

# **Opioid analgesia: modulation by drug interactions and glial activation**

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**ACADEMIC DISSERTATION**

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*"Do your work. Don't be stupid."*  
- Anonymous

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## List of abbreviations

ACC	Anterior cingulate cortex
AMY	Amygdala
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCE	Before common era
BDNF	Brain-derived neurotrophic factor
BG	Basal ganglia
BSA	Bovine serum albumin
CAMKII	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
CCD	Chronic compression of dorsal root ganglia
cDNA	Complementary DNA
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CX3CL1	Fractalkine
CYP	Cytochrome P450
DAP12	DNAX activation protein of 12kDa
DDD	Defined daily dose
DOR	Delta opioid receptor
DPA	Dynamic plantar aesthesiometer
EAAC1	Excitatory amino-acid carrier 1
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal–regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Fkbp5	FK506 binding protein 5

FPKM	Fragments per kilobase of transcript per million mapped reads
GABA	gamma-Aminobutyric acid
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GR	Glucocorticoid receptor
GRK	G protein-coupled receptor kinase
i.p.	Intraperitoneal
i.v.	Intravenous
IASP	International Association for the Study of Pain
IL-1	Interleukin 1
IL-1 $\beta$	Interleukin 1 beta
IL-6	Interleukin 6
Kcnn4	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4
KOR	Kappa opioid receptor
LC	Locus coeruleus
LD <sub>50</sub>	Half maximal lethal dose
LPS	Lipopolysaccharide
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MACS	Magnetic cell isolation and cell separation
MAPK	Mitogen-activated protein kinase
MOR	Mu opioid receptor
MPE%	Percentage of maximum possible effect
MR	Mineralocorticoid receptor
n.d.	No date
NMDA	<i>N</i> -methyl-D-aspartate
NOP	Nociceptin receptor

OIH	Opioid-induced hyperalgesia
P2RX4	P2X purinoceptor 4
P2RX7	P2X purinoceptor 7
PAG	Periaqueductal gray
PB	Parabrachial nucleus
PFC	Prefrontal cortex
P-gp	P-glycoprotein
PKA	Protein kinase A
PKC	Protein kinase C
RNA	Ribonucleic acid
RVM	Rostroventral medulla
s.c.	Subcutaneous
S1	Primary somatosensory cortex
S1P	Sphingosine-1-phosphate
S2	Secondary somatosensory cortex
TLR4	Toll-like receptor 4
TNF $\alpha$	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TRP	Transient receptor potential
UGT	Uridine 5'-diphospho-glucuronosyltransferase
USA	United States of America
VGCC	Voltage-gated calcium channel
VM	Ventral medulla
$\omega$ -CgTx	N-type calcium channel blocker $\omega$ -conotoxin GVIA

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Opioid analgesia: modulation by drug interactions and glial activation

Corrected part in **bold**

*Correction to page 15 reference, line 3: "Häkkinen et al., 2014"*

*Correction to Figure 2 legend: "...[J Clin Invest. **Central modulation of pain.**, Michael H. Ossipov et al., 2010. Year of copyright 2010];..."*

*Correction of typographical error page 26, line 22: "(Le Merrer et al., 2009)"*

*Correction of substitution error page 27, line 6: "...is a substance that binds to opioid receptors and produces **opium**-like effects,..."*

*Correction to reference page 27, line 5: "(Pain & Policy Studies Group, **n.d.**)"*

*Addition of a reference to a figure page 28, line 4: "...respectively, **(Fig. 4B)**"*

*Correction of typographical error page 33, line 8: "Section 2.4.2"*

*Correction of typographical error page 34, line 7: "Section 2.4.2"*

*Correction to reference page 48, line 4: "Poggioli et al."*

*Correction of typographical error page 70, line 13: "Study I: Results"*

*Correction of typographical error page 36, line 12: "Fig. 7"*

*Correction to reference page 37, line 8: "...as discussed by **Dumas et al. (2008)**:..."*

*Correction of typographical error page 39, line 17: "(Koppert, 2007.)"*

*Correction to reference page 51, line 2: "Häkkinen et al., 2014"*

*Correction of typographical error in Figure 10 legend: "Gurkoff et al.,..."*

*Correction to reference order in the bibliography: "Häkkinen, M., ..." should be located after the reference "Hutchinson, M.R., Zhang, Y ..."*

**Correction to reference format:** page 34 line 24: "Okura et al. **(2008)**", page 47 line 9: "Karst et al. **(2005)**", line 16: "Grenados-Soto et al. **(2015)**", 21: "Chu et al. **(1978)**", page 48 line 4: "Poggioli et al. **(1985)**", page 55 line 9: "Brennan et al. **(1996)**", page 59 line 10: "Penttinen et al. **(2016)**", Page 80 line 12: "Heiskanen et al. **(2008)**", page 81 line 19: "Omote et al. **(1996)**", page 82 line 6: "Shimizu et al. **(2004)**", page 84 line: "Hutchinson et al. **(2010)**", page 85 line 10: "Hutchinson et al. **(2009)**", line 12: "Zhang et al. **(2012)**", page 86 line 8: "Kobyashi et al. **(2016)**". **The references at the end of the respective sentences in the text are now unnecessary because they would be duplicate.**

## Abstract

Opioids are the most efficacious analgesics in the treatment of nociceptive pain. Opioid treatment can, however, be complicated by side effects and also tolerance and opioid-induced hyperalgesia. Therefore, drugs that potentiate opioid-induced analgesia and help reduce the required opioid dose can be beneficial as adjuvants in pain treatment. Interestingly, the mechanism of the attenuation of the analgesic effect of morphine in tolerance and opioid-induced hyperalgesia might be related to neuroinflammation in the central nervous system. The aims of this investigation were to study drug interactions that could potentiate the effects of opioids and prevent/reverse opioid tolerance, and also to assess the role of immunomodulating cells, microglia and astrocytes, in morphine tolerance and opioid-induced hyperalgesia in male Sprague-Dawley rats. Spironolactone, which has been suggested in the literature to potentiate the effects of morphine, did not display antinociceptive effects of its own in thermal tests, but acutely enhanced the morphine antinociception, most likely by inhibiting P-gp and increasing morphine brain concentrations. Spironolactone did not prevent the development of morphine tolerance. Spironolactone increased the antinociceptive effects and brain concentrations of oxycodone, probably via inhibition of metabolism. The increased brain disposition might be associated with P-gp inhibition.

Pregabalin enhanced the antinociceptive and sedative effects of both oxycodone and morphine in thermal tests. The interaction, however, differed between these opioids as it depended on the dose or/and temporal scheme of the drug administration. The behavioral results could not be explained by pharmacokinetic interaction at the central nervous system level. Pregabalin did not prevent or reverse morphine tolerance. Chronic morphine treatment induced tolerance and hyperalgesia. The associated immunohistochemically determined increase in microglia reactivity took place at the spinal but not supraspinal level. The transcriptome of the spinal microglia cells indicated

upregulation of many inflammation and pain-associated genes. The results of this thesis imply that spironolactone has a robust pharmacokinetic interaction with morphine and oxycodone, which could have implications in opioid therapy. The interaction between pregabalin and opioids, however, does not seem to involve pharmacokinetic interaction. Thus, the results suggest that the drug interaction between pregabalin and opioids at the CNS level is pharmacodynamic. The transcriptome study of the spinal microglia strengthens the hypothesis that microglial activation after chronic morphine treatment is similar to activation that occurs with pathological pain.

## List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

**I The mineralocorticoid receptor antagonist spironolactone enhances morphine antinociception.** Lilius, T.<sup>#</sup>, Jokinen, V.<sup>#</sup>, Neuvonen, M. S., Väänänen, A. J., Niemi, M., Rauhala, P. V., & Kalso, E. A. 2014. *European Journal of Pain*. 18, 3, 386-395. Included also in the doctoral thesis of Tuomas Lilius 'New insights into enhancing morphine analgesia: from glia to pharmacokinetics', 2014. <sup>#</sup> Authors contributed equally to the study.

**II Do Diuretics have Antinociceptive Actions: Studies of Spironolactone, Eplerenone, Furosemide and Chlorothiazide, Individually and with Oxycodone and Morphine.** Jokinen, V., Lilius, T., Laitila, J., Niemi, M., Kambur, O., Kalso, E., & Rauhala, P. 2017. *Basic & Clinical Pharmacology & Toxicology*. 120, 1, 38-45.

**III Pregabalin enhances the antinociceptive effect of oxycodone and morphine in thermal models of nociception in the rat without any pharmacokinetic interactions.** Jokinen, V., Lilius, T., Laitila, J., Niemi, M., Rauhala, P., & Kalso, E. 2016. *European Journal of Pain*. 20, 2, 297-306.

**IV Differential spinal and supraspinal activation of glia in a rat model of morphine tolerance.** Jokinen, V., Sidorova, Y., Viisanen H., Suleymanova I., Tiilikainen H., Li Z., Lilius T., Mätlik K., Anttila J., Airavaara M., Tian L., Rauhala P., & Kalso E. 2017. Submitted.

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# 1. Introduction

Opioid drugs constitute the cornerstone of the treatment of moderate-severe acute, postoperative, and cancer pain. Although the analgesic efficacy of opioids is not restricted by a ceiling effect, in practice the opioid dose and analgesia can be limited by side effects. Therefore, other drugs that potentiate opioid-induced analgesia and help reduce the required opioid dose can be beneficial as adjuvants in pain treatment. Besides beneficial modulation, previously unrecognized interactions between opioids and other drugs can affect the opioid response and compromise the treatment.

Interestingly, the literature suggests that diuretics might exert an antinociceptive effect (Granados-Soto et al., 2005; Poggioli et al., 1985; Sun et al., 2012) of their own and modulate the effects of opioids (Chu et al., 1978; Poggioli et al., 1985) in preclinical models. Antinociceptive mechanisms of individual diuretics include attenuation of microglial activation (Sun et al., 2012), antagonism of mineralocorticoid receptor (Dong et al., 2012), and blocking of the neural sodium-potassium-chloride co-transporter (Granados-Soto et al., 2005). The mechanism of the suggested interactions between diuretics and opioids remains unknown.

Gabapentinoids have reduced the amount of opioids needed to alleviate experimental nociceptive pain in healthy volunteers (Eckhardt et al., 2000) and in postoperative pain (Clarke et al., 2009; Fassoulaki et al., 2012; Mahoori et al., 2014; Tiippana et al., 2007; Yücel et al., 2011). Drug interactions can be either pharmacokinetic and take place at the level of absorption, distribution, metabolism, and elimination or pharmacodynamic and take place at the molecular level, governing the response of the body to the drug. To characterize the nature of the interaction, drug concentration measurements might be required. Indeed, although pregabalin is not known to have any drug interactions, a

pharmacokinetic interaction between pregabalin and opioids has not been excluded. The interaction between pregabalin and opioids is also interesting as both drugs have been shown to be co-abused in forensic cases (Häkkinen, 2014), indicating that abuse of pregabalin might predispose to opioid-induced respiratory depression.

Opioids also modulate their own analgesic effect by initiating adaptive processes like opioid-tolerance and opioid-induced-hyperalgesia (OIH), both of which can undermine the treatment (Watkins et al., 2009). Opioid tolerance and OIH might share a common neurobiological basis and the activation of microglia and related neuroinflammation has emerged as one possible mechanism contributing to the development of opioid tolerance and OIH (Cui et al., 2006; Grace et al., 2015; Raghavendra et al., 2002; Watkins et al., 2009).

In this thesis, pharmacological interactions between the diuretics spironolactone, eplerenone, furosemide, and chlorothiazide and the anticonvulsant pregabalin in co-administration with oxycodone and morphine were studied. In addition to drugs that could modulate opioid antinociception, we assessed the reactivity of microglia and astrocytes in morphine tolerance in spinal and supraspinal structures.

## 2. Review of the literature

### 2.1 Definition of pain

Pain is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International Association for the Study of Pain, IASP).

