeneous, and they account for 5–12% of its cells. Under normal conditions, microglia are considered inactivated, but they continuously sense their environment with their ramified processes (Nimmerjahn et al., 2005). Under any kind of brain damage or injury, microglia are activated and undergo morphological and functional changes. The most selective markers for microglia
are intracellular proteins CD11b (cluster of differentiation molecule 11b) and ionized calcium-binding adapter molecule 1 (Iba1, also known as allograft inflammatory factor 1, AIF1) (Fig. 7). In peripheral nerve injury, the spinal microglia become activated and their morphology changes from a ramified to an ameboid shape (Suter et al., 2007).

Figure 7. Iba1 immunohistochemical staining of microglial cells in the brain cortex of the rat. 200 × magnification. Iba1, ionized calcium-binding adapter molecule 1. (Lilius et al., unpublished)

Glia exert different types of functional changes upon neuronal injury or continuous painful stimulation. Many studies have defined glial activation as upregulation of glia-selective markers. This is often associated with morphological changes such as cell hypertrophy. Upregulation of these markers has been demonstrated in numerous experimental pain states, for example nerve injury, spinal cord injury, paw incision, joint arthritis, cancer, chemotherapy, and diabetes models; for reviews, see Grace et al. (2014) and Ji et al. (2013). Chronic morphine exposure has also been shown to result in considerable upregulation of the microglial markers Iba1 and CD11b (Daulhac et al., 2006; Wen et al., 2009; Zhou et al., 2010; Zhu et al., 2014), as well as the astrocyte marker GFAP (Song and Zhao, 2001) in the spinal cord.

Another indicator of glial cell activation is the upregulation of intracellular signaling cascade proteins such as the MAPK family. These pathways have an important role in the intracellular signaling of glia. The enhanced function of MAPKs by phosphorylation is essential for the development of chronic pain (Ji et al., 2009), and phosphorylation of p38, a MAPK family member, has been observed during chronic opioid treatment (Cui et al., 2006). Thirdly, many receptors and transporters having a role in glial intracellular signaling are regulated in painful states. At present, there is evidence for the upregulation of
purinergic P2X4 and P2X7 ion channel receptors in spinal microglia (Horvath and DeLeo, 2009; Zhou et al., 2010).

Toll-like receptors (TLRs) are important regulators of innate immunity (Nicotra et al., 2012) by sensing various xenobiotic substances, for example bacterial parts (lipopolysaccharide, LPS), as well as drugs such as opioids. Activation of the TLR4-MD2 (lymphocyte antigen 96) receptor complex has been shown to cause activation of both microglia and astrocytes. Morphine was suggested to induce glial responses via the TLR4-MD2 complex (Wang et al., 2012b; Watkins et al., 2009), and the blockade of TLR4-MD2 inhibited the development of opioid tolerance, withdrawal and hyperalgesia. Interestingly, glial activation via TLR4 was also observed when mu-opioid receptor inactive (+) opioid isomers were administered (Hutchinson et al., 2010a). In two studies, however, morphine-induced hyperalgesia was not altered in mice with Tlr4 gene knockout (Ferrini et al., 2013), and morphine tolerance developed similarly as in wild-type animals (Fukagawa et al., 2012).

The effects of opioids on glial cell activation have been reported in multiple studies, with Song and Zhao being the first to report the phenomenon (Song and Zhao, 2001). Particularly in the spinal cord, glial activation after chronic intrathecal and/or systemic morphine administration has been demonstrated by multiple methods, such as upregulation of the astroglial marker GFAP (Cui et al., 2006; 2008; Huang et al., 2012; Raghavendra et al., 2002; 2004; Song and Zhao, 2001; Zhou et al., 2010) and the microglia-selective markers Cd11b and Iba1 (Cui et al., 2006; Ferrini et al., 2013; Horvath et al., 2010; Liu et al., 2006; Raghavendra et al., 2002; Shen et al., 2011; Tai et al., 2009; 2006; Zhou et al., 2010). After morphine, a smaller number of studies have shown GFAP and/or Iba1 upregulation in multiple brain areas, such as the ventral tegmental area, nucleus accumbens, and frontal cortex (Garrido et al., 2005), the posterior cingulate cortex and hippocampus (Song and Zhao, 2001), PAG (Eidson and Murphy, 2013; Hutchinson et al., 2009), and midbrain (Harada et al., 2013). In several studies, glial activation has been indirectly observed by upregulation of glial cell membrane receptors such as P2X4 (Horvath et al., 2010), P2X7 (Zhou et al., 2010), and upregulation of the intracellular p38 protein (Cui et al., 2006; 2008; Liu et al., 2006).

Glial cells affect neural pain processing via soluble mediators that may be released for months after an injury or insult as a result of pathological activation (Milligan et al., 2006). Compared with classical neurotransmitters, glia-secreted immune modulators may modulate spinal cord neurotransmission at much lower

33
concentrations (Ji et al., 2013). Many immune modulators such as tumor necrosis factor (TNF), interleukin (IL)-1 beta, and interferon gamma (IFN-gamma) are capable of directly affecting excitatory transmission by increasing glutamate release from primary afferent neurons (Gao et al., 2009; Kawasaki et al., 2008; Nishio et al., 2013; Yan and Weng, 2013; Zhang et al., 2010). Another mechanism for increased synaptic transmission may be increased surface AMPA receptor expression or phosphorylation of the NMDA receptor (Meng et al., 2013; Stellwagen et al., 2005; Viviani et al., 2003; Zhang et al., 2008). Increased release of proinflammatory cytokines by glia after morphine has been documented (Shen et al., 2011; Tai et al., 2009; 2006). However, the activation of spinal cord glia has been much more extensively studied compared with different supraspinal areas. Furthermore, all of these studies have been conducted with morphine as the study opioid.

The efficacy of several glia-affecting drugs has been investigated in various models of opioid antinociception. Minocycline, a broad-spectrum tetracycline antibiotic and glial inhibitor, enhanced opioid-induced acute antinociception and attenuated morphine tolerance (Cui et al., 2008; Hutchinson et al., 2008a; 2008b). Interestingly, it also reduced opioid-induced respiratory depression (Hutchinson et al., 2008b). Ibudilast (AV411), a nonspecific phosphodiesterase (PDE) inhibitor, enhanced opioid antinociception both acutely and in models of opioid tolerance (Hutchinson et al., 2009; Ledeboer et al., 2006), as well as reducing spontaneous and precipitated opioid withdrawal periods (Hutchinson et al., 2009). The xanthine derivatives propentofylline and pentoxifylline, both inhibitors of PDE and antagonists of adenosine receptors, have shown promise in opioid antinociception in neuropathic pain and opioid tolerance (Mika, 2008; Mika et al., 2007; Raghavendra et al., 2004). For a more extensive review, see Watkins et al. (2009).

### 2.3.4 Opioids and the alpha-2-adrenergic receptors

Catecholamine receptors are G protein-coupled receptors divided into two categories, alpha- and beta-adrenoceptors. Alpha-adrenoceptors are further divided to subtypes 1A, 1B, 1D, 2A, 2B, and 2C. Alpha-2-adrenoceptors decrease intracellular adenylyl cyclase activity through G<sub>α</sub>, or they may also modify the activity of ion channels (Na<sup>+</sup>/H<sup>+</sup> antiport, Ca<sup>2+</sup> or K<sup>+</sup> channels). Adrenoceptors located in catecholaminergic neurons are called autoreceptors, and those located in non-adrenergic neurons are called heteroceptors. The autoreceptors may be located at presynaptic axon terminals, where they inhibit
the release of the neurotransmitter, whereas in the somatodendritic area they inhibit impulse discharge (Westfall and Westfall, 2011).

In the periphery, the main source of catecholamines is the postganglionic sympathetic nerve fibers, mainly releasing norepinephrine, and the adrenal medulla, mainly releasing epinephrine. In the DRG, the subtype alpha-2C is the most common, followed by alpha-2A receptors, whereas alpha-2B is rare (Shi et al., 2000). Whether the peripheral alpha-2-adrenoceptor system promotes pain facilitation or suppression strongly depends on the pain model and time course. In physiological conditions, peripheral norepinephrine has only minor effects on pain, but in conditions of inflammation and neuropathy it may aggravate pain (Davis et al., 1991; Drummond, 1995; Torebjörk et al., 1995). Peripheral nerve sprouting and the formation of new adrenoceptors may follow inflammation and injury, which may have a role in maintaining hyperalgesia (Baron, 2000). It is not exactly known which subtypes of adrenoceptors in the periphery contribute to the aggravation and which to the inhibition of pain.

In the CNS, the spinal cord and its dorsal horn is critical for the ascending nociceptive pathways. It receives noradrenergic innervation from the modulatory descending noradrenergic pathways. The source for the spinal norepinephrine is located in axons descending from noradrenergic nuclei in the pons and brainstem (Jones, 1991; Kwiat and Basbaum, 1992). These nuclei, in turn, receive important projections from PAG (Bajic and Proudfit, 1999). The descending noradrenergic fibers have sparse axon-axon contacts with central terminals of the primary afferent fibers (Hagihira et al., 1990); however, contacts with the descending noradrenergic axons and the spinal dorsal horn neurons are abundant (Doyle and Maxwell, 1991). It is also possible that the noradrenergic influence on spinal dorsal horn neurons is mainly non-synaptic, i.e. the transmitter acts by diffusion from the release site to the site of action (Rajaofetra et al., 1992; Zoli and Agnati, 1996). The alpha-2A-adrenoceptor is localized in the whole dorsal horn (Shi et al., 1999), especially on the primary afferent nociceptor nerve terminals (Stone et al., 1998). The alpha-2C receptors are probably located on the axon terminals of spinal excitatory interneurons (Olave and Maxwell, 2003), whereas the expression of the 2B-type receptor is only minute in postnatal animals.

In supraspinal areas, alpha-2-adrenoceptors are widely distributed. The subtype 2A is common throughout the brain, whereas subtypes 2B and 2C have specific localizations (2B in the thalamus and 2C prominently in the striatum) (Scheinin et al., 1994).
The most important mechanism for alpha-2-adrenoceptor-mediated pain regulation occurs in the spinal dorsal horn. Pain signaling is attenuated by reducing the release of excitatory amino acids from the primary afferent nerve fibers (Kawasaki et al., 2003; Pan et al., 2002), but also by inducing hyperpolarization by activating K$^+$ currents in spinal pain-relay neurons (Sonohata et al., 2004). It is likely that the 2A subtype of the alpha-adrenoceptors mediates both of these effects, as alpha-2-agonists failed to induce antinociception after the knockout of alpha-2A-adrenoceptors (Lakhlani et al., 1997; Stone et al., 1997). The activity of the spinal noradrenergic inhibitory pathways seems to be rather inactive in animals without sustained pain, because the knockout of any of the receptor subtypes had no effect on stimulus-evoked pain. In chronic pain, however, the alpha-2A receptor subtype in particular seems to be involved in noradrenergic feedback inhibition (Mansikka et al., 2004). At supraspinal sites, despite the fact that every major structure responsible for processing pain signaling has noradrenergic receptors, their regulatory role is not yet well established. The effects of alpha-2-antagonists have been contradictory, depending on the pain model and supraspinal area that has been under research.

The interplay between the alpha-2-adrenergic system and the opioidergic system has been under extensive research. Spinal administration of morphine or other opioids produces marked analgesia, which is mediated by opioid receptors located both pre- and postsynaptically in the terminals of the nociceptive primary afferent neurons and the dorsal horn neurons, respectively. During prolonged administration of either opioids or alpha-2-adrenoceptor agonists, pharmacologic tolerance to the drug action is observed (Milne et al., 1985; Quartilho et al., 2004; Reddy and Yaksh, 1980; Takano and Yaksh, 1992). Numerous in vivo studies investigating the functional interaction between opioid receptors and alpha-2-adrenoceptors have shown that animals tolerant to intrathecal morphine also show tolerance to alpha-2-agonist antinociception (Milne et al., 1985; Stevens et al., 1988), and opioid receptor antagonists can antagonize the antinociceptive action of spinal norepinephrine or other alpha-2-adrenoceptor agonists (Roerig et al., 1992; Sullivan et al., 1992). On the contrary, adrenoceptor antagonists, including the alpha-2-adrenoceptor antagonist yohimbine, have been shown to antagonize opioid-induced antinociception (Bentley et al., 1983; Browning et al., 1982). Furthermore, Aley and Levine (1997) showed that antagonists for mu-opioid receptors and alpha-2-adrenoceptors antagonized the effect of agonists activating the other receptor.
However, some recent findings concerning the interplay between the alpha-2-receptor and mu-opioid receptor render the interpretation of the supposed drug effects more complex. Milne et al. (2008) reported that ultralow doses (an ultralow dose is defined as a dose several log units lower than the dose required to produce functional antagonism at the respective receptor) of four structurally different intrathecally administered alpha-2-adrenoceptor antagonists (atipamezole, yohimbine, mirtazapine, and idazoxan) increased acute spinal morphine antinociception, attenuated the induction of acute and chronic tolerance, and also reversed the already established morphine tolerance in rats. The same group has also demonstrated an augmentation in clonidine and norepinephrine antinociception after coadministration of atipamezole (Milne et al., 2011). Moreover, ultralow doses of systemic or intrathecal naltrexone, an opioid receptor antagonist, administered systemically or intrathecally had a paradoxical additive effect on the antinociceptive effect of morphine in heat nociception and also blocked the development of tolerance (Powell et al., 2002). This has additionally been shown in a model of neuropathic pain in rats (Largent-Milnes et al., 2008). Similar effects were noted with ultralow doses of selective mu-, delta- and kappa-opioid receptor antagonists (Abul-Husn et al., 2007; McNaull et al., 2007).

G protein-coupled receptors normally act as monomeric cell surface receptors, but there is increasing evidence that G protein-coupled receptors may also form homo- and heterodimeric complexes. Interestingly, mu-opioid and alpha-2A receptors may form heterodimers at the plasma membrane of cultured cells as well as in primary spinal cord neurons (Jordan et al., 2003). The activation of either monomeric mu-opioid or alpha-2A-adrenoceptors normally mediates a parallel signaling effect, leading to antinociception at the spinal level. However, the simultaneous activation of both the mu-opioid- and alpha-2A-adrenoceptors in heterodimer complexes changes the cellular response, which is not an additive effect (Jordan et al., 2003). Activation of either mu-opioid or alpha-2A receptors leads to increased signaling, whereas activation of both receptors decreases the response. Moreover, an in vitro study (Vilardaga et al., 2008) has shown that in the heterodimer complexes, the mu-opioid and alpha-2A receptors communicate with each other through a cross-conformational switch that allows the direct inhibition of one receptor by the other. Binding of morphine to the mu-opioid receptor triggers a conformational change in the norepinephrine-occupied alpha-2A-adrenoceptor that inhibits its signaling. Norepinephrine has been shown to inhibit morphine-mediated G protein activation, suggesting that a conformational change may also take place in the opposite direction (Jordan et al., 2003).
Endogenous norepinephrine could inhibit the effect of morphine in heterodimers through these mechanisms. Supporting the alpha-2A and opioid receptor-interplay related nociceptive changes, the antinociceptive effect of morphine and especially tramadol was increased in alpha-2A-adrenoceptor knockout mice (Ozdoğan et al., 2006).

Another theory for the mechanism behind the paradoxical augmentation of opioid agonists by ultralow doses of opioid antagonists has been postulated. Wang et al. (2008) showed that cotreatment with 10 ng/kg naloxone prevented the chronic morphine-induced G\textsubscript{i}/G\textsubscript{o}-to-G\textsubscript{s} switch in mu-opioid receptor G protein coupling. While opioid receptors in a normal state preferentially bind to G\textsubscript{i} or G\textsubscript{o} proteins to inhibit adenyl cyclase (Laugwitz et al., 1993), chronic morphine treatment may induce a switch to G\textsubscript{s} coupling (Chakrabarti et al., 2005; Wang et al., 2008), leading to cAMP accumulation. A subgroup of mu-opioid receptors preferentially binding to G\textsubscript{s} proteins has also been proposed, but the naloxone dose of 10 ng/kg has been estimated to occupy only 1% of mu-opioid receptors, which is not sufficient for antagonizing the supposed G\textsubscript{s}-binding subpopulation of mu receptors (Lewanowitsch and Irvine, 2003). Moreover, an ultra-low dose of naloxone reduced G\textsubscript{s} coupling and restored levels of coupling to native G\textsubscript{i} or G\textsubscript{o} proteins (Wang et al., 2005). In further studies by the same group, naloxone was found to bind with high affinity to filamin A, a 300-kDa protein co-immunoprecipitating with mu-opioid receptors (Wang et al., 2008). Filamin A is known as a large cytoplasmic protein responsible for regulating cell signaling by interacting with various receptors and signaling molecules, including the mu-opioid receptor (Onoprishvili et al., 2003). The ultimate hypothesis is that naloxone binding to filamin A prevents morphine tolerance and hyperalgesia by inhibiting mu receptor coupling to excitatory G\textsubscript{s} proteins. Interestingly, filamin A may also inhibit the signaling cascades of the TLR4-MD2 receptor complex (Wang et al., 2012a). Whether ultralow antagonists of alpha-2-adrenergic receptors also have affinity for filamin A or other closely mu-opioid receptor related proteins remains to be shown. Table 2 presents the current evidence for the paradoxical enhancement of opioid antinociception by either opioid receptor or alpha-2-adrenoceptor antagonists.

In clinical studies, ultralow-dose naloxone combined with morphine in patient controlled analgesia (PCA) led to decreased nausea and pruritus, but did not affect analgesia or opioid requirements (Cepeda et al., 2004). When the administered naloxone concentration was ten times greater (6 µg/ml with an initial setting of 0.5 ml bolus per demand), the combination led to poorer pain relief than
morphine alone (Cepeda et al., 2002). Chindalore et al. (2005) reported that Oxytrex, a preparation that combines oxycodone with ultralow-dose naltrexone, enhanced and prolonged analgesia compared with oxycodone alone in patients suffering from osteoarthritis. Thus, it seems that in patients the choice of the ultralow dose may far more difficult than in laboratory animals.

Interestingly, the alpha-2-adrenoceptor is also expressed in glial cells (Hösli et al., 1982), but its role in glial reactivity has not been thoroughly researched and has produced some contradictory results. For example, Yan et al. (2011) demonstrated that dexmedetomidine, an alpha-2-adrenoceptor agonist, stimulated astrocyte activation and glial cell line-derived neurotrophic factor (GDNF) production in vitro. In vivo, the alpha-2-adrenoceptor antagonist yohimbine (at a dose large enough to antagonize morphine antinociception) was shown to reduce GFAP upregulation induced by chronic morphine administration (Alonso et al., 2007; Garrido et al., 2005).
(Table 2 continued)

<table>
<thead>
<tr>
<th>Drug studied</th>
<th>Pain model</th>
<th>Outcome</th>
<th>Route, subject</th>
<th>Reference</th>
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<td><strong>POSITIVE FINDINGS</strong></td>
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<tr>
<td>Nonselective opioid receptor antagonist <strong>naloxone</strong></td>
<td>Intrathecal morphine tolerance: thermal nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance, inhibition of Gs coupling and Gβγ signaling to adenylyl cyclase, increasing Gi/o coupling</td>
<td>i.t., rat</td>
<td>Wang et al., 2005</td>
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<td>Inhibition of 1) development of intrathecal morphine tolerance, 2) glutamate transporter downregulation, 3) NMDAR NR1 subunit phosphorylation 4) PKC-gamma expression, 5) glial cell activation, 6) excitatory amino acid release</td>
<td>i.t. infusion, rat</td>
<td>Lin et al., 2010</td>
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<tr>
<td>Remifentanil infusion-induced hyperalgesia during sevoflurane anesthesia</td>
<td>Reduction of needed minimum alveolar concentration (MAC) of sevoflurane</td>
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<td>i.v. infusion, rat</td>
<td>Aguado et al., 2013</td>
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<tr>
<td>Intrathecal PTX-induced thermal hyperalgesia</td>
<td>Preservation of morphine antinociception, suppression of PTX-induced excitatory amino acid release, inhibition of microglial activation, suppression of cytokine release</td>
<td></td>
<td>i.t. rat</td>
<td>Tsai et al., 2008</td>
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<td>Nonselective opioid receptor antagonist <strong>naltrexone</strong></td>
<td>Acute thermal and mechanical nociception</td>
<td>Prolongation of intrathecal and augmentation of systemic morphine antinociception</td>
<td>i.t. and s.c., rat</td>
<td>Powell et al., 2002</td>
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<td>Intrathecal or systemic morphine tolerance: acute thermal and mechanical nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance; restoration of morphine antinociceptive potency in developed tolerance</td>
<td>i.t., female and male SD and LE rats</td>
<td>Terner et al., 2006</td>
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<td>Intrathecal morphine tolerance: acute thermal nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance; restoration of morphine antinociceptive potency in developed tolerance</td>
<td>i.t., rat</td>
<td>Mattioli et al., 2010</td>
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<td>Inhibition of development of i.t. morphine tolerance, attenuation of microglial and astrocyte activation</td>
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<td>Intrathecal or oral oxycodone antinociception in spinal nerve ligation-induced hyperalgesia</td>
<td>Reduction of allodynia and hyperalgesia after SNL and oxycodone treatment, attenuation of Gs coupling</td>
<td>i.t., rat</td>
<td>Largent-Milnes et al., 2008</td>
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Table 2. Current evidence for the paradoxical enhancement of opioid- or alpha-2-adrenoceptor agonist antinociception by coadministration of **ultralow doses** (a dose several log units lower than the dose required to produce functional antagonism at the respective receptor) of either opioid receptor or alpha-2-adrenoceptor antagonists. CTOP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; CTAP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2; PTX, pertussis toxin; SD, Sprague-Dawley, LE, Long Evans.

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<tr>
<td>Alpha-2-adrenoceptor antagonists atipamezole, yohimbine, mirtazapine, and idazoxan</td>
<td>Acute thermal and mechanical nociception</td>
<td>Prolongation of intrathecal morphine antinociception</td>
<td>i.t., rat</td>
<td>Milne et al., 2008</td>
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<td>Intrathecal morphine tolerance: acute thermal and mechanical nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance; restoration of morphine antinociceptive potency in developed tolerance</td>
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<tr>
<td>Alpha-2-adrenoceptor antagonist atipamezole</td>
<td>Acute thermal and mechanical antinociception</td>
<td>Prolongation of intrathecal clonidine (tail-flick and hot plate) and norepinephrine (tail-flick only) antinociception</td>
<td>i.t., rat</td>
<td>Milne et al., 2011</td>
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<td></td>
<td>Intrathecal norepinephrine antinociceptive tolerance: acute thermal and mechanical antinociception</td>
<td>Inhibition of development of intrathecal norepinephrine antinociceptive tolerance</td>
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<td>Alpha-2-adrenoceptor antagonist efaroxan, (+) and (±) isomers but not the (-) isomer</td>
<td>Intrathecal morphine tolerance: acute thermal and mechanical nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance</td>
<td>i.t., rat</td>
<td>Milne et al., 2013</td>
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<td>Alpha-2-adrenoceptor antagonists efaroxan, atipamezole, and yohimbine</td>
<td>Low-dose intrathecal morphine thermal hyperalgesia</td>
<td>Inhibition of acute morphine thermal hyperalgesia</td>
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**POSITIVE FINDINGS**

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<th>Outcome</th>
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<tr>
<td>Mu-opioid receptor antagonists CTOP and CTAP and delta-opioid receptor antagonist naltrindole</td>
<td>Acute thermal and mechanical nociception</td>
<td>Prolongation of intrathecal morphine antinociception</td>
<td>i.t., rat</td>
<td>Abul-Husn et al., 2007</td>
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<td>Intrathecal morphine tolerance: acute thermal and mechanical nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance; restoration of morphine antinociceptive potency in developed tolerance</td>
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<tr>
<td>Mu-opioid receptor antagonist CTAP, delta-opioid receptor antagonist naltrindole, kappa-opioid receptor antagonist norbinaltorphimine and nonselective opioid receptor antagonist naltrexone</td>
<td>Acute thermal and mechanical nociception</td>
<td>Prolongation of intrathecal morphine antinociception</td>
<td>i.t., rat</td>
<td>McNaull et al., 2007</td>
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<td>Intrathecal morphine tolerance: acute thermal and mechanical nociception</td>
<td>Inhibition of development of intrathecal morphine tolerance</td>
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<td></td>
<td>Low-dose systemic and intrathecal morphine thermal hyperalgesia</td>
<td>Inhibition of acute thermal hyperalgesia</td>
<td>i.t. and s.c., rat</td>
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2.3.5 Access of morphine and other opioids to the central nervous system

Clinically, one interesting developing line of research is the study of opioid pharmacokinetics and drug–drug interactions for the improvement of opioid efficacy. The CYP450 family enzymes only have an effect on the metabolism of opioids, whereas several drug transporter proteins may affect the absorption, distribution, and excretion of opioid drugs (Mercer and Coop, 2011). Recent preclinical evidence suggests that the BBB efflux transporters may contribute to opioid efficacy and the development of tolerance. P-gp, a member of the ATP-binding cassette (ABC) superfamily, is one of the best characterized efflux transporters to date. It has functions in the extrusion of xenobiotics and uptake of nutrients (Hennessy and Spiers, 2007). In the brain, the expression of P-gp is high in the frontal cortex, choroid plexus, and PAG, with lower levels in the cerebellum and hippocampus (King et al., 2001).

A role of P-gp in the transport of opioids was first observed by Callaghan and Riordan in an in vitro system (Callaghan and Riordan, 1993). Since then, multiple in vivo and in vitro studies have been conducted with different experimental approaches to confirm this finding. In Caco-2 and L-MDR1 cells, morphine was acknowledged as a weak P-gp substrate with an efflux:influx ratio of 1.5 (Crowe, 2002; Wandel et al., 2002), a ratio that was supported by in situ studies (P-gp effect of 1.24) (Dagenais et al., 2004). In P-gp knockout mice, morphine antinociception was increased and prolonged (Thompson et al., 2000). Human evidence is sparse and it only consists of acute studies in healthy volunteer subjects. In the presence of the P-gp inhibitor quinidine, enhanced CNS effects were not experienced after an i.v. morphine dose (Skarke et al., 2003), whereas increased plasma concentrations but no influence on morphine pharmacodynamics after morphine p.o. administration were observed in another study (Kharasch et al., 2003). Finally, pharmacokinetic modeling for morphine has been employed, resulting in three major factors limiting the access of morphine to the brain: limited passive diffusion, active efflux, and low-capacity active uptake (Groenendaal et al., 2007). Interestingly, a variety of the P-gp gene ABCB1 polymorphisms have been discovered, and variability in pain relief in cancer patients was noted with the 3435C>T variant (Sai et al., 2006).

Much less information exists on the interaction of other opioids and P-gp. For the commonly used oxycodone, there are conflicting results (Boström and Simonsson, 2005; Hassan et al., 2007). Interestingly, a correlation for P-gp and the CYP3A substrate co-specificity exists (Schuetz et al., 1996; Wacher et al.,
1995), and oxycodone is an example of this phenomenon (Hassan et al., 2007; Lalovic et al., 2004). Loperamide, a peripherally restricted opioid often used as an anti-diarrheal drug, has been identified as a good P-gp substrate with an efflux:influx ratio of 10 (Wandel et al., 2002). In P-gp knockout mice, its concentration was increased 13-fold in the brain and doubled in plasma (Schinkel et al., 1995), suggesting that P-gp is a key factor in constraining loperamide to the periphery.

A number of studies have implicated the upregulation of the BBB P-gp in morphine tolerance (Aquilante et al., 1999; Hassan et al., 2009; King et al., 2001; Mercer and Coop, 2011; Yousif et al., 2012). Aquilante et al. (1999) reported that the whole brain P-gp content was doubled in morphine-tolerant rats. Oxycodone was also shown to induce P-gp up to 4-fold in the liver and 1.3-fold in brain tissues (Hassan et al., 2007), but in a transcriptome study, the P-gp (Abcb1) mRNA levels were not increased (Hassan et al., 2009). Furthermore, King et al. (2001) reported that P-gp antisense or mismatch treatment eliminated morphine tolerance. Interestingly, in all these studies, measurements were conducted at the withdrawal phase, pointed out by Yousif et al. (Yousif et al., 2012). In their study, the expression of P-gp and breast cancer resistance protein (Bcrp) after chronic morphine administration was not directly upregulated after the last morphine dose, but only after 9 hours of the last dose. They hypothesized that P-gp upregulation is not induced by chronic morphine administration itself, but by the following withdrawal period. Furthermore, the expression of P-gp and Bcrp was clearly downregulated by the administration of MK-801 (NMDA receptor antagonist) or meloxicam (cyclooxygenase-2 inhibitor) after the last morphine dose.