### 2.2 Physiology of nociception and pain

The neural process that encodes noxious stimuli (nociception - according to the International Association for the Study of Pain) that alerts the organism of an imminent threat to its structural integrity is well preserved within evolution in different species (Sneddon, 2004). The consequence of nociception - motor response to withdraw from noxious stimuli or motivation to let damaged tissue heal provides an obvious advantage for survival. In the last centuries, many theories attempting to explain the physiology of nociception have been presented. For example, in the *Specificity theory*, all sensory modalities, including pain, have dedicated and distinct pathways, whereas in the *Intensity theory* separate pathways do not exist and the intensity of all stimuli is encoded in the frequency of the nerve impulses (Moayedi and Davis, 2012). An important step forward was taken with the *Gate control theory*, which postulated that the transmission in the fibers conveying touch information can affect whether the fibers conveying noxious stimulation can relay their information onwards (Melzack and Wall, 1965; Moayedi and Davis, 2012). According to the current knowledge, the phases of nociception and pain can be functionally divided into transduction, transmission,

modulation, and perception, all of which take place in specific structures of the nervous system.

### 2.2.1 Nociceptors and nocireceptors

Nociceptors are the first neurons in the nociceptive pathway. The cells are pseudounipolar with their somas in the trigeminal ganglion (Fig. 1A) or dorsal root ganglion (Fig. 1B). According to the Erlanger Gasser classification (Whitwam, 1976), the nociceptors are further divided into A $\delta$  and C fibers based on the diameter of the axon. A $\delta$  fibers are myelinated and conduct action potentials fast (5-30 m/s) (Marchland, 2012a). A $\delta$  fibers evoke sharp, localized pain and respond to all noxious modalities (chemical, heat, mechanical) (type I) and to heat with relatively low threshold (type II) (Basbaum et al., 2009). A $\delta$  type II fibers convey rapid signaling of noxious heat (Basbaum et al., 2009). Most C fibers are also considered polymodal, but their activation produces more ill-localized pain and sufficient activation can evoke a burning sensation (Basbaum et al., 2009). Unlike A $\delta$ , C fibers are unmyelinated and conduct action potentials slowly (0.5-2 m/s) (Marchland, 2012a). In addition to the actual nociceptors, A $\beta$  fibers that in normal conditions convey impulses from innocuous mechanical stimuli can begin to contribute to allodynia ("Pain due to a stimulus that does not normally provoke pain", IASP) under conditions where the CNS is sensitized (Marchland, 2012a).

The distal ends of the axons on the nociceptors lay in the peripheral tissues or viscera. All nociceptors have unmyelinated, free nerve-endings, which contain the actual molecular receptors (transducers) of the noxious stimuli. The transducers are a heterogeneous group of membrane-bound-proteins that include metabotropic, enzyme-linked receptors, and ionotropic channels (Woolf and Costigan, 1999). Important individual group of transducers is transient receptor potential (TRP) channels,

for review, see Julius (2013). Unlike the other primary afferents, nociceptors are activated only by stimuli whose magnitude exceeds the high threshold, which can be attributed to the high activation threshold of the transducing molecules (Woolf and Ma, 2007).

Transducers begin the first phase of nociception by transducing the noxious stimuli into action potentials. The action potentials are generated and conducted by voltage-gated sodium channels (VGCC), of which  $Na_v1.7$ ,  $Na_v1.8$ , and  $Na_v1.9$  are particularly important (Dib-Hajj et al., 2010). The hereditary channelopathies which prevent the depolarization from amplifying into action potential can cause inability to experience pain (Cox et al., 2006). Propagation of the action potential along the axon leads to the *transmission* phase of nociception.

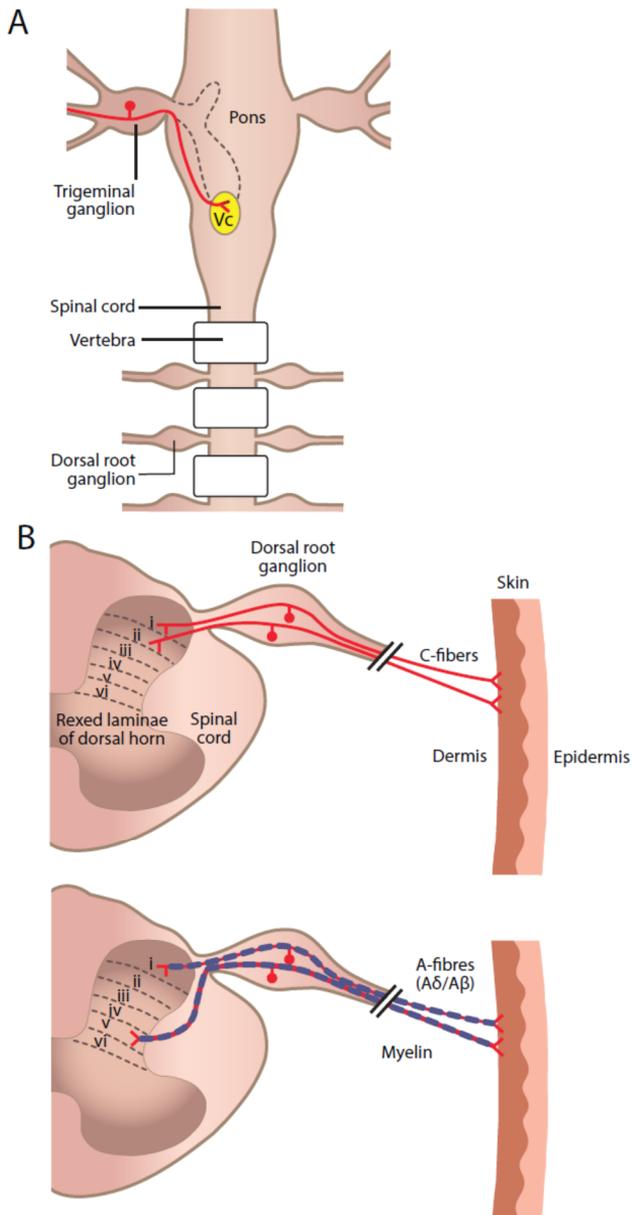


Figure 1. Schematic diagram of the anatomy of nociceptors from the head (A) and elsewhere from the body (B). Trigeminal nucleus (Vc). Republished and modified with permission of American Society for Clinical Investigation from [J Clin Invest. Nociceptors: the sensors of the pain pathway, Adrienen E. Dubin et al., 2010. Year of copyright 2010]; permission conveyed through Copyright Clearance Center, Inc.

### 2.2.2 Role of spinal cord and medulla oblongata in nociception

The proximal end of the nociceptor forms a synapse with the second order afferent neuron or interneuron either in the dorsal horn of the spinal cord or trigeminal nucleus in the brain stem. C fibers synapse in laminae I and II and A $\delta$  fibers in laminae I and V of the dorsal horn (Fig. 1) (Dubin and Patapoutian, 2010). The synapses are excitatory and use glutamate, neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P, and proteins, including brain-derived neurotrophic factor (BDNF) as neurotransmitters (Basbaum et al., 2009; Woolf and Ma, 2007). Interneurons can, for example, produce direct neuronal interlinks to the motor nerves and facilitate spinal reflexes, but also locally inhibit the transmission from the afferent fibers. The axons of second order neurons decussate to the contralateral side and ascend via the anterolateral system of the spinal cord to the thalamus (spinothalamic tract) in diencephalon or reticular formation (spinoreticular tract) in the pons (Westlund and Willis, 2015). From the trigeminal nucleus, the axons to the thalamus and reticular formation ascend via the trigeminothalamic (Henssen et al., 2016) and the trigeminoreticular tracts (Panneton et al., 2011), respectively. Both the spinal cord and the trigeminal nucleus also project to the amygdala via the parabrachial complex (Jasmin et al., 1997). The tracts projecting to the thalamus mediate the sensory-discriminative dimension of pain (Fig. 2), and the tracts to the amygdala the motivational-affective dimension (Ab Aziz and Ahmad, 2006). Projections for reticular formation mediate poorly localized pain (Patestas et al., 2016), but also induce changes in alertness (Mendoza, 2011) as a response to noxious stimuli.

The *modulation* phase has a significant role in nociception and pain. The modulation exists in the form of a descending modulatory circuit that can produce strong endogenous analgesia, but can also facilitate nociceptive transmission (Fig. 2) (Colloca and Grillon, 2014), for example, due to an emotional status. The descending modulation

gathers input from multiple areas, including the amygdala, the rostral anterior cingulate cortex, and the ascending tracts (Ossipov et al., 2010). Signals from the supraspinal structures converge to the periaqueductal gray (PAG) in the midbrain, which projects to the medulla (Ossipov et al., 2010) (Fig. 2). From the rostroventromedial medulla (RVM), the neurons of nucleus raphe magnus and nucleus reticularis gigantocellularis project signals to both the spinal and medullary dorsal horns directly or via interneurons to govern the nociceptive input from the periphery (Ossipov et al., 2010).

GABAergic and glycinergic neurotransmission from the ventral medulla to the spinal cord is antinociceptive (Hossaini et al., 2012; Kato et al., 2006), but the subtype of the serotonin receptor determines whether the serotonergic transmission evokes excitatory or inhibitory effects (Ossipov et al., 2010). Antinociceptive noradrenergic projections to the spinal cord descend from the locus coeruleus, which itself closely communicates with PAG and RVM (Llorca-Torrallba et al., 2016; Ossipov et al., 2010). Recent studies have also described direct descending modulatory circuits from the cortex to the dorsal spinal horn and trigeminal nucleus (Wang et al., 2015). The interneurons which can get input from both the peripheral afferents and descending tracts use, for example, enkephalinergic (François et al., 2017) and GABAergic/glycinergic transmission when inhibitory and glutamate when excitatory (Todd, 2010).

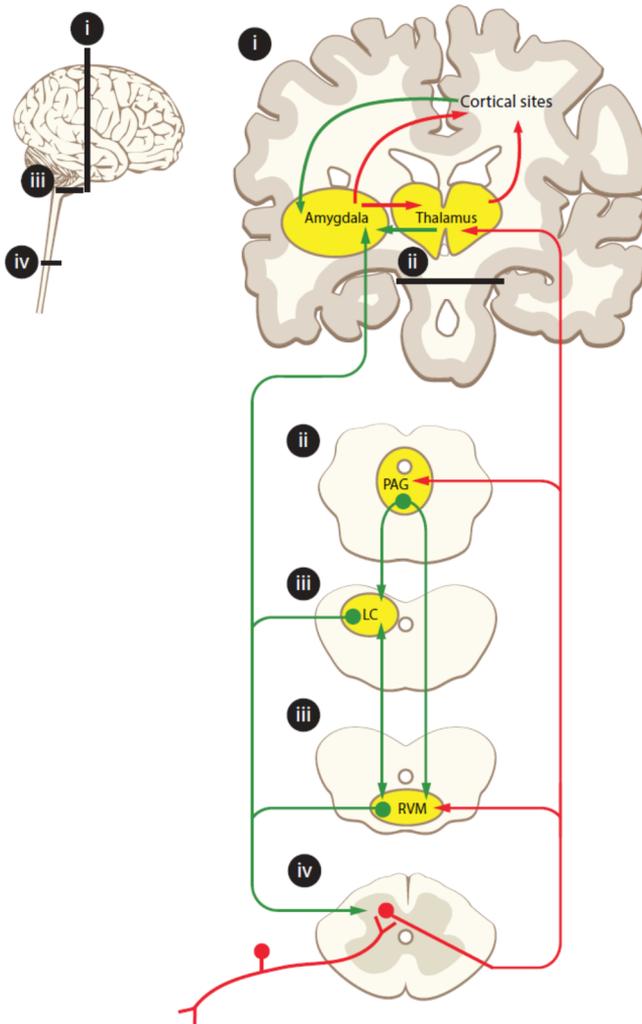


Figure 2. Schematic diagram of pain modularity circuitry. Periaqueductal gray (PAG), Locus Coeruleus (LC), Rostrovventral medulla (RVM). Republished and modified with permission of American Society for Clinical Investigation from [J Clin Invest. Nociceptors: the sensors of the pain pathway, Michael H. Ossipov et al., 2010. Year of copyright 2010]; permission conveyed through Copyright Clearance Center, Inc.