Only a few studies have reported other transporters than P-gp for morphine. Bcrp was not found to transport morphine, even though chronic morphine induced it (Tournier et al., 2010; Yousif et al., 2012). Like P-gp, multidrug resistance protein (Mrp) is a member of the ABC superfamily of transport proteins. In the brain, Mrp is expressed in the cerebellum, PAG, and hippocampus, and at lower levels in the frontal cortex and choroid plexus (Su and Pasternak, 2013). In the same study, morphine displayed increased antinociception after Mrp antisense administration. Dose–response studies have revealed a twofold-enhanced potency of morphine after Mrp downregulation. Furthermore, in the Mrp antisense group, morphine antinociception in repeated intracerebroventricular (i.c.v.) administration developed significantly more slowly, indicating slower transport from the cerebrospinal fluid to the blood. Rao et al.
(1999) utilized a choroid plexus (blood–cerebrospinal fluid barrier) model to suggest that Mrp and P-gp are working in series, with P-gp located subapically and Mrp situated basally. This might explain why downregulation of either transporter resulted in a similar modulation of opioid analgesia. Finally, Tzvetkov et al. (2013) showed that morphine uptake was fourfold increased in OCT1-overexpressing HEK293 cells and morphine uptake in human hepatocytes was reduced by 1-methyl-4-phenylpyridinium (MPP), an OCT inhibitor. Furthermore, the mean morphine AUC in OCT1 loss-of-function polymorphism carriers was significantly higher.

As noted in chapter 2.2.4, morphine is mainly metabolized in phase II metabolism to water-soluble glucuronide conjugates M3G and M6G in the liver by UGT2B7 in humans and Ugt2b1 in rats. Interestingly, human brain homogenates have also been shown to metabolize morphine to its glucuronides, and the M3G:M6G ratio produced has been associated with the morphine concentration, so that higher morphine concentrations lead to a higher M3G:M6G ratio (Yamada et al., 2003). M6G is an attractive analgesic, but its BBB permeability is very poor, with a permeability that is 7.5 times less than that of morphine and equivalent to that of sucrose (Yoshimura et al., 1973). Bourasset et al. (2003) found that the brain uptake of M6G was not increased in P-gp- or Mrp1-deficient mice, but suggested M6G to be a substrate of the organic anion transporter (Oatp) 2, as the brain M6G concentration was decreased when Oatp2 substrates digoxin and PSC833 were co-perfused. Oatp2 is known to exist on both the luminal and basolateral sides of the endothelium of the brain vasculature. Moreover, co-perfusion of D-glucose with M6G also led to decreased brain M6G, indicating that these compounds may use the same transporter, glucose transporter (GLUT) 1, expressed on the both sides of brain endothelial cells and used for facilitated diffusion of glucose to the brain. Interestingly, the binding of the opioid peptide enkephalin with β-D-glucose by an O-β-linkage has also been shown to enhance their brain access via the glucose transporter GLUT1 (Polt et al., 1994). Even less information exists on the transport of M3G, the main glucuronide metabolite formed in the Sprague-Dawley rat. In microdialysis studies, Xie et al. (2000) demonstrated that probenecid, a typical Oatp inhibitor, increased the brain:blood ratio of M3G. Current knowledge of the possible BBB transporters for morphine and its metabolites is presented in Figure 8.
2.4 Drugs used in the study

2.4.1 Atipamezole, an alpha-2-adrenoceptor antagonist

Atipamezole (4-[2-ethyl-2,3-dihydro-1H-inden-2-yl]-1H-imidazole) (Fig. 9) is an alpha-2-adrenoceptor antagonist (Scheinin et al., 1988; Virtanen, 1989). Its binding affinity for alpha-2 receptors (Kᵢ = 1.6 nM) is approximately 100 times higher than for alpha-1 receptors (Haapalinna et al., 1997; Virtanen, 1989). It has affinity for alpha-2A-, alpha-2B-, and alpha-2C-adrenoceptor subtypes, and also for the alpha-2D human receptor variant (Haapalinna et al., 1997; Renouard et al., 1994). It has no affinity for opioid (mu and delta) receptors. However, in an *in vivo* situation, the increase in norepinephrine release in the
CNS following the inhibition of alpha-2-adrenoceptors may result in the indirect activation of other systems (Haapalinna et al., 2000).

![Figure 9. Structural formula of atipamezole.](image)

In rodents, atipamezole is well tolerated. It is rapidly absorbed and distributed, and peak concentration levels in tissues are two- to threefold higher than the peak plasma concentration. Atipamezole undergoes first-pass metabolism and its elimination half-life is 1.3 hours. The LD₅₀ for mice and rats is >30 mg/kg after i.p., s.c. or i.v. administration (Haapalinna et al., 1997; 1999). In human phase I studies, atipamezole was also well tolerated after a single dose (10–100 mg). After the largest dose, some subjective drug effects (e.g. motor restlessness, coldness, sweating, increased salivation) were reported. It also increased the plasma norepinephrine concentration and both the systolic and diastolic blood pressure. At the dose of 30 mg/subject, no subjective or cardiovascular side effects were reported (Karhuvaara et al., 1990).

The majority of the brain noradrenergic neuron somas are based in the brainstem and medulla, and alpha-2-antagonists have extensive effects on the modulation of brain function. The locus coeruleus, the principal site for noradrenergic neurons, is affiliated with attention, memory, and learning (Berridge et al., 1993). Atipamezole, by stimulating endogenous norepinephrine release (presynaptic modulation), has potential effects on these parameters. The effects on cognitive performance have varied depending on atipamezole dose, stress experienced during the test, and the age of the animal. Low doses improved alertness, attention, planning, and recall of animals. At higher doses, the overactivation of the noradrenergic system has led to impairment in cognitive tasks; for a review, see Pertovaara et al. (2005). Atipamezole treatment has also shown promise in recovery after traumatic brain injury (Pitkänen et al., 2004) and enhanced the positive effects and reduced the adverse effects of dopaminergic drugs used for the treatment of Parkinson’s disease (Haapalinna et al., 2003).
As the noradrenergic system has a role in modulating pain in both the brain and spinal cord (see 2.3.4), atipamezole has also been studied in various pain models. Supporting the hypothesis that the activity in the spinal descending noradrenergic pain regulatory pathways is low in non-painful conditions, atipamezole has not been shown to have effects in baseline nociceptive latencies (Pertovaara, 1993). In sustained nociception (formalin and capsaicin models), atipamezole administration antagonized the spinal descending noradrenergic pathways and increased the nociceptive responses (Green et al., 1998; Mansikka et al., 2004). At the brainstem level, however, microinjection of atipamezole produced antinociception in a neurogenic inflammation model (Mansikka and Idänpään-Heikkilä, 1996). Therefore, atipamezole may have two opposing mechanism of action regarding pain in different areas of the CNS.

Interestingly, a recent study reported that intrathecal atipamezole (and other chemically distinct alpha-2-adrenergic antagonists) administered in ultralow doses increased the effect of morphine in thermal and mechanical nociception tests. The synergistic effect of atipamezole on morphine antinociception was seen at a dose (0.08 ng i.t.) lower than needed for blocking the spinal clonidine antinociception (5–10 µg i.t.). Atipamezole also inhibited the development of morphine tolerance and reversed the analgesic tolerance that had already developed (Milne et al., 2008; 2013). Clinically, alpha-2-agonists exert sedative and anesthetic properties, having similar effects to general anesthetics such as isoflurane. Currently, alpha-2-adrenoceptor agonists (e.g. medetomidine and dexmedetomidine) are in clinical use in both human and veterinary medicine. Atipamezole is registered for clinical use in veterinary medicine in several countries. It has proven effective in reversing the anesthesia induced by alpha-2-adrenoceptor activation by medetomidine. The animals are awake within minutes of administration of the antagonist, which is useful in minor veterinary surgical operations (Ewing et al., 1993; Jalanka, 1989; Vainio and Vähä-Vahe, 1990).

2.4.2 Ibudilast, a phosphodiesterase inhibitor

Ibudilast (3-isobutyryl-2-isopropylpyrazolo-[1,5-a]pyridine) (Fig. 10), also named AV411, MN-166, or KC-404, is used in Asia to treat bronchial asthma (Kawasaki et al., 1992), cerebrovascular disorders, and post-stroke dizziness (Armstead et al., 1988; Fukuyama et al., 1993; Shinohara and Kusunoki, 2002). These applications are based on the effect of ibudilast on tracheal smooth muscle contractility (Souness et al., 1994), cerebral blood flow improvement (Fukuyama
et al., 1993), and platelet aggregation (Kishi et al., 2000; Ohashi et al., 1986; Rile et al., 2001). The effects of ibudilast for its clinical implications are presumed to be related to PDE inhibition, which is key to the regulation of important second messengers, *i.e.* cAMP and cyclic GMP (cGMP). The inhibitory profile of ibudilast on human phosphodiesterases includes PDEs 3A, 4, 10 and 11, with a lesser inhibition on other families (Gibson et al., 2006). In addition, ibudilast is a weak adenosine receptor antagonist, but other target proteins have not been identified (Ledeboer et al., 2006; 2007).

![Figure 10. Structural formula of ibudilast.](image)

In addition, ibudilast has anti-inflammatory properties in the CNS. In cultured glial cells, it suppressed LPS-induced production of inflammation markers TNF, IL-1 and IL-6, nitric oxide (NO) and reactive oxygen species concentration-dependently, and increased the production of the anti-inflammatory cytokine IL-10 and neurotrophic factors GDNF and nerve growth factor (NGF) (Mizuno et al., 2004). It also decreased LPS-induced neuronal cell death in mouse neuron-glia co-cultures and glutamate-induced cell death in hippocampal neurons. The exact target protein for this effect of ibudilast on glial cells is not known. Inhibition of phosphodiesterases may additionally contribute to the anti-inflammatory effects of ibudilast in the CNS (Sebastiani et al., 2006; Suzumura et al., 1999). Another potential mechanism of action is the inhibition of the proinflammatory macrophage migration inhibitory factor (MIF) (Cho et al., 2010b). MIF is a proinflammatory cytokine itself (Alexander et al., 2012), but it is required for both IL-1 and TNF release and IL-1- and TNF-induced MAPK activation (Toh et al., 2006).

Concerning opioid treatment, ibudilast was shown to attenuate morphine-induced glial proinflammatory responses in rats (Hutchinson et al., 2009). Ibudilast also augmented the antinociceptive action of morphine and oxycodone in the tail-flick test without having noticeable effects of its own (Hutchinson et
al., 2009; Ledeboer et al., 2007). It attenuated chronic constriction injury (CCI) induced allodynia and was suggested to affect the development of opioid tolerance in CCI rats (Ledeboer et al., 2006). It has also been studied for the treatment of opioid dependency; for a review, see Coller and Hutchinson (2012). For a summary of findings on ibudilast in pain models, see Table 3.

In phase 1 human studies, ibudilast was shown to be well tolerated as a single 30-mg oral dose, as well as in repeated administration (30 mg p.o. b.i.d.). Adverse events were minor. The mean elimination half-life was 19 h (Rolan et al., 2008). It is currently in clinical trials for the treatment of migraine, medication overuse headache, metamphetamine dependence, multiple sclerosis, and opioid withdrawal (http://www.clinicaltrials.gov, searched on May 1, 2014).
Table 3. Studies on ibudilast in different *in vivo* pain models. *q.d.*, once daily; *b.i.d.*, twice daily.

<table>
<thead>
<tr>
<th>Research model</th>
<th>Outcome</th>
<th>Dosing, route</th>
<th>Other notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POSITIVE FINDINGS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCI and SNL models, rat</td>
<td>Alleviation of alodynia</td>
<td>10 mg/kg i.p. <em>b.i.d.</em>; 22 mg/kg p.o. <em>b.i.d.</em>; 25 μg i.t. single dose</td>
<td>Ibudilast is well distributed in the CNS; Ibudilast tolerability was rather good with only transient changes in the neurobehavioral tests</td>
<td>Ledeboer et al., 2006</td>
</tr>
<tr>
<td>Paclitaxel-induced alodynia, rat</td>
<td>Alleviation of alodynia</td>
<td>7.5 mg/kg i.p. <em>b.i.d.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine tolerance in CCI, rat</td>
<td>Attenuation of alodynia</td>
<td>7.5 mg/kg i.p. <em>b.i.d.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute morphine and oxycodone antinociception, rat</td>
<td>Augmentation of opioid effect</td>
<td>7.5 mg/kg i.p.</td>
<td>Plasma morphine levels were not altered by ibudilast</td>
<td>Hutchinson et al., 2009</td>
</tr>
<tr>
<td>Precipitated and spontaneous opioid withdrawal periods, rat</td>
<td>Decrease in withdrawal behavior</td>
<td>7.5 mg/kg i.p. <em>b.i.d.</em> along morphine treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine-induced dopamine release in nucleus accumbens, rat</td>
<td>Decreased dopamine release</td>
<td>7.5 mg/kg i.p. <em>b.i.d.</em> along morphine treatment</td>
<td>Ibudilast also reduced withdrawal symptoms</td>
<td>Bland et al., 2009</td>
</tr>
<tr>
<td>Spinal cord injury, rat</td>
<td>Decreased hyperalgesia</td>
<td>10 mg/kg i.p.</td>
<td>Sciatic nerve TNF-alpha ▼</td>
<td>Hama et al., 2012</td>
</tr>
<tr>
<td>CCI model, rat</td>
<td>Decreased central alodynia</td>
<td>10 mg/kg s.c. <em>q.d.</em></td>
<td></td>
<td>Ellis et al., 2014</td>
</tr>
<tr>
<td>T13/L1 level dorsal root avulsion</td>
<td>Decreased central alodynia</td>
<td>10 mg/kg s.c. <em>q.d.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NEGATIVE FINDINGS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCI model, rat</td>
<td>No effect</td>
<td>7.5 mg/kg p.o.</td>
<td>Low oral bioavailability</td>
<td>Ledeboer et al., 2006</td>
</tr>
</tbody>
</table>
2.4.3 Spironolactone, a mineralocorticoid receptor antagonist

Spironolactone (7α-acetyltio-3-oxo-17α-pregn-4-ene-21,17-carbolactone) (Fig. 11) is an old mineralocorticoid receptor antagonist with indications for the treatment of diseases associated with primary or secondary hyperaldosteronism (Doggrell and Brown, 2001). As it is a potassium-sparing diuretic, it can also be used to alleviate heart failure or to reduce ascites formation. Many patients diagnosed with acute heart failure or liver metastases are co-treated with spironolactone and morphine. For the management of treatment-resistant hypertension, the daily spironolactone doses are usually small, ranging from 12.5 to 50 mg/day, but patients suffering from ascites due to cancer may be prescribed high doses of up to 400 mg/day (Ginès et al., 2004). In the rat, spironolactone has been reported to potentiate the cataleptic effect of morphine (Chu et al., 1978). Possible mechanisms include the inhibition of efflux transporters leading to greater morphine disposition into the brain or the inhibition of morphine metabolism.

Figure 11. Structural formula of spironolactone.

Spironolactone has been shown to inhibit the production of TNF, IL-1, and several other proinflammatory cytokines in peripheral blood mononuclear cells. Whether this action is mediated by mineralocorticoid receptors is unknown. Spironolactone also shows promise in patients suffering from autoinflammatory diseases such as rheumatoid arthritis (Bendtzen et al., 2003; Hansen et al., 2004; Miura et al., 2006; Syngle et al., 2009). In an observational case series, spironolactone improved fibromyalgia-associated pain, stiffness, fatigue, depression, and mood (Wernze and Herdegen, 2014). Interestingly, spironolactone reversed corticosterone- and aldosterone-induced microglial
activation in vitro (Tanaka et al., 1997). In a cerebral stroke model (middle cerebral artery occlusion), the knockout of mineralocorticoid receptors led to reduced microglial activation and infarct volume (Frieler et al., 2011). Spironolactone also showed efficacy in the CCI neuropathic pain model (Jaggi and Singh, 2010) in peroral administration, and also when intrathecally administered in the chronic compression of the DRG model (Sun et al., 2012). In the same study, spironolactone decreased the expression and phosphorylation of the NMDA receptor, an important player in opioid tolerance and hyperalgesia. Interestingly, eplerenone, another more selective mineralocorticoid receptor antagonist, also reduced mechanical hypersensitivity in an inflamed DRG model (Dong et al., 2012), indicating that the mineralocorticoid receptors may play a role in the modulation of pain, probably via the inhibition of proinflammatory cytokine production (Chantong et al., 2012). The preclinical studies on spironolactone in different pain models are listed in Table 4.

Spironolactone inhibits human microsomal UGT2B7 (Knights et al., 2010), and could thus also inhibit morphine glucuronidation. Even though there is no direct evidence for inhibition of the rat homologue Ugt2b1 by spironolactone, the genes encoding the two enzymes have a high gene sequence similarity (Tukey and Strassburg, 2000). In rodents, the disposition of morphine in the CNS is regulated by P-gp (Letrent et al., 1999; Schinkel et al., 1995; Xie et al., 1999), an efflux protein at the BBB. Inhibition of P-gp could lead to increased access of morphine to the CNS. Spironolactone has been reported to inhibit the transcellular transport of the P-gp substrate digoxin in P-gp-overexpressing LLC-GA5-COL150 cells (Nakamura et al., 2001) and to increase the plasma concentration of the P-gp substrate digoxin in humans (Hedman et al., 1992), indicating a potential interaction with P-gp substrate drugs.

Taken together, spironolactone possesses three properties that make it interesting for combining with morphine in the treatment of pain. First, it may suppress the pathological activation of glial cells via an unknown mechanism; second, it may inhibit the formation of the pronociceptive morphine metabolite M3G; and third, it may increase the access of morphine to the brain and delay its elimination by the inhibition of P-gp.
Table 4. Studies on spironolactone in different *in vivo* pain models. CCI, chronic constriction injury; CCD, chronic compression of the dorsal root ganglion; DRG, dorsal root ganglion.

<table>
<thead>
<tr>
<th>Pain model</th>
<th>Outcome</th>
<th>Dosing, route</th>
<th>Other notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POSITIVE FINDINGS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptozotocin diabetes model, mouse</td>
<td>Attenuation of hyperalgesia</td>
<td>7 or 15 mg/kg p.o.</td>
<td></td>
<td>Khan et al., 2009</td>
</tr>
<tr>
<td>CCI, rat</td>
<td>Attenuated CCI-related pain</td>
<td>10 and 20 mg/kg p.o.</td>
<td>Sciatic nerve TNF-alpha ↓</td>
<td>Jaggi and Singh, 2010</td>
</tr>
<tr>
<td>CCD, rat</td>
<td>Attenuated CCD-related pain</td>
<td>3 ( \mu g ) i.t. b.i.d.</td>
<td>Synergistic effects with glucocorticoid receptor antagonists</td>
<td>Gu et al., 2011</td>
</tr>
<tr>
<td>CCD, rat</td>
<td>Attenuated CCD-related pain</td>
<td>3 ( \mu g ) i.t. b.i.d.</td>
<td>Spinal microglial reactivity ↓</td>
<td>Sun et al., 2012</td>
</tr>
<tr>
<td></td>
<td>behavior</td>
<td></td>
<td>Concentrations of IL-1( \beta ) and TNF-alpha in the spinal cord and DRGs ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMDA receptor expression and phosphorylation ↓</td>
<td></td>
</tr>
<tr>
<td><strong>NEGATIVE FINDINGS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCI, rat</td>
<td>No effect on hyperalgesia or</td>
<td>3 ( \mu g ) i.t. b.i.d.</td>
<td></td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td>Spinal morphine tolerance, rat</td>
<td>Allodynia</td>
<td></td>
<td></td>
<td>Lim et al., 2005</td>
</tr>
<tr>
<td>Vincristine neuropathy, rat</td>
<td>No alleviation of hyperalgesia</td>
<td>10 and 20 mg/kg p.o.</td>
<td></td>
<td>Jaggi and Singh, 2010</td>
</tr>
</tbody>
</table>
2.4.4 Ketamine, an N-methyl-D-aspartate receptor antagonist

Ketamine [2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone] (Fig. 12) is a phencyclidine (PCP) derivative available as a racemic mixture and also as an S(+) enantiomer in Europe. It is highly lipophilic and easily crosses the BBB (White, 1988). It is a general anesthetic that produces a state of dissociative anesthesia at high doses and analgesia at low doses (Sinner and Graf, 2008). The S-enantiomer is a 2–4 times more potent anesthetic and analgesic than the R-enantiomer (Arendt-Nielsen et al., 1996; Oye et al., 1992; White et al., 1980). The actions of ketamine are mainly based on noncompetitive antagonism of the NMDA receptor by binding to the PCP binding site (Fig. 5). Ketamine reduces the calcium influx and during a longer period modulates long-term potentiation, the wind-up phenomenon, and glutamate-mediated neurotoxicity. Ketamine has other possible interactions with sodium (Frenkel and Urban, 1992) and calcium channels (Sikand et al., 1995), the potassium/sodium hyperpolarization-activated cyclic nucleotide-gated (HCN) 1 channel (Chen et al., 2009), as well as the nicotinic (Scheller et al., 1996), muscarinic (Hustveit et al., 1995), and opioid receptors (Finck and Ngai, 1982; Hirota et al., 1999; Hustveit et al., 1995; Smith et al., 1980).

![Figure 12. Structural formula of ketamine.](image)

In humans, the elimination half-life of ketamine is two to three hours after oral administration and approximately 80% of the drug undergoes N-demethylation to norketamine by CYP3A4 and CYP2B6 enzymes (Hijazi, 2002; Sinner and Graf, 2008; Yanagihara et al., 2001). The bioavailability of ketamine is 17–24% in humans after oral administration (Chong et al., 2009; Clements et al., 1982). Norketamine and other metabolites are mainly excreted renally, and only small
amounts of ketamine are excreted unchanged (Geisslinger et al., 1993). Norketamine is an active metabolite of ketamine (Ebert et al., 1997; Holtman et al., 2008; Shimoyama et al., 1999). In a binding study, Ebert et al. (1997) reported that S-norketamine is an approximately five times weaker NMDA receptor antagonist than S-ketamine. Racemic norketamine was found antinociceptive in the rat (Holtman et al., 2008; Shimoyama et al., 1999) and it potentiated morphine antinociception in thermal, peripheral neuropathy, and tonic inflammatory pain models (Holtman et al., 2008).

At low doses, ketamine has been proven efficacious as an analgesic perioperatively or in chronic pain states (Bell et al., 2005; Laskowski et al., 2011; Visser and Schug, 2006). Empirical evidence also indicates that ketamine coadministered with morphine can significantly increase the efficacy of morphine in cancer pain management. However, there has been little clinical research to support this (Bell, 2012). The majority of preclinical and human experimental studies (Arroyo-Novoa et al., 2011; Carstensen and Møller, 2010; Honarmand et al., 2012; Suppa et al., 2012) have focused on the pharmacodynamic properties of ketamine as an NMDA receptor antagonist leading to decreased opioid tolerance and hyperalgesia.

Some preclinical research regarding the pharmacokinetic interaction between opioids and ketamine has been conducted. Pretreatment with ketamine caused cross-tolerance to morphine, but pretreatment with morphine caused an increase in the cataleptic response to ketamine. This finding was suggested to be caused by residual morphine in the brain after morphine treatment (Hance et al., 1989). A similar effect was also observed with the use of sufentanil, fentanyl, alfentanil, nalbuphine, and butorphanol in combination with ketamine (Benthuysen et al., 1989). Alfentanil infusion increased the brain concentration of ketamine and decreased its plasma AUC, indicating a greater distribution volume. On the other hand, the brain concentration of alfentanil was decreased in ketamine coadministration (Edwards et al., 2002). Interestingly, acute LPS treatment lowered plasma ketamine AUC and increased its elimination half-life, indicating an increase in BBB permeability and increased access of ketamine to the brain (Veilleux-Lemieux et al., 2012). In repeated administration, morphine may increase the permeability of the BBB (Sharma and Ali, 2006). This provides another interesting theory for the possibility of altered ketamine pharmacokinetics during chronic opioid administration. Regarding drug transporters, ketamine has not been identified as a substrate of P-gp (Doan, 2002; Tournier et al., 2010) or Bcrp (Tournier et al., 2010), but an older study
(Ullrich et al., 1993) listed ketamine as a candidate substrate for both organic cation transporter (OCT) and organic anion transporter (OAT) families. In HEK293 cells transfected with human or rat OCT1, OCT2, and OCT3, ketamine was found to inhibit radiolabeled 1-methyl-4-phenylpyridinium uptake (Amphoux et al., 2006), a finding later confirmed by Massmann et al. (2013) regarding OCT3.
3. AIMS OF THE STUDY

Drugs with different mechanisms of action such as ibudilast, atipamezole, spironolactone, and ketamine have shown promise in preclinical studies of offering new pain treatment possibilities. This study focused on these drugs by evaluating them in coadministration with morphine, the gold standard of opioids. The rat was used as the study subject. Studies were performed with special emphasis on both pharmacodynamic and pharmacokinetic properties of the studied drugs.

The specific aims of this thesis study were:

I. To investigate the antinociceptive and other nociception-modulating properties of ibudilast alone and in coadministration with morphine;

II. To assess the antinociceptive effects of ultralow doses of the alpha-2-adrenergic antagonist atipamezole as an adjuvant to morphine therapy in intrathecal and systemic administration;

III. To determine the effects of the mineralocorticoid receptor antagonist spironolactone in acute coadministration with morphine and in morphine tolerance with special emphasis on potential pharmacokinetic interactions;

IV. To assess the potential pharmacokinetic interactions between morphine and the NMDA receptor antagonist ketamine in acute and chronic morphine administration, with a focus on possible changes in brain drug and metabolite concentrations.
4. MATERIALS AND METHODS

4.1 Ethical considerations

The research procedures were conducted in accordance with the guidelines of the local authorities and the IASP (Zimmermann, 1983). The provincial government of Southern Finland had approved the study concept (Uudenmaan lääninhallitus, Hämeenlinna, Finland). For ethical reasons, the smallest possible number of animals and the smallest number of nociceptive tests per animal were used. In all nociceptive behavioral models, the animals were able to terminate the noxious stimulation. Cut-off latencies were used to avoid tissue damage.

4.2 Materials

4.2.1 Animals

Male Sprague-Dawley rats (Harlan, Horst, Netherlands, and Scanbur, Sollentuna, Sweden) weighing 180–300 g at the beginning of experiments) were used. Rats were housed in plastic cages in light- and temperature-controlled rooms (artificial 12 h/12 h light–dark cycle, temperature 23 ± 2 °C). Water and standard laboratory chow were available ad libitum. For the behavioral studies, the rats were habituated to the testing environment 60–90 min/day for at least three days. Behavioral measurements were performed in a randomized and blinded fashion in all studies. After the experiments, the rats were euthanized by decapitation and brain, liver and serum samples were collected.

4.2.2 Drugs

Morphine hydrochloride, racemic ketamine hydrochloride (Ketaminol vet®, Intervet, Boxmeer, The Netherlands) and medetomidine hydrochloride (Domitor, Orion Pharma, Espoo, Finland) were purchased from the University Pharmacy (Helsinki, Finland). Ibudilast was purchased from APAC Pharmaceutical, LLC (Columbia, MD, USA), and atipamezole hydrochloride was provided by Orion Pharma (Espoo, Finland). Spironolactone and loperamide hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Morphine and atipamezole were dissolved in physiological saline. Ibudilast was dissolved in 2% polysorbate 20 (Tween® 20; Fluka Chemika, Buchs, Switzerland) in physiologic saline. Spironolactone was diluted with 4% polysorbate 80 (Tween® 80, University Pharmacy, Helsinki, Finland) and loperamide with 2% polysorbate 80 in physiological saline. Ketamine and medetomidine were diluted to the administration concentrations with physiological saline.