### 2.2.3 Nociception and pain in the brain

*Perception*, the last phase of nociception, takes place in the brain (Fig. 3). Cognitive functions are required to feel pain, at least with regard to how pain is defined. Thus, pain is not only a sensory experience but a complex sensation, which is affected by personality, memories, mood, and expectations. Considering the multidimensional role of pain, it is easy to understand that nociceptive information from the spinal cord and trigeminal nucleus is relayed to multiple brain regions. Although not specific for pain (Iannetti and Mouraux, 2010), four cortical structures have a dominant role in the brain: primary and secondary somatosensory cortices are important in the sensory-discriminative dimension of pain, whereas insular cortex and anterior cingulate cortex participate in processing of the motivational-affective dimension (Marchland, 2012b) (Fig. 3). However, also subcortical structures like the amygdala have an important contribution to pain (Simons et al., 2012).

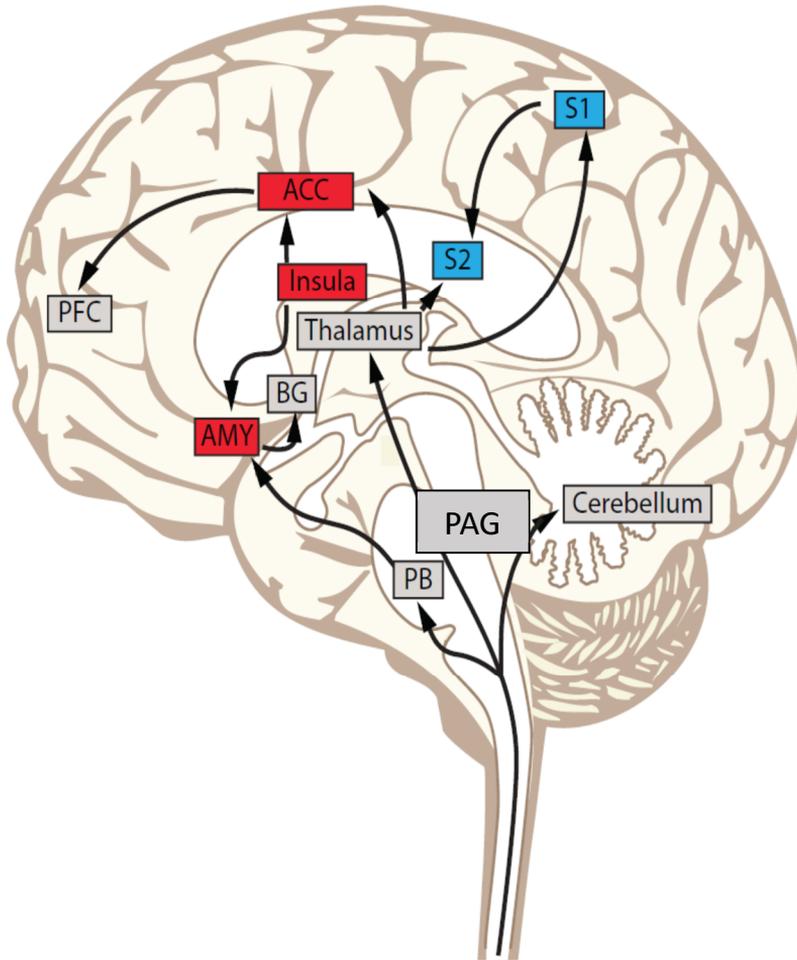


Figure 3. Schematic diagram of the brain areas involved in pain and nociception. Red labels indicate an association with the motivational-affective component of pain and blue labels a sensory-discriminative association. Prefrontal cortex (PFC), Anterior Cingulate Cortex (ACC), Amygdala (AMY), Basal ganglia (BG), Primary somatosensory cortex (S1), Secondary somatosensory cortex (S2), Parabrachial nucleus (PB), and Periaqueductal gray (PAG). Adapted by permission from Macmillan Publishers Ltd: [Nat. Rev. Neurosci., Cognitive and emotional control of pain and its disruption in chronic pain, Bushnell et al., 2013. Year of copyright 2013.]

## 2.3 Endogenous opioid system

The endogenous opioid system is an essential part of the central descending modulatory circuit. However, the endogenous opioid system also participates in peripheral analgesia, reinforcement networks, and modulation of stress and mood (Le Merrer et al., 2009; Vadivelu et al., 2011). The system consists of widespread but strategically concentrated opioid receptors and endogenous agonists, and it also mediates the effects of the exogenous opioid agonists, as is covered under Section 2.4 'Exogenous opioids'.

### 2.3.1 Opioid receptors and their endogenous agonists

The endogenous opioids comprise peptides in the  $\beta$ -endorphin, enkephalin, and dynorphin families (Benarroch, 2012). Enkephalins and dynorphins are expressed in interneurons, including those that participate in the modulation of nociception in the PAG-RVM-axis and the dorsal horn and trigeminal nucleus (Benarroch, 2012).  $\beta$ -endorphins also participate in the modulation of nociception, but they project widely across the CNS from the nuclei at the brain stem and diencephalon (Benarroch, 2012) and are also secreted to the circulation from the pituitary gland (van Den Burg et al., 2001). In addition, opioid peptides are secreted from immune cells in peripheral tissue, for review, see Kapitzke et al. (2005). The individual endogenous opioid peptides have differential affinity profiles to the opioid receptors (Kapitzke et al., 2005).

Four opioid receptors:  $\mu$ - (MOR),  $\kappa$ - (KOR)-, and  $\delta$  (DOR)-opioid receptors and opioid-like-receptor-1 (ORL-1) or nociceptin receptor (NOP), have been identified (Table 1). The opioid receptors belong to the family of seven transmembrane G protein-coupled receptors and are encoded by a single gene (OPRM1, OPRK1, OPRD1, and OPRL1, respectively) (Al-Hasani and Bruchas, 2011). Opioid receptors can further form dimers either with the same or a different opioid receptor type, increasing opioid receptor

variety (Al-Hasani and Bruchas, 2011). Subtypes of the different receptors produced by RNA splicing also exist, but their biological relevance remains unclear (Al-Hasani and Bruchas, 2011). Opioid receptors are highly conserved and are present in most species (Dreborg et al., 2008). However, the opioid system does not seem to be imperative for the development or survival of the individual, as genetically modified mice with no opioid receptors are fertile and stay alive at least in laboratory conditions (Kieffer, 1999).

Opioid receptors reside in critical locations of the descending pain modulation circuit, including the anterior cingulate cortex, PAG-RVM axis, and superficial dorsal spinal cord, although the distribution of the different receptors varies (Benarroch, 2012). Studies using knockout mice have demonstrated that MOR, KOR, and DOR have distinct roles in the physiological regulation of nociception and that their individual ablation causes subtle differences in nociceptive tests. For example, KOR has been shown to be involved in spinally mediated thermal nociception, and MOR elicited an effect in mechanical nociception at the supraspinal level (Martin et al., 2003). Interestingly, endogenous antinociception caused by conditioned fear was attenuated by intra-RVM injection of MOR but not KOR and DOR antagonists (Foo and Helmstetter, 1999), and intra-PAG injection of MOR but not KOR antagonist (Bellgowan et al., 1998). The results are in harmony with the activation of MOR being required for antinociceptive effect of potent exogenous opioids (Kieffer and Gaveriaux-Ruff, 2002).

Opioid circuits are also found in brain sites associated with reward-processing and addiction, including the ventral tegmental area, nucleus accumbens, and amygdala (Le Merrer et al., 2009)). In addition to their central functions, opioid receptors are a constitutive part of the peripheral nervous system, where their role has especially been studied in inflammation-related analgesia (Vadivelu et al., 2011).

Table 1. Opioid receptors and their agonist and antagonists.  $\mu$ -opioid receptor, MOR ( $\mu$ ),  $\delta$ -opioid receptor, DOR ( $\delta$ ),  $\kappa$ -opioid receptor, KOR ( $\kappa$ ), Nociceptin receptor (NOP). (Chartoff and Connery, 2014; Contet et al., 2004; Eguchi, 2004; Gaveriaux-Ruff and Kieffer, 2011; Land et al., 2008; Zaveri et al., 2005)

Opioid receptor	Endogenous agonist	Selective agonist	Selective antagonist	Effects of activation
$\mu$	$\beta$ -endorphin, Met-Leu enkephalin	DAMGO, JOM-5, ADAMB	MET-CAMO, DOC-CAM, CYPRODIME	analgesia, euphoria, addiction
$\delta$	$\beta$ -endorphin, Met-Leu enkephalin	BM373U86, JOM-13, SL-3111	Indolomorphinan naltrindole, Naltriben, Portoghese	analgesia
$\kappa$	Dynorphin	TRK-820, Scopes 1992, CI-977	nor-bintorphan, JD1c, 5'-GNTI	dysphoria, analgesia
NOP	Nociceptin	hexahydroppyrrrolopyrrole	J-113397	modulation of opioid receptors, immunomodulation

## 2.4 Exogenous opioids

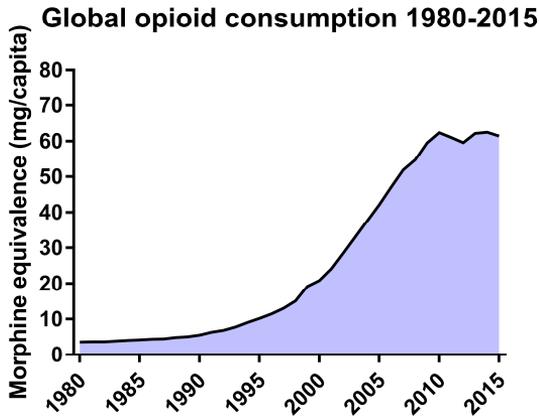
### 2.4.1 Clinical use

Opioid is a substance that binds to opioid receptors and produces morphine-like effects, whereas the term opiate refers to compounds found in the opium poppy plant. Opiates have been used for thousands of years. Archeological studies implicate the use of opium already in 14th century BCE in Minoan civilization (Askitopoulou et al., 2002). Morphine was extracted from opium first in 1805, and since then many synthetic or semi-synthetic (synthesized from opiates) opioid agonists have been developed. In the modern world, opioids are still the analgesics known to have the highest efficacy in the treatment of nociceptive pain.

The global consumption of opioids has been on the rise during the past three decades, but reached a plateau between 2010 and 2015 (Pain & Policy Studies Group, 2015; Silbermann, 2011) (Fig. 4A). The use, however, is highly dissimilar between countries; in

2008, countries with 15% of the world's population consumed 89% of the global morphine (Silbermann, 2011). In Finland in 2015, the consumption of strong opioids and weak opioids was 3 and 11 defined daily doses (DDD)/1000 inhabitants/day (Fimea, n.d.), respectively. The main indication for the use of opioids is moderate to severe pain. Opioids are widely used to treat acute nociceptive (Ramsay, 2000) and cancer pain (Caraceni et al., 2012). Opioids are also used in the management of neuropathic pain, although they are not the first-line choice (Dworkin et al., 2010). The careless use of opioids in non-malignant chronic pain can easily lead to problems due to the reinforcing effect of opioids on drug-seeking behavior (Chou et al., 2009). From the clinical perspective, opioids can be classified as weak, intermediate, or strong. In Finland, the following opioids are used as analgesics outside hospital in clinical practice: codeine and tramadol (weak opioids), buprenorphine (intermediate opioid), and morphine, oxycodone, fentanyl, methadone, and hydromorphone (strong opioids).