The injection volumes were 2 ml/kg subcutaneously and 10 ml/kg (Study I) or 5 ml/kg (Study III) intraperitoneally. In the intrathecal experiments, drugs were administered in separate phases using 50-µl Hamilton microsyringes in injection volumes of 10 µl. Between the drug phases, 1 µl of air was used to prevent ex vivo mixing of the solutions. Finally, 10 µl of saline was administered to flush the catheter.

4.3 Methods

4.3.1 Intrathecal cannulation

The catheter implantation (Study II) was performed according to the modified method of Yaksh and Rudy (Yaksh and Rudy, 1976). Each rat was anesthetized with subcutaneous (s.c.) 0.4 mg/kg medetomidine (Domitor®, Orion Pharma, Espoo, Finland) and 60 mg/kg ketamine hydrochloride (Ketaminol vet®, Intervet, Boxmeer, The Netherlands). The rats were placed prone in a stereotactic frame, and the membrane of the cisterna magna was surgically exposed and punctured with care. A polyethylene cannula (8 cm, PE-10) was inserted through the opening and advanced caudally to the level of the lumbar enlargement. The cannula was fixed to the paravertebral muscles, and the skin was closed with 3-0 sutures. The cannula was then flushed with 0.9% saline. Rats showing any neurological symptoms such as movement of extremities or muscle twitches indicating potential CNS trauma leading to neurological deficits were immediately euthanized. Five days after cannulation, the placement of the cannula was verified by administering 10 µl of 10 mg/ml lidocaine intrathecally (Lidocain®, Orion Pharma, Espoo, Finland). Only rats with reversible symmetrical paralysis of both hindlimbs after injection were accepted for testing. To enable recovery from the cannulation operation and anesthesia, rats were allowed to rest for at least 8 days before any nociceptive tests.
4.3.2 Morphine tolerance treatment schemes

In all studies, morphine tolerance was induced with a 4-day scheme. In studies I, II and III, the rats received 2 daily s.c. injections at 10:00 am and 8:00 pm. The individual doses were 10 mg/kg on day 1, 15 mg/kg on day 2, 20 mg/kg on day 3, and 30 mg/kg on day 4. In the intrathecal experiments in study II, rats received intrathecal injections of morphine twice daily for four days. The individual dose was 15 µg each day. In experiment IV, Alzet minipumps pumping 10 µl/h for 4 days (model 2ML1, Durect, Cupertino, CA, USA) were used to provide a continuous morphine exposure. Under brief isoflurane anesthesia (induction in a chamber with 4–5% isoflurane at a flow rate of 1.5 l/min of air and maintenance with 2% at 1.5 l/min), a small incision was made between the scapulae of the rat and a subcutaneous pocket was created. The minipumps had been previously filled with vehicle or morphine hydrochloride to release 6 mg of morphine base per day. The pumps were installed subcutaneously and the wound was closed with 3-0 sutures.

4.3.3 Pain measurement in acute nociception

Hot plate tests (Woolfe and Macdonald, 1944) (Studies I–IV) were performed with a Harvard Apparatus Ltd hotplate apparatus (Edenbridge, Kent, United Kingdom). In the test, the rats were put onto a circular transparent plastic ring on the hot plate (52 ± 0.2 °C). Licking or brisk shaking of the hindpaw or jumping was considered a sign of thermal nociception. Latency to the first reaction was measured. To avoid tissue damage, the cutoff time was set to 60 seconds.

Tail-flick latencies (D’Amour and Smith, 1941) (Studies I–IV) were assessed with a Ugo Basile 37360 (Comerio, Italy) tail-flick apparatus. In the test, the rats were kept in hard plastic tubes covered with a dark cloth. After accustomization, an infrared beam (radiant heat) was directed in turn to three different points on the middle third of the tail of the test subject. The intensity was adjusted to produce a baseline latency of approximately 3.5 s. A flick of the tail was considered as a sign of thermal nociception and it automatically stopped the timer of the apparatus. The measurements were repeated three times at each time point, and the mean of the values was used as the result. To avoid tissue damage, the cutoff was set at 10 s. If an individual measurement reached the cutoff time, no further tests were performed for the particular time point.
The paw pressure test (Study II) (Randall and Selitto, 1957) was performed with the Ugo Basile paw pressure device (Comerio, Italy). Rats were gently wrapped in a towel during the test where the left hindpaw was placed under a pivot and the force applied to the paw was gradually increased. A brisk withdrawal of the hind paw terminated the measurement. A cutoff of 500 g was used.

At each time point, the tail-flick test was performed first followed by the hot plate and/or paw pressure tests. A short waiting period was used between each test. The predrug (baseline) latencies were measured separately for each experiment day immediately before the administration of any drugs.

### 4.3.4 Assessment of motor function

A rotarod apparatus (Palmer electric recording drum; United Kingdom; diameter, 80 mm; speed, 27 rpm (Study I) and 12 rpm (Study IV)) was used to assess the actions of the test drugs on motor coordination. A rat was placed on the rotating rod, and the time the rat stayed there was measured. Training on the rod was allowed on three consecutive days. On the fourth day, animals that remained for at least 60 s on the rotating rod before drug administration were accepted to the test, and 60 s was also used as a cutoff time in the test proper.

Possible effects of the drugs on spontaneous locomotor activity (Study I) were tested in a measurement box (70 × 70 × 35 cm; Kungsbacka Regler- & Mätteknik, Kungsbacka, Sweden) isolated from sound and light. Photocells were located at two different levels (2 and 12 cm) above the floor of the box to automatically detect movements of the animal. A 30-min measurement period was started 15 min after the drug injections.

### 4.3.5 Drug concentration measurements

The drug concentrations of morphine, M3G, M6G, normorphine (Study III), and ketamine and norketamine (Study IV) were determined with LC-MS/MS (liquid chromatography-tandem mass spectrometry) analyses as previously described (Zheng et al., 1998) with some modifications. An Agilent 1100 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) coupled to an API 3000 tandem mass spectrometer (AB Sciex, Toronto, ON, Canada) operating in a positive turbo ion spray mode was used. The chromatographic separation was achieved on an Atlantis HILIC Silica column (3 µm particle size, 2.1 × 100 mm I.D.) (Waters, Milford, MA, USA) using a gradient elution of the mobile phase consisting of
acetonitrile with 10 mmol/l ammonium formate in 0.2% formic acid (v/v) (Study III) or 20 mmol/l ammonium acetate (pH 3.0, adjusted with formic acid) (Study IV). An aliquot (7 µl, Study III; 5 µl, Study IV) was injected at a flow rate of 250 µl/min (Study III) or 200 µl/min (Study IV) to give a total chromatographic run time of 18 min (Study III) or 24 min (Study IV). Oxycodone served as an internal standard for morphine and its metabolites. Deuterium-labeled internal standards were used for ketamine and norketamine (Study IV). The following ion transitions were monitored: morphine, m/z 286 to m/z 152; M3G and M6G, m/z 462 to m/z 286; normorphine, m/z 272 to m/z 152; and oxycodone, m/z 316 to m/z 241. The limit of quantification was 1.0 ng/ml for morphine, M3G, and M6G, and 0.5 ng/ml for ketamine and norketamine. A signal-to-noise ratio (S/N) of 20:1 was used as the limit of detection for normorphine, and the quantities were given in arbitrary units relative to the ratio of the peak area of normorphine to that of the internal standard. The day-to-day coefficients of variation (CV) were below 15% at relevant concentrations for all analytes.

4.3.6 Experimental design of Studies I–IV

The experimental designs of the studies are described in brief in Table 5 and in detail in the original publications (Studies I–IV, Methods).
Table 5. Designs of studies I-IV in brief.

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<tr>
<th>Question addressed</th>
<th>Experimental design</th>
<th>Methods used</th>
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<tr>
<td><strong>Study I</strong></td>
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<td></td>
</tr>
<tr>
<td>• Antinociceptive effects of ibudilast alone or in combination with morphine</td>
<td>Ibudilast with or without morphine administered to drug-naive animals</td>
<td>Tail-flick Hot plate</td>
</tr>
<tr>
<td>• Effects of ibudilast coadministration on the development of morphine tolerance</td>
<td>Ibudilast co-treatment with the morphine tolerance scheme for four days; nociceptive tests on day 5; observations of weight change during withdrawal</td>
<td>Tail-flick Hot plate</td>
</tr>
<tr>
<td>• Effects of ibudilast on morphine withdrawal</td>
<td>Morphone tolerance treatment protocol, drug coadministration and nociceptive tests on day 5, continuation of drug coadministration and repeated nociceptive tests on day 9</td>
<td>Tail-flick Hot plate</td>
</tr>
<tr>
<td>• Effects of acute coadministration of ibudilast and morphine in morphine-tolerant animals</td>
<td>Ibudilast alone and in coadministration with morphine</td>
<td>Rotarod Locomotor boxes</td>
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<tr>
<td><strong>Study II</strong></td>
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<tr>
<td>• Effects of subcutaneously administrated low-dose atipamezole on subcutaneous morphine antinociception</td>
<td>Morphine combined with three atipamezole doses</td>
<td>Tail-flick Hot plate</td>
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<tr>
<td>• Effects of subcutaneously administrated low-dose atipamezole on subcutaneous morphine antinociception in opioid tolerance</td>
<td>Morphine combined with three atipamezole doses in opioid-tolerant animals</td>
<td>Tail-flick Hot plate</td>
</tr>
<tr>
<td>• Effects of intrathecally administered low-dose atipamezole on intrathecal morphine antinociception</td>
<td>Morphine combined with three atipamezole doses</td>
<td>Tail-flick Paw pressure</td>
</tr>
<tr>
<td>• Effects of intrathecally administered low-dose atipamezole on intrathecal morphine antinociception in opioid tolerance</td>
<td>Morphine combined with three atipamezole doses in opioid-tolerant animals</td>
<td>Tail-flick Paw pressure</td>
</tr>
<tr>
<td>Question addressed</td>
<td>Experimental design</td>
<td>Methods used</td>
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<tr>
<td><strong>Study III</strong></td>
<td></td>
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<tr>
<td>• Effects of systemical spironolactone on morphine-induced antinociception</td>
<td>Two doses of morphine combined with spironolactone</td>
<td>Tail-flick, Hot plate</td>
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<tr>
<td>• Effects of acute morphine with or without spironolactone in morphine-tolerant rats</td>
<td>Four-day morphine tolerance scheme with or without spironolactone cotreatment followed by drug coadministration on day 5</td>
<td>Tail-flick, Hot plate</td>
</tr>
<tr>
<td>• Effects of repeated spironolactone administration on morphine-induced tolerance</td>
<td>Tail-flick, Hot plate, High performance liquid chromatography</td>
<td></td>
</tr>
<tr>
<td>• The concentrations of morphine and its metabolites in the brain, liver, and serum after morphine and spironolactone coadministrations</td>
<td>Tail-flick, Hot plate</td>
<td></td>
</tr>
<tr>
<td>• Effects of spironolactone on the antinociceptive properties of loperamide, a peripherally restricted P-glycoprotein substrate opioid</td>
<td>Tail-flick, Hot plate, High performance liquid chromatography</td>
<td></td>
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<tr>
<td><strong>Study IV</strong></td>
<td></td>
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<tr>
<td>• Effects of acute small dose of ketamine on morphine antinociception and tissue ketamine, norketamine and morphine concentrations in opioid-tolerant animals</td>
<td>Preceding morphine pump treatment for four days; administration of ketamine on day 5</td>
<td>Tail-flick, Hot plate, Rotarod, High performance liquid chromatography</td>
</tr>
<tr>
<td>• Effects of acute small dose of ketamine on morphine antinociception and tissue ketamine, norketamine and morphine concentrations in opioid-tolerant animals under withdrawal</td>
<td>Removal of morphine pumps after day 5, withdrawal for two days, acute doses of ketamine and/or morphine on day 7</td>
<td>Tail-flick, Hot plate, Rotarod, High performance liquid chromatography</td>
</tr>
<tr>
<td>• Effects of ketamine and/or morphine administration on observed antinociception and drug tissue concentrations</td>
<td>Acute tests using small doses of morphine and/or ketamine</td>
<td>Tail-flick, Hot plate, Rotarod, High performance liquid chromatography</td>
</tr>
</tbody>
</table>
4.4 Statistical analysis

The hot plate and tail-flick results are expressed as a percentage of the maximum possible effect (MPE%), calculated as MPE% = [(postdrug latency – baseline latency)/(cutoff time – baseline latency)] × 100%, which takes into account the differences in baseline nociceptive latencies. In the text and figures, results are presented as means of the groups (± SEM). The behavioral data were tested for statistically significant differences in mean values by two-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple comparisons (group × dose or group × time) (Studies I and II) or followed by a Holm-Sidak correction for multiple comparisons (group × dose or group × time) (Studies III and IV). For the concentration data in Studies III and IV, a two-tailed t-test or one-way analysis of variance followed by the Holm-Sidak correction was used. For the nonparametric rotarod test data (Study IV), the Kruskal-Wallis test followed by Dunn’s multiple comparison was used. The difference was considered significant at P < 0.05 in both the analysis of variance and the post hoc test. The data were analyzed using GraphPad Prism, versions 4.0c (Study I), 5.0b (Study II), 6.0a (Study III), and 6.0c (Study IV) for Macintosh (GraphPad Software, Inc., San Diego, CA, USA).
5. RESULTS

5.1 Morphine

5.1.1 Effects of morphine in different models of nociception

Morphine was used as the study opioid in all studies. The peak antinociception was measured at 30 minutes after s.c. administration, when it had an ED$_{50}$ of 3.1 mg/kg in the tail-flick test and 3.7 mg/kg in the hot plate test (Study III) (Fig. 13). After i.t. administration, 1.5 µg morphine was needed to produce over 50% MPE. The peak antinociception was measured at 30 min in both the tail-flick and paw pressure tests (Study II, Fig. 1).

The tissue concentrations of morphine and their corresponding antinociceptive responses were determined (Study III). After 4 mg/kg of s.c. morphine, significant antinociception was observed in the tail-flick test at 30 and 90 minutes after administration and in the hot plate test at 30 min after administration (Fig. 14A and B). The brain morphine concentrations were 406 ± 22 nmol/kg and 258 ± 9 nmol/kg at 30 and 90 minutes, respectively (n.s.). In serum, the morphine concentration at 90 min was significantly lower than at 30 min (2000 ± 210 nmol/l vs. 410 ± 22 nmol/l, P < 0.001) (Fig. 14C).