**A**



**B**

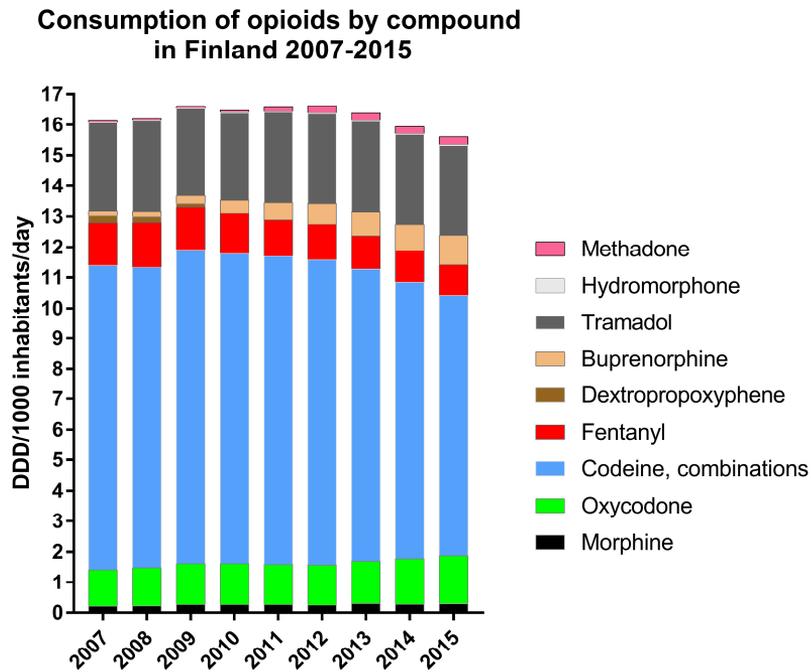


Figure 4. Consumption of opioids globally (A) and in Finland (B). Defined daily doses (DDD). (Fimea, 2016, 2012, 2010, n.d.; Pain & Policy Studies Group, n.d.)

## 2.4.2 Opioid-induced analgesia

Opioid-induced analgesia is elicited via activation of the endogenous opioid system (Fig. 5). Analgesia comprises three system-level mechanisms: 1) emotional detachment from the pain, which has been shown to be due to MOR-mediated activity in the limbic system of the brain (Lee et al., 2014; Leppä et al., 2006.), 2) facilitation of the descending inhibitory system, and 3) inhibition of transmission of peripheral nociceptive input in the dorsal horn of the spinal cord and trigeminal nucleus.

Interaction with the MOR as their mechanism of action is common to all exogenous opioid agonists (later only opioids) (Drewes et al., 2013). MOR is the most abundant opioid receptor in the supraspinal level, including the brainstem, amygdala, and thalamus (Benarroch, 2012). Specifically, the PAG-RVM axis, which has been shown to have high density of MOR (Commons et al., 2000), and intra-PAG injection of MOR antagonist have been demonstrated to attenuate the antinociceptive effect of systemic morphine (Bernal et al., 2007; Lewis and Gebhart, 1977). MOR comprises also 70% of the total opioid receptors in the spinal level, where the opioid receptors are concentrated presynaptically (~70%) in nociceptors and postsynaptically (~30%) in interneurons or projection neurons (Besse et al., 1990). Activation of MOR by opioid agonist causes the dissociation of the G-protein subunits  $G\beta\gamma$  and  $G\alpha$ . The  $G\beta\gamma$  activates inwardly rectifying potassium channels in the post-synaptic nerve terminal (Zylbergold et al., 2010), and deactivates voltage-dependent  $Ca^{2+}$ -channels presynaptically (Dolphin, 1998).  $G\alpha_i$  inhibits adenylate cyclase, and thus, formation of cAMP (Hsia et al., 1984). The described events lead to inhibition of neuronal activity. In addition, activation of MOR is followed by intracellular mechanisms that desensitize the receptor signaling and could be interpreted as adaptation aiming to preserve homeostasis. The process is mediated via MOR phosphorylation and  $\beta$ -arrestin binding, which disturb the cell signaling and might lead to MOR internalization (Groer et al., 2006). Opioid-induced

analgesia is effectively blocked with the MOR antagonist naloxone (Markowitz et al., 1976).

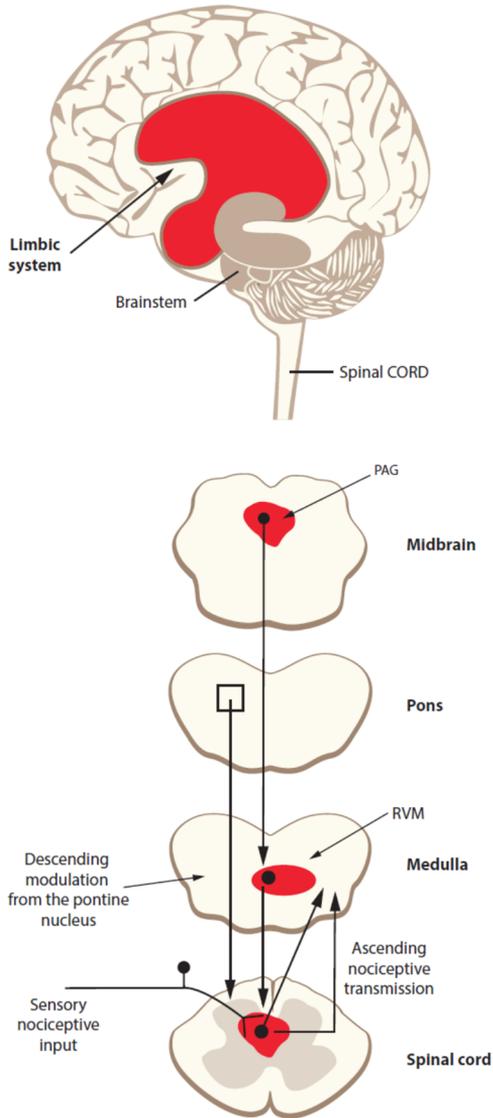


Figure 5. Schematic diagram of opioid action sites. Periaqueductal gray (PAG), Rostroventral medulla (RVM).

### 2.4.3 Adverse effects

Opioid administration can cause adverse effects that limit dosing and thus effectiveness of the opioid treatment. In a meta-analysis of non-malignant chronic pain patients, the most common adverse effects and their incidence were constipation (33%), nausea (26%), and sedation (23%) (Kalso et al., 2004). Opioids can also elicit other adverse effects, including pruritus and bladder dysfunction (Benyamin et al., 2008). Opioid-induced immunomodulating effects via alteration of the endocrine system or immune cells are also possible, but their clinical relevance is less clear (Welters, 2003). Typically, patients experience adverse effects at the beginning of opioid treatment or during dose escalation, but these effects might continue also during the maintenance dose in chronic treatment (Benyamin et al., 2008; Smith and Laufer, 2014). The most severe complication of opioids is respiratory depression. Respiratory depression is the prevalent mechanism of death in opioid overdose in drug addicts (White and Irvine, 1999), but also the legal use of an opioid can predispose to respiratory depression; prescribed opioids were the most significant drugs involved in drug overdose fatalities in the USA in 2015 (CDC, 2016).

The adverse effects are mediated via both peripheral and central mechanisms. Constipation, for example, is caused by the activation of MOR and DOR in the submucosa and myenteric plexuses of the gastrointestinal tract, leading to inhibition of motility of the small and large intestine (Nelson et al., 2016). The specific mechanisms of the opioid central adverse effects are less clear. Opioid-induced nausea and vomiting probably encompasses activation of MOR in the chemoreceptor trigger zone in the medulla and vestibular apparatus in the inner ear (Smith and Laufer, 2014). Opioid-induced sedation seems to arise from central anticholinergic effects, but also from parallel inhibitory effects on cerebral activity (Vella-Brincat and Macleod., 2007). Studies indicate that the mechanism of respiratory depression is associated with the opioid actions at the bulbar

respiratory network (Lalley et al., 2014). Other drugs, such as anti-emetics and peripheral opioid antagonists, are often needed to alleviate opioid-induced adverse effects (Smith and Laufer, 2014).

#### 2.4.4 Morphine

Morphine is the classical MOR agonist (Pasternak and Pan, 2013) and although other opioids are replacing it in clinical medicine it has an established place as the reference drug in pharmacology. The pharmacodynamic mechanism of morphine is covered under Section 2.3.2 'Opioid-induced analgesia'. Morphine is glucuronidated to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) mainly in the liver by UGT2B7 (Coffman et al., 1997) and N-demethylated to normorphine by CYP3A4 (Projean et al., 2003; Hasselström and Säwe, 1993) (Fig. 6). The rat, however, produces no M6G from morphine (Kuo et al., 1991). M3G is inactive at MOR, but M6G possesses potent analgesic efficacy, which is considered to contribute significantly to morphine analgesia (De Gregori et al., 2012). Bioavailability of orally administered morphine is about 30% (Hasselström and Säwe, 1993) in humans, and of the total morphine dose, 9% (i.v.) and 2% (p.o.) is excreted in urine as morphine, 44% (i.v.) and 42% (p.o.) as M3G, and 11% (i.v.) and 3% (p.o.) as M6G (Osborne et al., 1990).

Drug transporters can exert significant effects in governing morphine and its metabolite disposition in the CNS. P-gp actively transports morphine out of the CNS across the blood-brain-barrier (BBB), and blocking P-gp pharmacologically and removing its expression from the BBB has increased morphine brain concentrations in the rat (De Gregori et al., 2012; Letrent et al., 1999; Xie et al., 1999). The P-gp blockers quinidine (Kharasch et al., 2003) and itraconazole (Heiskanen et al., 2008) did not, however, appear to have a significant effect on morphine brain disposition, but probably by inhibiting P-gp in the periphery they increased morphine plasma concentrations. Other

relevant drug transporters at the BBB are the probenecid-sensitive organic anion transporter, which facilitates transport of M3G (Xie et al., 2000), and the organic cation transporter 2, which facilitates transport of morphine (Mashayekhi et al., 2010).

#### 2.4.5 Oxycodone

Oxycodone is a semi-synthetic MOR agonist that is increasingly used in clinical medicine (Lemberg et al., 2006). The pharmacodynamic mechanism of oxycodone is covered under Section 2.3.2 'Opioid-induced analgesia'. Oxycodone is metabolized in the liver mainly to noroxycodone, oxymorphone, and noroxymorphone (Fig. 6). The CYP3A *N*-demethylates oxycodone to noroxycodone and oxymorphone to noroxymorphone, whereas CYP 2D6 *O*-demethylates oxycodone to oxymorphone, but also noroxycodone and oxymorphone to noroxymorphone (Lalovic et al., 2006; 2004; Samer et al., 2010). The role of the metabolites in oxycodone-induced analgesia is considered negligible (Lemberg et al., 2009), although intrathecal administration of noroxymorphone has shown potent analgetic efficacy in the rat (Lemberg et al., 2008), and oxymorphone itself is used as an opioid analgesic (Babalonis et al., 2016). Oxycodone has a bioavailability of 60% (Pöyhia et al., 1992), and of the total orally administered dose of oxycodone, 9% is excreted in urine, 22% as noroxycodone, 11% as oxymorphone, and 14% as noroxymorphone (all presented as sum of free and conjugated proportions) (Lalovic et al., 2006).

The role of transporter molecules in the oxycodone disposition across the blood-brain barrier is incompletely understood. Boström et al showed using microdialysis that oxycodone concentrations in the brain were disproportionately high relative to blood, indicating involvement of active oxycodone transport in the rat BBB (Boström, 2006; Boström et al., 2008). Interestingly, Okura et al. have suggested that oxycodone intake in the CNS is mediated via an organic cation transporter responsible for pyrilamine

transport in vitro and in situ in the rat (Okura et al., 2008), which could explain the results of Boström et al. Whether oxycodone is a substrate to P-gp is unclear. Co-administration of P-gp substrate PSC833 failed to alter oxycodone brain concentrations in the rat, suggesting that P-gp might not effectively participate in oxycodone efflux from the CNS (Bostrom et al., 2005). On the other hand, the investigation conducted in P-gp knock-out mice showed that presence of P-gp reduced concentrations of oxycodone in the brain (Hassan et al., 2007). Of the active oxycodone metabolites, oxymorphone has been shown to be actively transported to the CNS, but still the concentrations are probably too low to contribute to analgesia (Sadiq et al., 2013). Noroxymorphone is likely unable to cross the BBB due to its low lipophilicity (Lemberg et al., 2008).