![Figure 13. ED$_{50}$ graphs for morphine in the tail-flick and hot plate tests 30 minutes after subcutaneous administration. n = 8.](image-url)
5.1.2 Development of morphine tolerance in different models

A 4-day scheme for inducing morphine tolerance was used both in s.c. and i.t. models. On day 5, morphine-tolerant and morphine-naïve rats received 5 mg/kg (Studies I and II) and 4 mg/kg (Study III) s.c. morphine as the test dose, which resulted in significant antinociception in morphine-naïve rats, whereas only minor (n.s.) antinociception was observed in morphine-pretreated rats (Table 6). In the i.t. model, the test dose was 1.5 µg (Study II).

In Study IV, osmotic minipumps delivering a steady dose of morphine were used. After 24 hours of minipump implantation, significant antinociception was observed. Tolerance to morphine antinociception developed on the fifth day of pump treatment (Study IV, Fig. 2).

In Studies I–IV, no opioid-induced hyperalgesia (reduction of nociceptive thresholds below baseline after morphine administration) was observed (Lilius et al., unpublished finding).

In Study III, brain morphine concentrations were measured 90 min after an acute 8 mg s.c. dose in morphine tolerant and morphine-naïve rats. No difference in the mean brain morphine concentration was observed (202 ± 8 ng/g in tolerant rats vs. 200 ± 10 ng/g in naïve rats, n = 4–8) (Lilius et al., unpublished data).
Table 6. Antinociceptive effects of a test dose of morphine (Mo) administered after the induction of morphine tolerance. B.i.d., twice daily; MPE%, percentage of the maximum possible effect; TF, tail-flick; HP, hot plate; n/a, data not available.
* Statistically significant difference compared with the vehicle-administered group. n = 7–8.

<table>
<thead>
<tr>
<th>Study</th>
<th>Dosing</th>
<th>Test dose</th>
<th>Mo-naïve</th>
<th>Tolerant</th>
<th>Mo-naïve</th>
<th>Tolerant</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>s.c. b.i.d.</td>
<td>5 mg/kg s.c.</td>
<td>81*</td>
<td>26</td>
<td>71*</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>s.c. b.i.d.</td>
<td>5 mg/kg s.c.</td>
<td>98*</td>
<td>34</td>
<td>83*</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
<td>s.c. b.i.d.</td>
<td>4 mg/kg s.c.</td>
<td>56*</td>
<td>18</td>
<td>34*</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>i.t. b.i.d.</td>
<td>1.5 μg i.t.</td>
<td>53*</td>
<td>22</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

5.2 Ibudilast (Study I)

5.2.1 Antinociceptive effects of ibudilast

Antinociception was assessed with the tail-flick (Fig. 15A) and hot plate (Fig. 15B) tests after the administration of four different doses of ibudilast with or without morphine to drug-naïve rats. An antinociceptive effect of ibudilast administered alone at doses of 7.5 and 22.5 mg/kg i.p. was observed. Morphine 2.5 mg/kg s.c. caused significant antinociception in both tests, and ibudilast and morphine had an additive effect in the tests.

In a separate experiment, at 30 minutes after drug administrations, ibudilast (22.5 mg/kg i.p.) did not modify the mean brain concentrations of morphine (4 mg/kg s.c.) (Lilius et al., unpublished data).
Figure 15. The antinociceptive dose–response curve for ibudilast in the tail-flick (A) and hot plate (B) tests with or without 2.5 mg/kg s.c. morphine at 30 minutes after administration. The mean of the maximum possible effect (MPE%) ± SEM is plotted. For the ibudilast groups: *, ** Statistically significant difference ($P < 0.05$, $P < 0.01$, respectively) compared with the group that was given the vehicle alone. For the morphine groups: # Statistically significant difference ($P < 0.05$) compared with the group that was given morphine without ibudilast. § Statistically significant difference ($P < 0.05$) compared with the group that was given the same dose of ibudilast without morphine. $n = 7$.

5.2.2 Effects of ibudilast cotreatment on the restoration of morphine antinociception

Ibudilast was studied in several models of opioid tolerance. The exact treatment schemes are presented in Study I, Table 1. When ibudilast was acutely coadministered with morphine to opioid-tolerant rats on day 5, it restored the antinociceptive effect of morphine in the tail-flick test (Fig. 16). However, in the hot plate test, ibudilast had antinociceptive effects of the same magnitude in opioid tolerant rats as in naïve animals (Study I, Fig. 5).

From this point, the cotreatment of morphine and ibudilast twice daily was continued for four more days and the rats were retested on day 9 after drug coadministration. Morphine alone produced no significant antinociception, but ibudilast 7.5 mg/kg i.p. combined with morphine enhanced morphine antinociception without having antinociceptive effects of its own (Study I, Fig. 6).
Figure 16. Effects of acute ibudilast treatment on morphine antinociception in morphine-tolerant rats in the tail-flick test. Morphine tolerant rats were given two different doses of ibudilast and/or a test dose of morphine. The mean of the maximum possible effect (MPE%) ± SEM is shown. *, P < 0.05 compared with the vehicle. #, P < 0.05 compared with the morphine-tolerant, morphine-administered group (black bar). §, P < 0.05 between selected groups.

In a separate experiment, three different doses of ibudilast (0.83, 2.5 and 7.5 mg/kg) administered to rats twice daily under the four-day morphine tolerance scheme to assess the potential of ibudilast in inhibiting the development of morphine antinociceptive tolerance. However, in the hot plate test, pretreatment with 7.5 mg/kg ibudilast increased the development of tolerance to an acute test dose of 5 mg/kg morphine (Study I, Fig. 2B).

5.2.3 Effects of ibudilast on motor coordination

As ibudilast showed sedative effects in routine handling of the rats, the effects of acutely administered ibudilast were investigated in a measurement box measuring spontaneous locomotor activity in a novel environment. The rats were placed inside the box 15 min after drug administration for a total of 30 minutes. At doses of 2.5 and 7.5 mg/kg i.p., compared with vehicle, ibudilast significantly reduced spontaneous locomotor activity during the 30-min observation period (Fig. 17A). In a separate experiment, rats first received a 4-day pretreatment of 20 mg/kg i.p. ibudilast twice daily and locomotor experiments were conducted.
on day 5, demonstrating that no tolerance to the observed effect had developed (Lilius et al., unpublished data, Fig. 17B).

![Graph A](image1.png)

**Figure 17.** Effects of acute ibudilast (A) and ibudilast pretreatment (B) on spontaneous locomotor activity. The activity counts (number of photocell crossings in 5-min periods) over time ± SEM are shown 15 min after drug administration. *, **, *** Statistically significant difference (P < 0.05, P < 0.01, P < 0.001, respectively) compared with the vehicle group. n = 6.

The effects of ibudilast on motor coordination were also examined using the rotarod test. On the first treatment day, all rats (100%) that had received vehicle achieved the cutoff time (60 s) at 30 and 120 min. Ibudilast i.p. in doses of 0.83 and 2.5 mg/kg caused no significant differences (52.3 ± 7.7 and 53.0 ± 7.0 s, respectively) compared with vehicle. After administration of 7.5 mg/kg ibudilast, the mean survival time was reduced to 11.2 ± 4.0 s at 30 min and 31.5 ± 12.6 s at 120 min (P < 0.05 compared with vehicle). These measurements were repeated on day 4 after three preceding days of ibudilast treatment (0.83, 2.5, and 7.5 mg/kg i.p. twice daily), and the same significant results were achieved as on day 1, indicating that tolerance to the observed effect did not develop during four days of treatment (Study I, Results).
5.3 Atipamezole (study II)

5.3.1 Effects of atipamezole coadministration in acute morphine antinociception

The choice of the initial intrathecal morphine dose was based on pilot experiments, in which 1.5 µg of morphine produced approximately 50 MPE% in the tail-flick test. The studied ultralow i.t. doses of atipamezole did not produce antinociception in any of the nociceptive tests on their own.

Morphine was administered in the same syringe in a separate phase with different doses of atipamezole (0.01, 0.1 and 1 ng) or vehicle (Fig. 18). Morphine alone produced significant antinociception at 30 (P < 0.05) and 60 min (P < 0.01) after drug administration. Morphine combined with 1 ng atipamezole produced significantly greater antinociception than the combination of morphine and vehicle 30 minutes after drug administration in the tail-flick test (P < 0.05). However, a longer-lasting effect of the drug combination was not observed. In the paw pressure test, morphine alone produced significant antinociception from 15 to 120 minutes after administration, but atipamezole did not augment this effect (Study II, Results). In further studies, intrathecal morphine doses of 0.5 and 5 µg produced significant antinociception at 30 and 60 min, but the 1-ng dose of intrathecal atipamezole did not have an additive effect on the observed antinociception (Study II, Results).
Results

Figure 18. Effects of intrathecal atipamezole on intrathecal morphine antinociception in the tail-flick test. Rats were administered three different doses of atipamezole (At) or vehicle (Veh) with morphine (Mo) 1.5 μg. The mean of the maximum possible effect (MPE%) ± SEM is plotted as a function of time. * Statistically significant difference (P < 0.05) compared with the group that was administered morphine and vehicle. n = 6–7, except n = 4 in the vehicle group.

5.3.2 Effects of spinal atipamezole coadministration in morphine tolerance

Spinal morphine tolerance was induced during four days by administering 15 μg of intrathecal morphine twice daily. On day 5, in the tail-flick test, intrathecal 1.5 μg morphine produced significant antinociception at 30 and 60 minutes (P < 0.001 and P < 0.01, respectively) (Fig. 19), whereas the same dose in morphine-pretreated animals did not cause significant antinociception compared with vehicle. At the intrathecal dose of 10 ng but not 1 ng, atipamezole coadministered with morphine partly restored morphine antinociception at 30 minutes (P < 0.05).
Results

Figure 19. Effects of intrathecal atipamezole on intrathecal morphine antinociception in the tail-flick test in morphine-tolerant rats. Morphine-tolerant rats were administered two different doses of atipamezole (At) or saline (Sal) with morphine (Mo) 1.5 μg. As a control, morphine was also administered to previously morphine-naïve animals. The mean of the maximum possible effect (MPE%) ± SEM is plotted as a function of time. *, **, *** Statistically significant difference (P < 0.05, P <0.01, P < 0.001 respectively) compared with the morphine-tolerant group that received acute morphine only. n = 6–9.

5.3.3 Effects of systemic administration of atipamezole with morphine

As systemic administration is the preferred route for the ease of drug use, the effects of systemic atipamezole on systemic acute morphine antinociception and morphine tolerance were also evaluated. Because in the pilot experiments atipamezole doses of 30 and 300 μg/kg s.c. induced motor restlessness, the atipamezole doses chosen were 0.03, 0.3, and 3 μg/kg s.c. Morphine 2.5 mg/kg s.c. produced significant antinociception in the tail-flick test 30 min after administration (P < 0.05) and in the hot plate test at 30, 75, and 120 minutes (P < 0.05 at all time points and P < 0.001 at 75 minutes). In acute tests, however, none of the coadministered atipamezole doses significantly increased the effect of morphine in either of the tests (Study II, Results).
5.4 Spironolactone (Study III)

5.4.1 Effects of spironolactone in acute morphine antinociception and morphine tolerance

Spironolactone 50 mg/kg i.p. had no antinociceptive effects of its own, but it increased the antinociceptive potency of 2.5 and 5.0 mg/kg of s.c. morphine in both the tail-flick and hot plate tests (Study III, Fig. 1). In morphine-tolerant rats, the magnitude of increased antinociception observed was at the same level as in naïve animals (Study III, Fig. 2).

Chronic spironolactone pretreatment alone (50 mg/kg i.p. twice daily for four days) did not affect antinociception by acute morphine alone. To study the effects of spironolactone treatment on the development of morphine tolerance, one group of the morphine tolerance-treated rats was cotreated twice daily with 50 mg/kg i.p. spironolactone, and acute 4 mg/kg s.c. morphine alone was given on day 5. Antinociception observed in cotreated animals was minimal and did not differ from rats that had received the morphine tolerance treatment without spironolactone (Study III, Results).

5.4.2 Effects of spironolactone on morphine and morphine-3-glucuronide concentrations in the brain

In acute coadministration, spironolactone administered 60 min and 0 min before morphine 4 mg/kg s.c. significantly increased morphine antinociception at 30 and 90 min (Fig. 20A). In the brain, coadministration increased the mean morphine concentration by 69% and 290% at 30 and 90 min, respectively (Fig. 20B). At 30 minutes, serum morphine concentrations were essentially at the same level, but the liver concentrations were increased in the group that was coadministered two doses of spironolactone (Fig. 20C and D). Continuing to 90 minutes, morphine concentrations in all tissues were elevated in the presence of spironolactone. Spironolactone did not, however, reduce the formation of M3G in the liver (Study III, Fig. 3G).
Figure 20. Effects of 50 mg/kg i.p. spironolactone treatment 60 min before morphine (SPR 50) or 60 min before and coadministered with morphine (SPR 50 × 2) in the hot plate test 30 and 90 min after morphine administration (A). The mean of the maximum possible effect (MPE%) ± SEM is shown. The corresponding brain (B), serum (C), and liver morphine concentrations (D) are presented (mean ± SEM). *, ***, Statistically significant difference (P < 0.05, P < 0.001, respectively) compared with the vehicle-administered group. #, ##, ### Statistically significant difference (P < 0.05, P < 0.01, P <0.001, respectively) between the selected groups. MO, morphine. n = 10 at 30 min and n = 5 at 90 min in behavioral experiments; n = 5 per timepoint in concentration studies.

5.4.3 Antinociceptive effects of the P-glycoprotein substrate loperamide combined with spironolactone

To assess the effects of spironolactone on BBB transporter proteins, spironolactone (50 mg/kg i.p.) was administered 60 minutes before and concomitantly with a peripheral opioid, P-gp substrate loperamide (10 mg/kg s.c.). In coadministration, loperamide showed significant antinociceptive
properties in the hot plate test 30 and 90 minutes after administration, indicating potential changes in the drug transporter function of the BBB induced by spironolactone (Study III, Fig. 4).