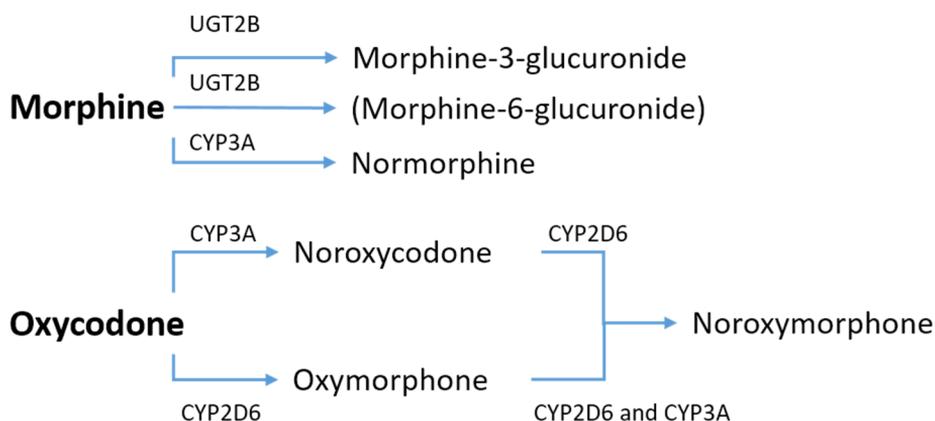


Figure 6. Schematic presentation of metabolism of morphine and oxycodone. Rat produces no morphine-6-glucuronide. Uridine 5'-diphospho-glucuronosyltransferase (UGT), Cytochrome P450 (CYP). (Coffman et al., 1997; Hasselström and Säwe, 1993; Kuo et al., 1991; Lalovic et al., 2004; Projean et al., 2003).

### 2.4.6 Loperamide

Loperamide is a synthetic opioid used to decrease gastrointestinal motility. Peripherally administered loperamide does not distribute to the CNS, thus demonstrating no central effects, but intrathecal (Kumar et al., 2012) administration produces MOR-dependent antinociception. The restriction of loperamide to the periphery is considered to be mediated via strong P-gp efflux at the BBB (Wandel et al., 2002). In accordance, the P-gp inhibitor quinidine has increased loperamide brain concentration in the rat (Dagenais et al., 2004), and in P-gp knock-out mice the radioactivity of the brain tissue after administration of radio-labeled loperamide increased 12.5-fold compared with wild-type mice (Schinkel et al., 1995).

### 2.4.7 The molecular structure of morphine, oxycodone, and loperamide

The molecular structures of the opioids used in the present study (morphine, oxycodone and loperamide) are presented in Fig 7. The alkaloids morphine, oxycodone, and loperamide have molecular weight of 285, 315, 477 grams/mole and molecular formula of  $C_{17}H_{19}NO_3$ ,  $C_{18}H_{21}NO_4$ , and  $C_{29}H_{33}ClN_2O_2$ , respectively.

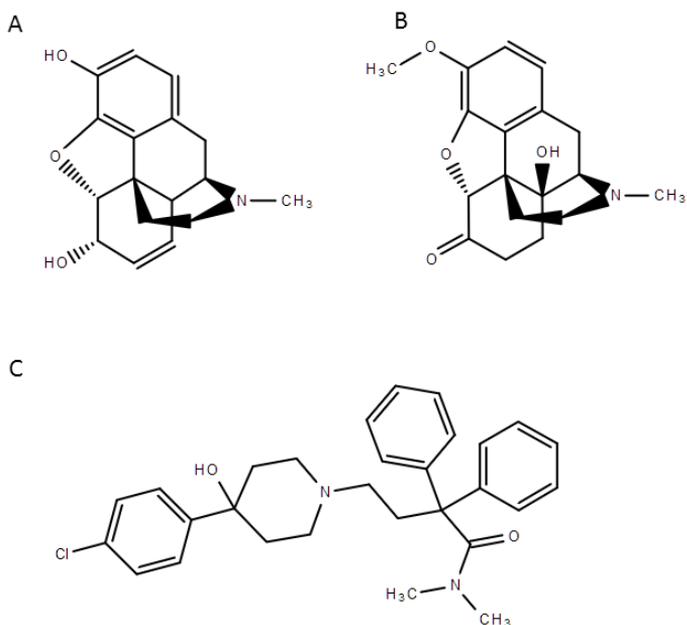


Figure 7. Molecular structure of morphine (A), oxycodone (B), and loperamide (C).

#### 2.4.8 Opioid tolerance and opioid-induced hyperalgesia

Opioid tolerance refers to acquired tolerance, which can be defined as loss of opioid efficacy following exposure to the drug. Tolerance has behavioral, pharmacokinetic, and pharmacodynamic dimensions (Collett, 1998). Behavioral tolerance refers to the individual being adapted to functioning under the influence of a substance (Collett, 1998). The relevance of the pharmacokinetic dimension of tolerance is considered somewhat unclear (Dumas et al., 2008): it seems that opioids do not markedly auto-induce biotransformation enzymes of phase I (Fredheim et al., 2012) or II (Rane et al., 1983), however, Oxycodone (Hassan et al., 2007) and morphine (Aquilante et al., 2000) have been shown to induce the expression of the drug transporter P-gp, which could

alter the distribution of the P-gp substrate opioids in the brain, and in theory, contribute to opioid tolerance.

Despite extensive research, the pharmacodynamic tolerance remains poorly understood. Studies indicate that the phenomenon encompasses multiple mechanisms, which seem to be differentially activated in response to different MOR agonists (Allouche et al., 2014). Numerous studies have demonstrated that binding of MOR agonist induces phosphorylation of MOR by many protein kinases, including G protein-coupled receptor kinases (GRKs), protein kinase C (PKC) and A (PKA), Ca<sup>2+</sup>/camodulin-dependent protein kinase II (CaMKII), and mitogen-activated protein kinases (MAPKs) (Liu and Anand, 2001). Notably, the degree of the receptor phosphorylation has been shown to correspond to the opioid ligand efficacy (Yu et al., 1997). The phosphorylation of MOR is followed by binding of  $\beta$ -arrestines, which causes a steric block to the involved G-protein, disturbing the signaling to the effector molecule (Williams et al., 2012). Phosphorylation of MOR by itself is not sufficient to disturb the signaling to the inwardly rectifying potassium channels and to desensitize the receptor, but requires the binding of arrestines (Kovoor et al., 1997). The desensitized MOR is often internalized, dephosphorylated, and recycled resensitized to the cell membrane (Williams et al., 2012). It is also possible that the internalized MOR is alternatively routed to degradation in lysosomes (Williams et al., 2012). The downregulation of the opioid receptors is, however, not considered necessary for the development of tolerance, as the expression of the opioid receptors has been shown to both upregulate and downregulate in chronic opioid treatment (Allouche et al., 2014). Particularly, the downregulation of MOR is not considered relevant in morphine-induced tolerance, as morphine differs from many other opioid agonists in that it is not considered to undergo MOR internalization (Dumas and Pollack, 2008; Williams et al., 2012). Morphine, however, also induces MOR phosphorylation (Mann et al., 2014), and deletion of  $\beta$ -arrestin has led into decreased development of morphine tolerance (Bohn et al., 2000). Failure of internalization of

MOR after morphine treatment might explain the reduced MOR recovery from desensitization after chronic morphine treatment, thus contributing to morphine tolerance (Dang and Williams, 2004). Other theories of opioid tolerance include glia activation, are covered in Section 2.4.9 'Role of glia in opioid analgesia'.

Opioid-induced hyperalgesia (OIH) refers to a phenomenon where administration of opioids provokes rather than alleviates pain. Although OIH is clinically acknowledged (Tompkins and Campbell, 2011), no publications asserting its epidemiology exist, probably because it is difficult to discern OIH from the development of opioid tolerance and progression of the underlying disease in patients (Lee et al., 2011).

Indeed, in addition to their analgesic effects, opioids can cause sensitization to pain. The *Opponent Process Theory* can be used to conceptualize how these separate counteracting processes determine the opioid response (Koppert, 2007). The analgesic effect of opioid arises if the opioid-induced antinociceptive processes surpass the concomitant opioid-induced pronociceptive processes. The recruitment of the pronociceptive processes reduces the analgesic efficacy of opioids and if the relative strength of these processes overcomes the antinociceptive processes, analgesia reverses to pain (Koppert, 2007.). It is therefore possible that the diminished antinociceptive effect of an opioid in developed tolerance is a sum of two components: desensitization to the antinociceptive effect of the opioid and enhanced recruitment of opioid-induced pronociceptive processes (Koppert, 2007).

Opioid-induced tolerance and pronociception apparently share common mechanisms, of which the activation of the excitatory glutaminergic system in the synapse between the first and second order afferent seems the most prominent (Lee et al., 2011). Antagonism of glutamate-sensitive NMDA receptors has attenuated both OIH and tolerance (Mao et al., 1994; Mert et al., 2009), and morphine-induced downregulation

of synaptic glutamate uptake transporters EAAC1 and GLAST has been demonstrated to have a correlation with the development of morphine tolerance and thermal hyperalgesia in the rat (Mao et al., 2002). In accordance, amitriptyline has reversed morphine tolerance while increasing the expression of glutamine uptake transporters GLAST and GLT-1 and decreasing the concentrations of glutamate in the CFS (Tai et al., 2007). In both the studies of Mao et al. and Tai et al., the occurrence of hyperalgesia and/or tolerance was prevented by protein kinase C inhibitor, indicating its pivotal role in the regulation of the glutamatergic system in response to opioids. The increased dorsal horn activity after opioid treatment might relate also to direct increase of glutamate release from the subpopulation of TRPV1-expressing spinal neurons and associated strengthening of the synapse via long-term potentiation (H.-Y. Zhou et al., 2010). Increase of glutamate release from MOR-containing neurons in opioid tolerance has also been detected in the nucleus raphe magnus, possibly facilitating spinal nociception (Bie and Pan, 2005). The emerging role of glia in synaptic modulation in opioid-induced tolerance and pronociception is discussed in the next section.

Another possible mechanism for opioid tolerance and pronociception is increased production of dynorphins. The role of dynorphins in the development of chronic pain has been established by many investigations, for review, see Podvin et al. (2016). Indeed, dynorphin antiserum injection blocked MOR agonist DAMGO-induced tactile allodynia and reversed thermal hyperalgesia in the rat (Vanderah et al., 2000). Other theories include the opioid-induced expression of cholecystinin octapeptide, which has been shown to reduce the antinociceptive effect of opioids (Han et al., 1993), and upregulation of the  $\beta$ 2-adrenergic signaling system (Liang et al., 2007).

#### 2.4.9 Role of glia in pain and opioid analgesia

The term 'glia', glue in Greek, reflects the passive and structural role in the CNS that glial cells (oligodendrocytes, satellite-glia, Schwann cells, microglia, and astrocytes) were first thought to play. What is now, however, well recognized is that microglia and astrocytes actively participate in the function of the nervous system by modulating neurotransmission reciprocally with the neurons and affecting the development and maintenance of synapses (Auld and Robitaille, 2003; Salter and Beggs, 2014). Specifically, the glia-neuron interfaces may also be involved in the chronic pain development, as many studies have revealed that glia-derived neuromodulators can affect the function of the spinal neurons related to nociceptive signaling, for review, see Grace et al. (2014). Interestingly, also opioids may facilitate many of the same pronociceptive glia-to-neuron mechanisms, and they can oppose opioid-induced analgesia, thus contributing to opioid tolerance and OIH (Fig. 8) (Watkins et al., 2005).