5.5 Ketamine (Study IV)

5.5.1 Ketamine coadministration to morphine-tolerant rats leads to increased brain concentrations of morphine, ketamine, and norketamine

The effects of ketamine in morphine tolerance have been suggested to be mediated via a pharmacodynamic interaction. In this study, the possibility of a pharmacokinetic interaction between the drugs was investigated. Subcutaneous minipumps delivering morphine 6 mg/day induced tolerance (Study IV, Fig. 2), and on day 5 rats received a small dose of ketamine (10 mg/kg s.c.). In morphine-naïve rats, ketamine caused no antinociception, whereas in morphine-tolerant rats it caused significant antinociception, peaking at 90 minutes (54 MPE% increase, Fig. 21A and B). In the rotarod test, ketamine induced motor dysfunction at 30 min and the effect was enhanced by the chronic morphine treatment. At peak antinociception, however, the rotarod performance of all groups had returned to normal (Fig. 21C).

In morphine-tolerant ketamine-treated rats, the mean morphine, ketamine and norketamine brain concentrations were 2.1-, 1.4-, and 3.4-fold higher, respectively, compared with the rats that received the morphine tolerance treatment or ketamine only (Fig. 19). In the liver of morphine-tolerant ketamine-treated rats, the mean ketamine concentration was six-fold higher and the norketamine concentration two-fold higher than in morphine-naïve rats. Hence, morphine tolerance affects the pharmacokinetic properties of ketamine, while on the other hand, acute ketamine affects the pharmacokinetic properties of morphine.

In a separate experiment, the effect of morphine withdrawal on morphine and ketamine drug concentrations was investigated. The morphine pump treatment lasted for 5 days, after which the pumps were removed. On day 7, the rats were administered the same low ketamine dose (10 mg/kg s.c.). As noted on day 5, during withdrawal ketamine and norketamine concentrations were also increased, although significantly only in the liver (Study IV, Fig. 4).
5.5.2 Effects of combined ketamine and morphine in acute models of nociception

As ketamine administered to morphine-tolerant rats caused notable increases in the brain concentrations of both ketamine and morphine, it was of interest whether this interaction would also be observable in an acute experiment. Morphine (2.5 mg/kg s.c.) was administered 15 minutes before ketamine (10 mg/kg s.c.). In behavioral experiments, the coadministration of morphine and ketamine caused significant motor coordination impairment compared with morphine only at 30 min, which was also reflected in the results of the hot plate test (Study IV, Fig. 5A–C). Morphine coadministration had no effect on ketamine or norketamine brain concentrations 90 min after ketamine administration, but ketamine slightly increased the mean morphine concentration in the brain and liver (Study IV, Fig. 5D–I).

Figure 21 (next page). Effects of an acute small dose of ketamine on antinociception in morphine-tolerant rats. Rats undergoing s.c. morphine (6 mg/day, Mo) or vehicle (Veh) pump treatment were administered an acute s.c. dose of ketamine (10 mg/kg, Ket) or vehicle (Veh) on day 5. Antinociception was measured using tail-flick (A) and hot plate (B) tests. The mean of the maximum possible effect (MPE%) ± SEM is plotted. In the rotarod test (C), the mean (± SEM) survival time (seconds) is plotted. From separate animals with the same pretreatments, whole brain, serum, and liver samples were collected 90 min after ketamine administration. The concentrations of the experimental drugs and their main metabolites (MO, morphine; M3G, morphine-3-glucuronide; KET, ketamine; NORKET, norketamine) were quantified. The mean tissue concentrations (± SEM) are plotted in graphs D–I. The increase or decrease in tissue concentrations between the treatment groups is shown in percentages for each substance. n = 7–8. **, *** Statistically significant differences (P < 0.01, P < 0.001, respectively) compared with the vehicle control group. #, ##, ### Statistically significant differences (P < 0.05, P < 0.01, P < 0.001, respectively) compared with the morphine-pump-treated group that received acute vehicle. §§, §§§ Statistically significant differences (P < 0.01, P < 0.001, respectively) compared with the vehicle-pretreated group that received acute ketamine.
**Results**

**Day 5 (tolerance): Behavioral data**

A. Tail-flick latency  
B. Hot plate latency  
C. Time on rotarod

**Concentration data 90 min after ketamine administration**

**Brain**

D. Concentration (ng/g)  
E. Concentration (ng/g)  

**Serum**

F. Concentration (ng/mL)  
G. Concentration (ng/mL)

**Liver**

H. Concentration (ng/g)  
I. Concentration (ng/g)  

Veh pumps  
Mo pumps  
Ket 10 mg/kg s.c.  
Veh
6. DISCUSSION

6.1 Methodological considerations

The use of animals for the study of pain is necessary and justified because of the complex pain and nociception processing systems. Studies on the complex processing of nociception and pain require a neuronal network with the supraspinal parts of the CNS. The struggle to alleviate pain and suffering and to improve existing pain remedies is one of the most important and humane areas of scientific research. Current animal models used for the study of pain have proven invaluable when they are used carefully and with attention to supporting data from in vitro, in silico, and other methods (Mogil et al., 2010). In the future, when evaluating changes (i.e. synaptic plasticity and potential glial activation) in the CNS during opioid treatment, the development of noninvasive imaging techniques such as magnetic resonance imaging may especially help in reducing the number of animals needed and also enable more versatile studies in humans. All animal treatment procedures of this study were carefully conducted following the guidelines of local authorities and the IASP (Zimmermann, 1983). The suffering of the animals was minimized and the number of animals used was reduced by using a washout period when applicable.

Of the thermal nociceptive tests used, the hot plate test reflects supraspinal antinociception, whereas the tail-flick reaction is a spinal reflex. Both of these are widely used and have been found predictive in opioid research (Le Bars et al., 2001). For the intrathecal experiments (Study II), the method for measuring spinal nociception was extended to also cover mechanical nociception measured by the Randall-Sellitto paw pressure test.

6.2 Ibudilast in coadministration with morphine

Study I demonstrated that ibudilast restored the antinociceptive effect of morphine in developed morphine tolerance after single and repeated administration. However, when ibudilast treatment was given together with the morphine tolerance treatment, the development of tolerance could not be attenuated.

Previous studies (Hutchinson et al., 2009; Ledeboer et al., 2007) have demonstrated the ability of ibudilast to augment morphine and oxycodone
antinociception. However, in these studies, the administration of ibudilast alone at a dose of 7.5 mg/kg i.p. was reported not to produce any change in hind paw or tail-flick latencies over a 100-minute time course after administration (Hutchinson et al., 2009). In Study I, already the 7.5-mg/kg dose of ibudilast caused a marginal (significant in one experiment) increase in the tail-flick, but a marked increase in hot plate latencies 30 and 120 min after acute administration. As the hot plate test involves supraspinal nociceptive processing (Le Bars et al., 2001), it may be more sensitive to unspecific behavioral effects such as sedation. Indeed, ibudilast decreased locomotor activity at doses of 2.5 and 7.5 mg/kg and impaired motor coordination at 7.5 mg/kg. Supporting this, 7.5 mg/kg ibudilast has previously been reported to cause transient sedation and decreased reactivity to touch (Ledeboer et al., 2007). Therefore, the observed ibudilast-induced antinociception is most likely caused by sedation and impaired motor coordination, especially in the hot plate test. However, the mechanisms by which ibudilast reduces locomotor activity and impairs motor coordination are not known. It is unlikely that these effects are mediated through actions on glial cells, because they are thought not to be reactive in healthy animals. The routine screening of ibudilast binding to various target proteins did not reveal any target proteins that could mediate the decreased locomotor activity (Ledeboer et al., 2007). In humans, however, sedation has not been reported to be a major problem after acute (30 mg p.o.) or subchronic (30 mg p.o. b.i.d.) administration regimen (Rolan et al., 2008).

The development of morphine dependence and tolerance has been classically linked to the cAMP pathway (see page 25). Activation of mu-opioid receptors leads to the inhibition of adenylate cyclase (Sharma et al., 1975; Wang et al., 1994). However, repeated administration of morphine may lead to upregulation of adenylate cyclase, increased intracellular cAMP concentrations, and activation of PKA. This may contribute to the development of tolerance via cyclic adenosine monophosphate response element-binding protein (CREB), a transcription factor (Montminy and Bilezikjian, 1987) that regulates genes responsible for the development of physical dependence (Lane-Ladd et al., 1997). Ibudilast, as an inhibitor of PDE, should counteract acute opioid effects by increasing intracellular cAMP levels. Therefore, it seems likely that the demonstrated effect of ibudilast is not linked to PDE inhibition in opioid receptor-containing neurons. In glial cells, however, the effect of PDE inhibition on the development of opioid tolerance may be the opposite. In an Alzheimer’s disease model, PDE4B, a cAMP-specific phosphodiesterase, was
upregulated by the administration of amyloid beta peptide leading to increased production of proinflammatory TNF (Sebastiani et al., 2006). After spinal cord injury, the PDE4 B2 isoform was upregulated from 24 h to 1 week after injury, at peak microglial activation (Ghosh et al., 2012). Thus, in microglial cells, the inhibition of PDE may lead to beneficial anti-inflammatory effects. Supporting this, various PDE inhibitors also show anti-inflammatory properties when treating peripheral diseases such as asthma and chronic obstructive pulmonary disease (theophylline and roflumilast; for a review, see Gavaldà and Roberts (2013)). An important observation, however, is that AV1013, an amino analog of ibudilast, shares many of the same anti-inflammatory properties as ibudilast, but lacks PDE inhibitory capabilities (Cho et al., 2010a). This suggests that the tolerance-suppressive effects of ibudilast may also result from alternative mechanisms, proposedly the inhibition of MIF (Cho et al., 2010a; 2010b) or antagonism of TLR4 receptors (Hutchinson et al., 2010b).

6.3 Morphine combined with ultralow doses of atipamezole

The main findings of Study II are that atipamezole, an alpha-2-adrenergic antagonist, had no independent antinociceptive effect at the studied ultralow doses, but it increased the antinociceptive effect of spinal morphine in the tail-flick test in both morphine-naïve and morphine-tolerant rats. The administration of systemic atipamezole did not alter systemic morphine antinociception in either morphine-naïve or morphine-tolerant animals.

Intrathecal atipamezole increased the peak antinociceptive potency of morphine in morphine-naïve animals, but no prolongation of the effect was observed. Milne et al. (2008) demonstrated that atipamezole decreased the acute antinociceptive effect of morphine during the first 30 minutes after administration, but after this period, it dramatically prolonged the antinociceptive response, resulting in an increase in the AUC of antinociception over time during 180 minutes after administration. Moreover, in morphine-tolerant rats, the same study demonstrated a much more pronounced augmentation of the morphine antinociceptive effect in comparison with the results of Study II. Differences in experimental design might at least partly explain the observed differences in antinociceptive responses. In Study II, the optimal dose of spinal morphine was 10 times lower (1.5 µg in comparison with 15 µg), while a 10 times higher dose of atipamezole (10 ng) was needed to achieve a significant augmentation of the morphine effect. Similar variation
between studies in the optimal dose of ultralow opioid antagonists has been observed (Mattioli et al., 2010). The rats in our laboratory appeared to be more sensitive to morphine (or more resistant to the development of acute tolerance) because the 15-µg morphine dose used by Milne et al. caused a long-lasting 100% MPE (unpublished results).

There were also several minor differences in study design. In the atipamezole study by Milne et al. (2008) and other reports from the same laboratory, morphine sulfate was used in contrast to morphine hydrochloride in Study II. Study II also used slightly longer tail-flick baseline response times and different anesthetics (ketamine vs. halothane) for the intrathecal cannulation operation. The intrathecal cannulation procedure itself may induce glial activation (DeLeo et al., 1997), and as previously noted, ketamine may have neuroprotective effects leading to decreased glial activation. This could also explain the better efficacy of morphine in our laboratory. Previously, i.t. morphine doses of 1 to 5 µg in 10 µl provided antinociception for several hours (Lemberg et al., 2008), in line with the observed responses in Study II. The best intrathecal morphine dose for evaluating the possible augmentative effects of atipamezole was 1.5 µg, resulting in approximately a 50% MPE at 30 min after administration.

The mechanism by which alpha-2-adrenoceptor antagonists at doses considerably below those producing blockade of the alpha-2-adrenoceptor increase opioid antinociception is not known. One proposal is the alpha-2-adrenoceptor-mu-opioid receptor heterodimer theory (see 2.3.4). The simultaneous activation of both the mu-opioid and alpha-2A-receptors in heterodimer complexes may change the cellular response (Jordan et al., 2003): activation of either mu-opioid or alpha-2A receptors leads to increased parallel intracellular signaling, whereas activation of both receptors decreases the response. An explanation for the paradoxical effect could be that the affinity of heterodimer-involved alpha-2-adrenoceptors for atipamezole would be greater than their monomeric counterparts. In the administration of ultralow doses, only the receptors in heterodimers would be manned, leaving the monomeric receptors unmanned to bind endogenic norepinephrine. However, this theory remains to be investigated. Interestingly, ultralow doses of opioid antagonists have shown similar properties to alpha-2-antagonists. For naloxone, a novel binding site in the cytoplasmic protein filamin A has been discovered, and it remains to be seen whether similar novel binding sites for alpha-2-adrenergic antagonists will be discovered. However, Ozdoğan et al. (2006) demonstrated
that morphine and especially tramadol analgesia were augmented in alpha-2A-adrenoceptor knockout mice, a finding defending the involvement of the alpha-2A receptor binding site in the augmentation of opioid antinociception.

### 6.4 Spironolactone for the treatment of opioid tolerance

The results of Study III indicate that spironolactone had no antinociceptive effect of its own in thermal models of nociception, but it dose-dependently increased the mean brain morphine concentration and corresponding antinociception in both tail-flick and hot plate tests. Spironolactone did not prevent the development of morphine tolerance, but in acute coadministration with morphine it restored the effect of morphine in tolerant rats. The peripheral μ-opioid agonist and P-gp substrate loperamide showed significant antinociception when combined with spironolactone. The results suggest that spironolactone increased the mean morphine brain concentration by inhibiting an efflux transporter, most likely P-gp, at the BBB.