Reinforcement of nociception by glia in pathological pain is based on 1) initiators (such as ATP and chemokines) leading to 2) glia activation (via signaling routes ERK and p38, for example) , and consequently to 3) secretion of glia-derived soluble mediators (such as interleukins and tumour necrosis factor) (Grace et al. 2014). Notably, the described steps in pathological pain, such as neuropathic pain, seem to be similar in the pathogenesis of opioid tolerance (Watkins et al., 2005). Specifically, opioid-associated initiators include substances such as fractalkine (CX3CL1) (Johnston, 2004), sphingosine-1-phosphate (S1P) (Muscoli et al., 2010), and probably adenosine triphosphate (ATP) (Horvath et al., 2010; D. Zhou et al., 2010). Opioid-induced activation of glia has been shown to involve mitogen-activated protein kinase (MAPK) signaling routes ERK and p38 in microglia and astrocytes (Cui et al., 2006; Z. Wang et al., 2010a; 2009).

The mechanism how opioids activate glia is, in part, unclear. Microglia and astrocytes may express MOR, KOR, and DOR (Chao et al., 1997; Horvath et al., 2010; Turchan-Cholewo et al., 2008), and migration of microglial cells has been shown to depend on  $\mu$ -opioid receptor activation in vitro (Horvath et al., 2010). On the other hand, recent studies indicate that the  $\mu$ -opioid receptor is not expressed in the microglia or astrocytes in the spinal dorsal horn (Corder et al., 2017; Kao et al., 2012). The other possible receptor that mediates glia activation is Toll-like receptor 4 (TLR4), which is expressed on microglia, but is inducible by inflammation also in astrocytes (Grace et al., 2015). Many opioids, including M3G, the metabolite of morphine, have been shown to possess TLR-4 signaling activity (Hutchinson et al., 2010).

Blockade or removal of TLR-4 has enhanced both morphine and oxycodone analgesia and diminished morphine tolerance (Eidson and Murphy, 2013; Hutchinson et al., 2012). Similarly with these, compared with the TLR-4 knock-out mice, only the wild-type exposed to M3G exhibited hyperalgesia (Due et al., 2012). The role of TLR-4 in pronociception and tolerance has also been argued, because its removal from the genome in mice failed to enhance opioid antinociception and attenuation of tolerance (Mattioli et al., 2014). In addition to direct mechanisms suggested, the opioid-induced activation of glia can arise via neuron-derived substances. Chronic morphine treatment promotes the expression of substance P and CGRP (Powell et al., 2000), both of which heighten glial cell activation (Reddington et al., 1995; Zhu et al., 2014).

Fractalkine is a product of neurons and astrocytes (Lindia et al., 2005), but its receptor is expressed in microglia (Verge et al., 2004). Suppression of fractalkine receptor has enhanced acute morphine analgesia and attenuated the development of tolerance and hyperalgesia (Johnston, 2004). Also of interest is that the effects of fractalkine may be, at least in part, IL-1-mediated, because fractalkine induces IL-1 release in the spinal cord, and co-administration of IL-1 receptor antagonist with morphine reverses hyperalgesia and prevents tolerance development (Johnston, 2004). Injection of fractalkine into

periaqueductal gray has diminished the acute antinociceptive effect of opioid agonists (Chen et al., 2007). Sphingosine-1-phosphate is a product of ceramide metabolism. Microglia express both the S1P receptors and the enzyme sphingosine kinase1, which catalyses S1P upregulation (Nayak et al., 2010; Tham et al., 2003). Morphine has been demonstrated to upregulate S1P in the spinal cord, and inhibition of ceramide and S1P blocks development of tolerance and OIH while preventing the production of the cytokines TNF- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6 (Muscoli et al., 2010). Moreover, administering S1P to activated microglia in vitro has elevated TNF- $\alpha$  and NO production (Nayak et al., 2010). The effects of S1P probably are not, however, glia-exclusive as S1P has been shown also to affect neuronal cAMP synthesis in the dorsal horn (Coste et al., 2008). Moreover, purinergic transmission (including ATP) may be vital because inhibition of its receptors P2X4 (Horvath et al., 2010) and P2X7 (Zhou et al., 2010) in microglia has attenuated morphine tolerance.

The soluble mediators augment excitatory synaptic transmission, primarily by enhancing glutamate release or suppression of inhibitory synaptic transmission or both (Grace et al., 2014). The precise mechanisms include IL-1 $\beta$ -evoked glutamate release from the primary afferents via activation of functionally coupled presynaptic IL-1 $\beta$  and NMDA receptors (Yan and Weng, 2013), TNF $\alpha$ -induced release of glutamate from the primary afferents (Kawasaki et al., 2008), and TNF $\alpha$ -induced disinhibition of spinal synaptic transmission by reducing GABAergic neuron activity (Zhang et al., 2010). One important function of astrocytes especially is the uptake of glutamate from the synapses by GLAST and GLT-1 transporters (EAAT1 and 2 in humans) (Anderson and Swanson, 2000); their downregulation might also contribute to hyperalgesia (Weng et al., 2005).

The research regarding opioid-induced glia activation has focused mainly on the spinal cord, and multiple studies have demonstrated increased reactivity of microglial cells in the dorsal horn after morphine treatment (Cui et al., 2008.; Holdridge et al., 2007;

Mattioli et al., 2010; Wang et al., 2010b). The reactivity of supraspinal glia in chronic morphine treatment is less acknowledged, however.

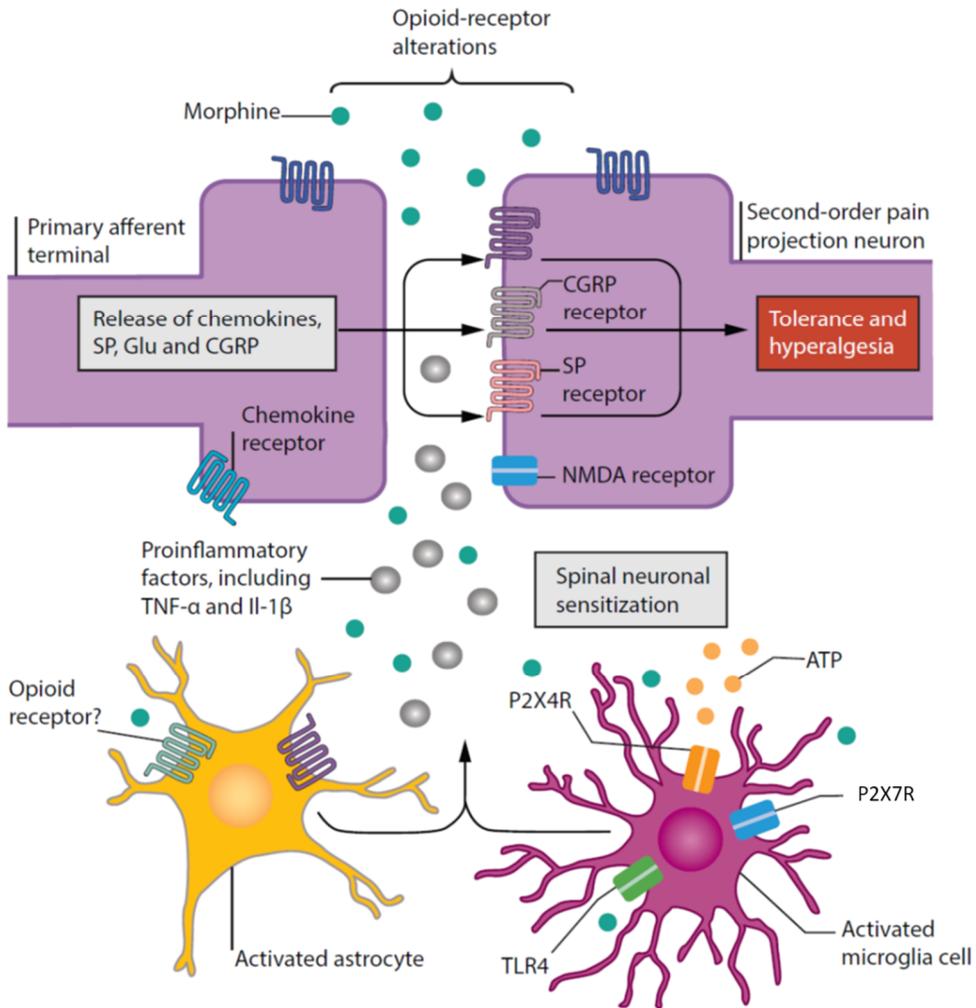


Figure 8. The role of neuroinflammation in morphine tolerance and hyperalgesia. Adapted by permission from Macmillan Publishers Ltd: [Nat. Rev. Neurosci., Opioid and chemokine receptor crosstalk: a promising target for pain therapy? Mélik et al., 2015, Year of copyright 2015]

## 2.5 Diuretics used in the present study

Diuretics are drugs that promote the production of urine used in edematous diseases, hypertension, and heart failure. The literature suggests that the diuretics studied in this thesis, however, could exert antinociceptive effects in the rat via various mechanisms, including antagonism of mineralocorticoid receptor (Dong et al., 2012) and neuronal sodium-potassium-chloride cotransporter (Granados-Soto et al., 2005). In addition, spironolactone (Chu et al., 1978), furosemide (Chu et al., 1978), and chlorothiazide (Poggioli et al., 1985) have also been suggested to potentiate the effects of the opioids via unknown mechanisms.

### 2.5.1 Spironolactone

After the discovery of aldosterone and its role in several edematous diseases, spironolactone was developed in the 1950s to antagonize the effect of aldosterone on mineralocorticoid receptors (Delyani, 2000). Spironolactone competes with aldosterone for binding to the mineralocorticoid receptor (MR) in the renal tubule, which decreases the retention of sodium and water and increases retention of potassium, but also in arterioles, where it antagonizes aldosterone-dependent vasoconstriction (Qavi et al., 2015; Roush and Sica, 2016). Clinically, spironolactone is used for such conditions as congestive heart failure and liver cirrhosis (Qavi et al., 2015).

In addition to its established indications, studies suggest that spironolactone can exert beneficial effects in experimental neuroinflammation and pain. Sun et al. (2012) showed that spironolactone attenuates pain behavior in a CCD model while inhibiting the immunoreactivity of microglia, decreasing the production of IL-1 $\beta$  and TNF- $\alpha$  and depressing the expression and phosphorylation of NMDA receptors. Supporting the hypothesis that spironolactone can diminish microglia reactivity, Tanaka et al. (1997)

demonstrated that spironolactone inhibits corticosterone-induced activation of microglia in cells isolated from the forebrain of neonatal rats. Furthermore, spironolactone has decreased the concentration of TNF- $\alpha$  in the sciatic nerve and attenuated pain behavior in chronic constriction injury, but not in the vincristine-induced neuropathic model (Jaggi and Singh, 2010). In contrast, Wang et al. (2004) failed to detect any beneficial effect of spironolactone on pain behavior in a chronic constriction injury model. In addition to its own effects, spironolactone has been shown to enhance the analgesic effect of GR agonist dexamethasone in a CCD model in rats (Gu et al., 2011).

The study by Chu et al. (1978) indicates that a significant interaction can arise between spironolactone and an opioid, as co-administration of spironolactone and morphine in morphine-tolerant rats was demonstrated to be lethal. Spironolactone inhibits UGT2B7 (Knights et al., 2010), the enzyme responsible for the metabolism of morphine in humans, and could thus decrease the production of M3G, a potent glial activator (Hutchinson et al., 2010). Evidence also indicates that spironolactone might inhibit P-gp (Nakamura et al., 2001) and could affect the disposition of morphine (Schinkel et al., 1995; Xie et al., 1999) and oxycodone (Hassan et al., 2007).

### 2.5.2 Eplerenone

Eplerenone, like spironolactone, is a competitive mineralocorticoid receptor antagonist indicated for the treatment of hypertension and congestive heart failure (Muldowney et al., 2009). Eplerenone, however, possesses greater mineralocorticoid receptor specificity, and thus lacks the androgen-receptor and progesterone-receptor related side effects of spironolactone (Muldowney et al., 2009; Struthers et al., 2008).