During coadministration of spironolactone, the brain:serum ratio of morphine was increased, indicating an increased distribution of morphine into the brain. Of the known BBB transporters, morphine is a substrate of P-gp (Letrent et al., 1999; Schinkel et al., 1995; Xie et al., 1999) and Mrp (Su and Pasternak, 2013), transporters that move their substrates from endothelial cells to the blood (see 2.3.5). Spironolactone is a P-gp inhibitor (Nakamura et al., 2001), and the augmentation of the mean brain morphine concentration may therefore be due to the inhibition of P-gp. The concentrations of the substances in the spinal cord were not measured in the study. However, parallel results were achieved in the tail-flick and hot plate tests, indicating a probable increase in also the spinal cord concentrations of morphine. In the elimination phase, the mean serum morphine concentration was increased after spironolactone co-treatment, indicating a probable decrease in the elimination of morphine. The higher serum morphine concentration as well as the possible P-gp inhibition additively increased the brain morphine disposition, leading to enhanced and prolonged antinociception in spironolactone-co-treated rats. The P-gp substrate loperamide only had an antinociceptive effect when coadministered with spironolactone, suggesting that spironolactone is an effective P-gp inhibitor at the studied doses. In the study, however, no direct evidence for the inhibition of P-gp was shown.

Morphine is mostly eliminated in the liver by metabolism and to a small extent by excretion in the kidney (Andersen et al., 2003). It is metabolized to
M3G by Ugt2b1 and to normorphine by CYP3A4 in humans and CYP3a1 in rats, and is mainly excreted in its glucuronide forms (Yaksh and Wallace, 2011). In spironolactone-cotreated animals, the mean M3G concentration in the liver was not decreased. However, it is difficult to evaluate the inhibitor effects of spironolactone on the Ugt2b1 enzyme, because spironolactone reduced morphine elimination, leading to increased morphine concentrations in the liver and thus an increased amount of substrate for the morphine glucuronidization reaction. In the presence of spironolactone, the concentration of M3G was vastly increased.

Spironolactone had no effect by itself in acute thermal nociceptive tests. It is interesting, however, that intrathecal spironolactone alleviated neuropathic pain in the chronic compression of the dorsal root ganglion (CCD) model in rats (Sun et al., 2012). The spironolactone-induced antinociceptive effect in the CCD model was suggested to be mediated through the inhibition of activated glial cells, as the expression of proinflammatory cytokines and the phosphorylation of NMDA receptor subunit NR1 was decreased. In Study III, acute pain was measured in healthy animals in which glial cells are not supposed to be pathologically activated. Therefore, no effects of spironolactone were expected. In line with this, spironolactone did not have any effect on its own when the contralateral (healthy) paws of the animals were investigated (Sun et al., 2012).

We examined whether spironolactone co-treatment with morphine could attenuate the development of tolerance. This effect could hypothetically have been mediated through the inhibition of M3G formation or inhibition of the pathological activation of glial cells. Importantly, 4-day spironolactone co-treatment during the morphine tolerance scheme did not affect the response of an acute test dose of morphine. Regarding M3G, spironolactone treatment did not lead to the desired results, as M3G concentrations even increased as a consequence of decreased morphine elimination. The study did not directly evaluate the possible activation of glial cells, because the pharmacokinetic interaction between spironolactone and morphine led to vastly increased brain morphine concentrations in co-treated animals, rendering the comparison between the groups unfeasible. Analysis of glial activation would have required alterations in experiment design, such as the intrathecal administration of morphine or administration of drugs by turns in order to avoid a pharmacokinetic interaction. Interestingly, the development of morphine tolerance was not attenuated by intrathecal spironolactone (Lim et al., 2005), supporting the conclusion that the augmentation of the antinociceptive effect of morphine also observed in study III is mainly due to the increased CNS concentrations of morphine. Even though it is
difficult to directly extrapolate the results of animal studies to patient treatment, previous studies have demonstrated that inhibition of the efflux transporter P-gp may contribute to the effects of morphine and other opioids in humans. Spironolactone may be administered in high doses to patients with liver cirrhosis, and these patients could be exposed to respiratory depression if given morphine to treat pain or dyspnea. Therefore, the clinical relevance of spironolactone in co-treatment with morphine in patients should be investigated.

6.5 A novel pharmacokinetic interaction between morphine and ketamine

Study IV demonstrated that a single dose of ketamine (10 mg/kg s.c.) did not cause antinociception in drug-naïve animals, but in rats under chronic morphine pump treatment it caused long-lasting antinociception up to 150 minutes after administration. At the time point of maximum antinociception, co-treatment caused significant increases in the brain morphine, ketamine and norketamine concentrations, indicating that morphine and ketamine have pharmacokinetic interactions during chronic morphine treatment. After two days of morphine withdrawal, the synergistic antinociceptive effect of ketamine and morphine in coadministration was still observable, but to a smaller extent. Acute co-treatment with ketamine and morphine in drug-naïve rats did not have such effects, suggesting that chronic morphine pump treatment may modify ketamine pharmacokinetics.

The most prominent co-treatment-induced increases in the brain concentrations of morphine, ketamine, and norketamine were observed during chronic morphine administration, during which the increase in antinociception was also marked. The mean brain norketamine concentration was most notably elevated (3.4-fold) in morphine-tolerant rats 90 min after ketamine administration, and the concentration exceeded that of ketamine. Norketamine has been identified as a potent NMDA receptor antagonist (Ebert et al., 1997; Holtman et al., 2008; Shimoyama et al., 1999; see also 2.4.4), and it can therefore be assumed that the vastly increased brain norketamine concentration contributed to the increased antinociception.

After ketamine administration to rats under chronic morphine treatment, the brain concentration of morphine was doubled at maximum antinociception, while the serum concentrations were essentially unchanged, implicating an
increased brain:serum ratio. Morphine is an acknowledged substrate of P-gp (Letrent et al., 1999; Schinkel et al., 1995; Xie et al., 1999) and possibly also Mrp (Su and Pasternak, 2013; see also 2.3.5), both of which are efflux proteins at the BBB. The inhibition of such efflux proteins by ketamine could underlie the decreased efflux and increased disposition of morphine in the brain. However, it is not known whether ketamine inhibits or is a substrate of P-gp (Doan, 2002; Varma et al., 2005) or Mrp. Thus, the mechanism behind the ketamine-induced increase in the brain:serum ratio of morphine remains undetermined.

Ketamine increased morphine concentrations in the liver in all experiments. In acute coadministration, the M3G:morphine metabolic ratio was significantly decreased in the liver, supporting the inhibition of Ugt2b1 by ketamine. Furthermore, when an acute high dose of ketamine was combined with morphine in pilot studies, this effect was even more pronounced (Lilius et al., unpublished data). Indeed, ketamine has previously inhibited UGT2B7 in vitro (Qi et al., 2010; Uchaipichat et al., 2011). However, decreased elimination did not consistently lead to increased serum concentrations. Thus, the inhibition of efflux proteins rather than decreased metabolism may explain the increased brain morphine concentrations after ketamine treatment.

Chronic morphine treatment caused a significant accumulation of ketamine and norketamine in the liver, serum, and brain compared with morphine-naïve rats. This effect was also to a lesser extent present during morphine withdrawal, and it was independent of an acute morphine dose. Similar accumulation could not be seen after acute coadministration in morphine-naïve animals, indicating long-term induced changes in the function of metabolic enzymes, transporter proteins or plasma drug carrier proteins that affect the pharmacokinetics of ketamine. The results did not support significant morphine-induced changes in ketamine metabolism. Thus, morphine tolerance-induced ketamine accumulation could lie behind the downregulation of a ketamine (or its metabolite) efflux transporter, or the upregulation of a reuptake transporter.

More research is needed to clarify the mechanisms of morphine tolerance-induced changes in ketamine pharmacokinetics. These findings also imply that possible changes in brain concentrations of ketamine and morphine should be taken into account in future clinical and preclinical studies that involve the coadministration of these drugs. This novel pharmacokinetic drug interaction could explain the variation in results when treating opioid tolerance in cancer pain management, where high doses of morphine or other opioids are used.
6.6 Future perspectives

The studies of this thesis demonstrate that tolerance to antinociceptive effects of systemic and intrathecal morphine develops rapidly and reliably in in vivo models. Opioid tolerance in animals has usually been suggested to be predominantly pharmacodynamic, time- and dose-dependent, and reversible (Collett, 1998). The first proposed mechanism for the development of tolerance was the upregulation of adenylyl cyclase inhibition by repeated administration of opioids (Collier, 1980), but tolerance has subsequently been shown to be a far more complex phenomenon, involving neuronal changes at both the cellular level and neuronal network level. In preclinical models, recent evidence has brought glial cells and drug transporters into the spotlight of opioid tolerance research.

In the clinic, pain is difficult to study due to problems in the quantification of pain and pain relief, the influence of psychological factors in its perception, and variability in the sensitivity of individuals to pain and to opioids. Tolerance may often be difficult to dissociate from disease progression and/or opioid-induced hyperalgesia when assessing the reasons for inadequate analgesia and the need for escalating the opioid dose. The continuous progress in noninvasive imaging techniques may provide new possibilities for the study of glial responses in humans; for reviews, see Gerhard (2013), Inglese and Petracca (2013), and Obenaus (2013).

The results of this thesis show that when two drugs are studied in coadministration, possible pharmacokinetic interactions must be considered, even when the studied drugs or drug combinations also have pharmacodynamic interactions. Increasing knowledge of drug transporters, their substrates, and potential genetic influences may aid in personalizing drug therapy and choosing better drug combinations while avoiding harmful ones. The pharmacokinetic interactions described in Studies III and IV may be advantageous, but one also has to be aware of potential adverse effects. An unidentified drug interaction is the most dangerous interaction. The undisputed advantage of animal research regarding pharmacokinetics is the possibility to determine the drug and metabolite concentrations in situ, i.e. the CNS. In humans, CNS drug concentrations can be estimated with pharmacokinetic modeling, whereas indirect radiological measurements have so far only been performed with a few radiolabelled drugs using fluorine magnetic resonance spectroscopy (Strauss et al., 1998).
The data of Studies III and IV and previous information indicate that spironolactone and ketamine may enhance morphine analgesia \textit{via} both pharmacodynamic and pharmacokinetic mechanisms. NMDA antagonists have pharmacodynamically advantageous effects that may also indirectly affect the pharmacokinetics of morphine, e.g. by attenuating the upregulation of P-gp at the BBB, not by directly binding to the target P-gp protein but by antagonizing the effects of excess glutamate (Zhu and Liu, 2004), linking pharmacodynamic and pharmacokinetic interactions inseparably together.

Ibudilast has already been proven safe in the treatment of asthma in Asian countries. Its multiple anti-inflammatory actions (inhibition of PDE and MIF as well as antagonism of TLR4 signaling) make it an interesting molecule for the treatment of various CNS diseases with inflammatory properties. It would be of great value to carefully analyze all the target proteins of ibudilast to fully understand its mechanism of action and to eliminate possibly harmful properties in the development of upcoming ibudilast-derived molecules.

Almost all preclinical studies regarding opioid-induced pathological activation of glia have focused on morphine, the gold standard opioid agonist, even though the clinical use of other opioids, especially that of oxycodone, has increased. Thus, more attention should be paid to the basic pharmacology of oxycodone. The contribution of M3G, the pronociceptive metabolite of morphine, to glial activation warrants further study, despite the difficult synthesis process of the molecule (Moreira et al., personal communication). M3G has been suggested to be a very potent activator of the TLR4-MD2 complex (Lewis et al., 2010), but the clinically important oxycodone and its metabolites have not yet been characterized in this respect. An interesting molecule, PTI-609 (Wang et al., 2012a), combines opioid receptor agonism with the inhibition of TLR4-MD2 signaling by binding to filamin A, an intracellular cytoskeleton protein (Burns and Wang, 2010). The development of new molecules with such properties may allow the positive analgesic actions of mu-opioid receptor activation while avoiding pronociceptive xenobiotic-induced central immune signaling.

Ultralow doses of various alpha-2-adrenoceptor antagonists and opioid receptor antagonists have shown promise for the treatment of acute opioid antinociception and also tolerance (Table 2, page 42). As a nonselective alpha-2-adrenoceptor antagonist, atipamezole is not ideal for dissecting the pharmacology behind the phenomenon, but on the other hand, its pharmacology is rather well characterized in veterinary medicine and also in human studies.
More research is needed to understand the role of dimerization of alpha-2-adrenoceptors and opioid receptors in opioid analgesia, bearing in mind the possibility of other novel binding sites for alpha-2-antagonists.
CONCLUSIONS

The conclusions for the specific aims set in Aims of the study are as follows:

I. Acutely administered ibudilast decreased spontaneous locomotion and impaired motor coordination. Ibudilast did not inhibit the development of opioid tolerance, but it restored the antinociceptive effect of morphine in morphine-tolerant animals after single and repeated administration. It did not prevent the development of opioid tolerance in a four-day tolerance model.

II. When administered subcutaneously, ultralow doses of atipamezole failed to augment morphine antinociception in acute administration or in morphine tolerance. In intrathecal administration, ultralow nanogram-scale doses of atipamezole augmented morphine antinociception both acutely and in morphine tolerance.

III. Spironolactone dose-dependently increased the antinociceptive effect of morphine. The effect was mediated via increased concentrations of morphine in the CNS. Spironolactone did not inhibit the development of the pronociceptive morphine metabolite M3G, and it did not prevent the development of opioid tolerance. Loperamide, the peripheral opioid and P-gp substrate, showed antinociceptive properties when coadministered with spironolactone.

IV. In acute cotreatment, a small dose of ketamine increased the brain access of morphine only marginally, whereas the same dose of ketamine administered to rats under chronic morphine treatment led to markedly increased brain concentrations of morphine, ketamine, and norketamine compared to the situation in which the drugs were administered alone. Moreover, chronic morphine treatment caused increased accumulation of ketamine, particularly in the liver.
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REFERENCES


References


Haapalinna, A., Leino, T., Heinonen, E. (2003). The α2-adrenoceptor antagonist atipamezole potentiates anti-Parkinsonian effects and can reduce the adverse cardiovascular effects of dopaminergic drugs in rats. *Naunyn-Schmiedeberg’s Arch Pharmacol* 368, 342–351.


References


References


in a manner parallel to endotoxin. *Proc Natl Acad Sci USA* 109, 6325–6330.


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