MR and GR are expressed in various tissues and are often co-located in the same cell. The receptors can initiate both genomic and rapid, non-genomic responses via

intracellular signaling pathways (Funder, 2012; Gomez-Sanchez and Gomez-Sanchez, 2014; Oakley and Cidlowski, 2013). Activation of MR can promote pro-inflammatory signaling that depresses the anti-inflammatory response of GR activation (Ibrahim et al., 2016). Indeed, eplerenone has been shown to reduce pain-related behavior and excitability of afferent neurons in a back-pain model in the rat (Dong et al., 2012; Ye et al., 2014). In addition, eplerenone has been shown to function in concert with glucocorticoid 6- $\alpha$  methylprednisolone reducing pain behavior in a low-back pain model (Ye et al., 2014)

Karst et al. demonstrated that MR mediated non-genomic effects of glutamate transmission in the hippocampal neurons within minutes (Karst et al., 2005). Hippocampus has been shown to affect acute pain processing in the rats (Ford et al., 2011), rendering the hippocampal MRs a possible site of action for eplerenone-induced antinociception.

### 2.5.3 Furosemide

Furosemide is a potent diuretic that inhibits the sodium-potassium-chloride symporter (NKCC) in the loop of Henle in the kidneys (Hannaert et al., 2002). Granados-Soto et al. showed that both centrally and peripherally administered furosemide along with another NKCC blocker bumetanide reduced pain behavior in a formalin-induced inflammation model in the rat (Granados-Soto et al., 2005). In accordance, bumetanide has reduced pain behavior in rats following spinal cord injury (Hasbargen et al., 2010). In the study of Chu et al. furosemide restored the effect of morphine to attenuate characteristic turning behavior in brain-lesioned morphine-tolerant rats (Chu et al., 1978).

#### 2.5.4 Chlorothiazide

Chlorothiazides are weak diuretics that inhibit reabsorption of sodium and chloride by acting on the sodium-chloride symporter in kidney tubules (Duarte and Cooper-DeHoff, 2010). A study by Poggioli suggested that chlorothiazides possess weak antinociceptive activity and that they would enhance the antinociceptive effect of morphine in the rat by an unknown mechanism (Poggioli et al., 1985). The expression of the thiazide-sensitive sodium-chloride-transporter is probably renal-specific (Gamba, 2009).

#### 2.5.5 The molecular structure of the diuretics used in the present study

The molecular structures of the diuretics used in the present study are presented in Fig. 9. Spironolactone, eplerenone, furosemide, and chlorothiazide have molecular mass of 417, 414, 330, and 296 grams/moles and molecular formula of  $C_{24}H_{32}O_4S$ ,  $C_{24}H_{30}O_6$ ,  $C_{12}H_{10}ClN_2O_5S$ , and  $C_7H_6ClN_3O_4S_2$ , respectively.

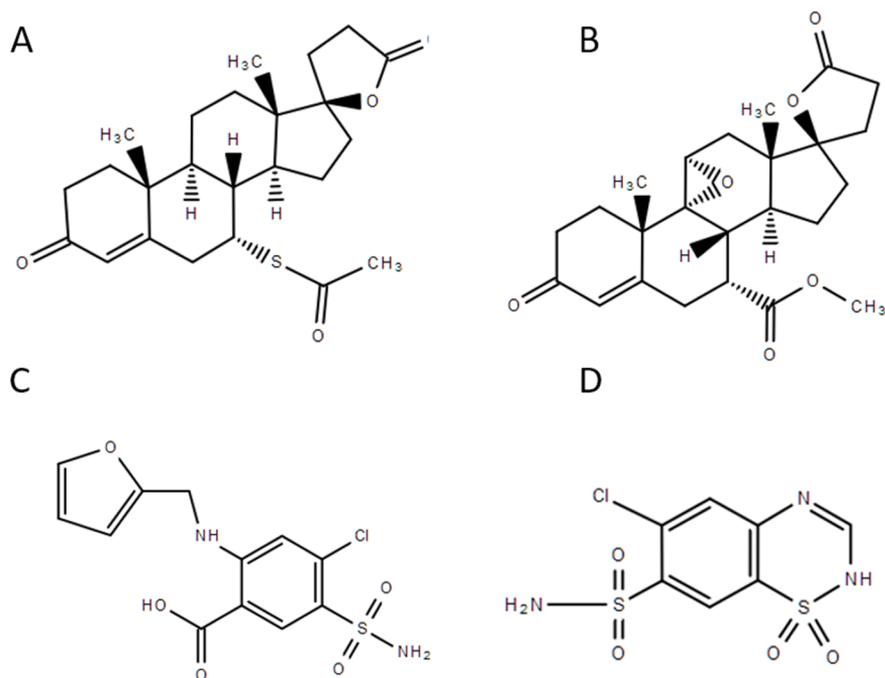


Figure 9. Molecular structure of spironolactone (A), eplerenone (B), furosemide (C), and chlorothiazide (D).

## 2.6 Anticonvulsant used in the present study

### 2.6.1 Pregabalin

Pregabalin is a synthetic molecule with an indication in the treatment of epilepsy, neuropathic pain, and generalized anxiety disorder. Pregabalin is the first-line drug with the combined norepinephrine and serotonin reuptake Inhibitors in neuropathic pain (Dworkin et al., 2010; Finnerup et al., 2015). It is a successor to gabapentin, which has a similar mechanism of action, but has saturable, and thus, unpredictable, absorption

(Bockbrader et al., 2010b). Pregabalin is considered to exert its effects via binding to the  $\alpha 2\text{-}\delta 1$  subunit of voltage-gated calcium channels (VGCC) (Field et al., 2006) (Fig. 10), which are widely distributed in the nervous system. Pregabalin also binds to subunit  $\alpha 2\text{-}\delta 2$  (Z. Li et al., 2011), which has been hypothesized to be associated with the adverse effects (McDonough, 2012). The precise role of the  $\alpha 2\text{-}\delta$  subunit in the VGCC has not yet been elucidated, although the evidence suggests that it regulates the expression of the VGCC in the plasma membrane (Dolphin, 2016). Inhibition of the VGCC depresses presynaptic calcium influx and reduces the release of neuromediators, including glutamate, substance P, and CGRP (Fehrenbacher et al., 2003; Fink et al., 2002). Other suggested mechanisms of action for pregabalin include decrease of synaptic transmission by modulation of  $K_{ATP}$  channels and increasing trafficking of glutamate transporter EAAT3 to post-synaptic cell membranes (Verma et al., 2014). Pregabalin is not active at opioid receptors (Pfizer, n.d.a), and although it is a GABA -analog in its chemical structure, it is inactive at GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Taylor et al., 2007).

As an accompanying drug, gabapentinoids have enhanced the antinociceptive effects of opioids in acute nociceptive thermal tests (Meymandi et al., 2006; Shimoyama et al., 1997) and in neuropathic pain (Christoph et al., 2011) in rats. In addition, gabapentinoids have reduced the consumption of opioids in humans in experimental nociceptive pain in healthy volunteers (Eckhardt et al., 2000) and in postoperative pain (Clarke et al., 2009; Tiippana et al., 2007; Yücel et al., 2011.).

In clinical use, pregabalin is generally well-tolerated. Its use is not associated with serious adverse effects, but individuals starting pregabalin can experience transient adverse effects related to coordination and cognition in a dose-response dependent manner (Gajraj, 2007; Zaccara et al., 2011). Pregabalin can, however, cause euphoria, which has raised concern about its misuse and abuse (Schifano, 2014). A history of opioid abuse is a particular risk factor for pregabalin abuse (Evoy et al., 2017). A recent study showed



## 2.6.2. Molecular structure of pregabalin

The molecular structure of pregabalin is presented in Fig. 11. Pregabalin has molecular mass of 159 grams/moles and molecular formula of  $C_8H_{17}NO_2$ .

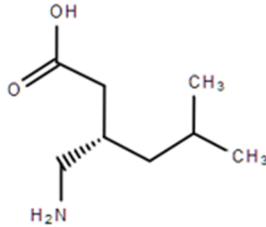


Figure 11. Molecular structure of pregabalin.

Table 2. Pharmacokinetic parameters of the studied diuretics, loperamide, and pregabalin. <sup>(1)</sup>(Brunton et al., 2011.) <sup>(2)</sup>(Welling, 1986) <sup>(3)</sup>(Maron and Leopold, 2008) <sup>(4)</sup>(Baker, 2007) <sup>(5)</sup>(Prandota and Witkowska, 1976) <sup>(6)</sup>(Pichette and Souich, 1996) <sup>(7)</sup>(Sica, 2005) <sup>(8)</sup>(Cook et al., 2002).

Drug	Target protein	Bound in plasma (%)	Bioavailability (%)	Urinary excretion (%)	Metabolism
<b>Spirolactone</b>	Mineralocorticoid-, androgen-, and progesteron receptor	>90 <sup>(1)</sup>	80-90 <sup>(7)</sup>	<1 (p.o. dose) <sup>(1)</sup>	Extensive metabolism: deacetylation, dethiolation, and thiomethylation <sup>(1,3)</sup>
<b>Eplerenone</b>	Mineralocorticoid receptor	33-60 <sup>(1)</sup>	67 <sup>(3)</sup>	7 (p.o. dose) <sup>(1)</sup>	Extensive metabolism: 6beta- and/or 21-hydroxylation and 3-keto reduction. Enzyme involved CYP3A4. <sup>(3,7,8)</sup>
<b>Furosemide</b>	Na-K-2Cl co-transporter (NKCC2)	99 <sup>(1)</sup>	71 <sup>(1)</sup>	30-50 (p.o. dose) <sup>(5)</sup>	Poorly understood, metabolism probably at kidney <sup>(6)</sup>
<b>Chlorothiazide</b>	Na(+)-Cl(-) cotransporter	70 <sup>(2)</sup>	15-30 <sup>(1)</sup>	almost entirely <sup>(2)</sup>	-
<b>Loperamide</b>	Opioid receptors	97 <sup>(4)</sup>	0.3 <sup>(4)</sup>	2-10 <sup>(4)</sup>	Extensive metabolism: enzymes involved CYP2C8 and CYP3A4 <sup>(4)</sup>
<b>Pregabalin</b>	$\alpha$ 2- $\delta$ subunit of voltage-gated calcium channels	0 <sup>(1)</sup>	>90 <sup>(1)</sup>	almost entirely <sup>(1)</sup>	-

### 3. Aims of the study

Drugs that modulate opioid-induced analgesia can be beneficial adjuvants in the treatment of pain. On the other hand, morphine modulates its own analgesic effect by tolerance and opioid-induced hyperalgesia, which can complicate opioid treatment. Therefore, we aimed to study drugs that could potentiate the effects of the opioids and prevent/reverse opioid tolerance, and also to assess the role of neuroinflammation in chronic morphine treatment using the rat as a model organism. Specific aims in this thesis were as follows:

- I. To clarify potential pharmacological interaction between the diuretics spironolactone, eplerenone, furosemide, and chlorothiazide and the opioids oxycodone and morphine in thermal tests of acute nociception.
- II. To characterize effects of the anticonvulsant pregabalin on the antinociceptive and sedative effects of oxycodone and morphine in thermal tests of acute nociception and coordination test and also to exclude pharmacokinetic interactions between the drugs in the brain using concentration measurements.
- III. To assess glial reactivity in the spinal and supraspinal structures in morphine tolerance using immunohistochemistry, flow cytometry, and transcriptomics.

## 4. Materials and methods

### 4.1 Materials

#### 4.1.1 Animals

Male Sprague–Dawley rats (Scanbur, Sollentuna, Sweden, Harlan, Horst, Netherlands) were used. The rats weighed 200-300 g at the beginning of the experiments. They were accommodated in groups of four in transparent cages in light- and temperature-controlled rooms. Water and standard laboratory chow were available ad libitum. The rats were habituated to the testing conditions for 3 days before the experiments. The animals were euthanized by decapitation or intracardiac perfusion in deep anesthesia. The National Animal Experiment Board of Finland approved the study protocols.

#### 4.1.2 Drugs

Morphine hydrochloride, oxycodone hydrochloride trihydrate solution (Oxanest<sup>®</sup>, Leiras Takeda, Helsinki, Finland), furosemide (Furesis<sup>®</sup>, Orion Pharma, Espoo, Finland), eplerenone (Inspra<sup>®</sup>, Pfizer, New York City, NY, USA) and pregabalin capsules (Lyrica<sup>®</sup>, Pfizer, New York City, NY, USA) were purchased from the University Pharmacy (Helsinki, Finland). Spironolactone, loperamide, and chlorothiazide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Morphine hydrochloride was dissolved and oxycodone hydrochloride trihydrate and furosemide diluted in physiological saline. Spironolactone and chlorothiazide were diluted with 4% polysorbate 80 (Tween<sup>®</sup> 80, University Pharmacy, Helsinki, Finland) and loperamide with 2% polysorbate 80 in physiological saline. Pregabalin was dissolved in

0.5% methylcellulose (University Pharmacy, Helsinki, Finland) in physiological saline. Eplerenone tablets were mechanically pulverized and dissolved in 4% v/v Tween® 80 in physiological saline. The injection volumes and routes of administration were 2 mL/kg subcutaneously and 5 mL/kg intraperitoneally.

## 4.2 Methods

### 4.2.1 Model of postoperative pain

The rats were deeply anesthetized using isoflurane and a short longitudinal incision with a sharp scalpel was made through the skin, fascia, and muscle of the plantar side of the hind paw, as previously described by Brennan et al. (Brennan et al., 1996). The inflicted wound was sutured with two absorbable knob sutures, and the animal was allowed to recover one day before the behavioral experiments. The wound on the paw was carefully observed during the study for signs of infection, which would have been an exclusion criterion.

### 4.2.2 Measurement of acute nociception

*Tail-flick latencies* (Studies I-IV) were assessed using a tail-flick apparatus (Ugo Basile 37360, Comerio, Italy). In the test, the rats were restrained in plastic tubes. At each time point, three different sites of the middle third of the tail were exposed to radiant light. A flick of the tail automatically stopped the timer of the apparatus and the mean of the results was calculated. The cut-off was set at 10 s to avoid tissue damage. When an individual measurement reached the cut-off, no further tests were performed for that particular time point on that animal.

*Hot plate tests* (Studies I-III) were performed with a Harvard Apparatus Ltd. hot plate device (Edenbridge, Kent, UK). In the test, the rat was put inside a transparent circular cage on the hot plate ( $52 \pm 0.2^\circ\text{C}$ ). Licking or stomping the hind paw was considered as a sign of thermal nociception. Latency to the first sign was measured, after which the animal was instantly removed from the hot plate. To avoid tissue damage, the cut-off was set at 60 s.

*Mechanical thresholds* were assessed using either the Ugo Basile 37450 Dynamic Plantar Aesthesiometer (DPA) (Comerio, Italy) (Study IV) or the Imada DPS-1 digital force gauge (Northbrook, IL, USA) (Study II). When using DPA, the rats were placed on a metal mesh in plastic cages and habituated for 10 min before testing. A dull metal monofilament with a diameter of 0.5 mm was applied perpendicularly to the middle of the hind paw. The pressure was gradually increased until a rapid withdrawal or shaking of the paw occurred. The filament withdrew rapidly after the reaction and the apparatus stored the threshold of the force needed to cause the motor reaction. A 40-g cut-off was used. When using the force gauge in the model of postoperative pain, the rats were placed on a metal mesh covered with a transparent cage to settle down for 2 min. Skin 2 mm proximal to the wound was gently touched with a metallic monofilament with a diameter of 0.3 mm when the animal was sitting on its four limbs. The force of the filament against the skin was steadily increased until the nociceptive behavior, either rapid withdrawal or brisk shaking, occurred. A cut-off of 50 g was used to allow the animal to withdraw the paw from the filament effortlessly.

*Cold thresholds* (Study IV) were measured with the Bioseb T2CT Cold/Hot plate apparatus (Vitrolles, France) as adapted from Allchorne et al (Allchorne et al., 2005). The temperature of the plate was set to  $0^\circ\text{C}$ , which was kept constant during the measurements. The rat was set free onto the apparatus plate in a transparent cage. The

latency to the first stomp or lift of the hind paw was recorded. Lift of the paw that was involved in coordinated movement of all four limbs was excluded. A cut-off of 180 s was used.

#### 4.2.3 Assessment of motor functions

The rotarod test was performed using a rotarod apparatus (Ugo Basile 47700, Comerio, Italy) (Study III). The test measures motor performance. The time that the rat stayed on a rubber-coated rod that was rotating at a fixed speed of 20 rpm was measured. The cut-off limit was set at 30 s.

#### 4.2.4 Determination of drug concentration

The non-perfused whole-brain and liver samples were snap-frozen in liquid nitrogen. The arterial blood samples gathered in decapitation were allowed to coagulate at room temperature for 60 min and centrifuged at 4900 g for 20 min at 23°C. (Studies I-III). The determinations of morphine (Studies I-III), M3G (Studies I-III), M6G (Study I), normorphine (Study I), oxycodone (Studies II and III), noroxycodone (Studies II and III), oxymorphone (Studies II and III), and pregabalin (Study III) were performed using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with API 3000 tandem mass spectrometry (AB Sciex, Toronto, ON, Canada) that operated in a positive turbo ion spray mode. Before the analysis, spiked calibration standards and quality control samples were prepared in rat brain tissue homogenates for all the treatment compounds at appropriate concentrations that ranged from 1.0 to 2250 ng/mL (Study II). The LC-MS/MS analyses of morphine, M3G, and pregabalin were carried out separately as previously described (Dominguez-Ramirez et al., 2006; Oertel et al., 2009) with some modifications.

The chromatographic separations were achieved on an Atlantis HILIC Silica column (3- $\mu\text{m}$  particle size, 100 mm  $\times$  2.1 mm I.D., Waters, Milford, MA, USA) using a gradient elution of mobile phase consisting of acetonitrile and 10 mmol/L ammonium formate in 0.2% formic acid (v/v). Oxycodone served as an internal standard for morphine and M3G, and the limit of quantification was 1.0 ng/mL for both analytes (Studies I-III). Gabapentin was used as an internal standard for pregabalin analysis (Study II). The determination of oxycodone and its metabolites was performed as previously described (Neuvonen and Neuvonen, 2008). The limit of quantification for oxycodone, noroxycodone, and oxymorphone was 1.0 ng/mL (study III). The detection limit for oxycodone and its metabolites was 5 ng/g in brain tissue (Study II). The following ion transitions were monitored (Study I): 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; oxycodone, m/z 316 to m/z 241, and the limit of quantification for morphine and M3G was 1.0 ng/mL. A signal-to-noise ratio 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 the normorphine to that of the internal standard (Study I). The detection limit of oxycodone and its metabolites was 5 ng/g in brain tissue (Study II). The day-to-day coefficients of variations for all the methods described were below 15% for the concentrations of all analytes. Original protocol kindly provided by M.Sc. Jouko Laitila.

#### 4.2.5 Immunohistochemical staining

Animals were deeply anesthetized using isoflurane and perfused intracardially with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) (Study IV). Collected tissues were fixed with 4% PFA overnight and embedded in paraffin (Study IV). Sections of 10  $\mu\text{m}$  were prepared from the selected regions of the brain and lumbar regions of the spinal cords. Sections were probed with antibodies for Iba1 (1:1000, Cat N 019-

19741, Wako, Richmond, VA, USA), GFAP (1:400, Cat G-3893, Sigma-Aldrich, St. Louis, MO, USA), CGRP (1:10,000, Cat. No. T-4032, Peninsula Laboratories, San Carlos, CA, USA), or substance P (1:10,000, Cat. No. T-4107, Peninsula Laboratories). Bound antibodies were visualized using anti-rabbit and anti-mouse biotinylated secondary antibodies and the VECTASTAIN ABC HRP Kit (Cat PK-6101, PK-4002 Vector Laboratories, Burlingame, CA, USA) as described by the kit manufacturer. Slides were imaged using the 3DHISTECH Scanner (3DHISTECH Ltd, Budapest, Hungary). Scanned images were analyzed with Matlab R2014b software (Mathworks, Natick, MA, USA). The number of Iba1 or GFAP-positive cells was quantified using manually set size and intensity thresholds as described by Penttinen et al (Penttinen et al., 2016). Original protocol kindly provided by Dr. Yulia Sidorova.

#### 4.2.6. Flow cytometry

Animals were deeply anesthetized using isoflurane and perfused intracardially with 100 ml of phosphate-buffered saline (PBS). The L4-L6 segments of the spinal cord, medulla, and primary somatosensory cortex were dissected. Dissected tissues were weighed, cut into tiny pieces, and gently homogenized through 70- $\mu$ m cell strainers (Fisher Scientific, Waltham, MA, USA) in PBS/1% FBS. Single-cell suspensions prepared from ~25 mg of tissue per sample were blocked with 5% normal rat serum, and stained with a co-use of anti-rat markers, including Granulocyte-FITC (clone HIS48, eBioscience, San Diego, CA, USA), CD172a-PE (clone OX41, BioLegend, San Diego, CA, USA), MHCII-PerCP-eFluor 710 (clone OX17, eBioscience), and CD11b/c-eFluor 660 (clone OX42, eBioscience) with light protection at 4°C for a 60-min continuous rotation. The cells were washed and resuspended in 2 mL of PBS/1% FBS/0.02% NaN<sub>3</sub> buffer, and acquired on a 2-laser, 6-color Gallios cytometer (Beckman Coulter, Brea, CA, USA) under a live gate of CD11b/c<sup>+</sup>. Kaluza flow analysis 1.3 software (Beckman Coulter) was used to analyze the flow

cytometry data. Microglia were defined as CD11b/c<sup>+</sup>granulocyte<sup>-</sup> total, MHCII<sup>+</sup>, and CD172a<sup>+</sup> subtypes. Original protocol kindly provided by Dr. Zhilin Li.

#### 4.2.7 Microglia RNA isolation (CD11b-positive cells)

Rats were perfused intracardially with 200 mL of 0.9% saline solution, and a L1-L6 spinal lumbar segment was collected for the isolation of CD11b-immunopositive microglia/macrophages by magnetic-activated cell sorting (MACS). The collected tissue was dissociated using a Neural tissue dissociation kit (T) (Miltenyi Biotec, San Diego, CA, USA; cat# 130-093-231) and the gentleMACS Dissociator (Miltenyi Biotec). After the dissociation process, the cells were suspended in 0.5% bovine serum albumin (BSA) in PBS and incubated with Myelin Removal Beads II (Miltenyi Biotec; cat# 130-096-733; 1:10 dilution) for 15 min at 4°C. After the incubation, the cells were washed once and resuspended in 0.5% BSA in PBS and filtered through an LS column (Miltenyi Biotec; cat# 130-042-401) using a QuadroMACS Separator (Miltenyi Biotec). The total effluent was gathered and resuspended in 0.5% BSA with 2 mM EDTA in PBS. The cells were incubated with mouse anti-CD11b:FITC antibody (AbD Serotec, Puchheim, Germany; Cat# MCA275FB; 1:10 dilution) at 4°C for 10 min. The cells were then washed, resuspended in 0.5% BSA with 2 mM EDTA in PBS, and incubated with anti-FITC MicroBeads (Miltenyi Biotec; cat# 130-048-701; 1:10 dilution) for 15 min at 4°C. The cells were washed and resuspended in 0.5% BSA with 2 mM EDTA in PBS. The cell suspension was placed on an LS column, put on a QuadroMACS Separator, and the anti-CD11b-labeled cells were collected from the column. RNA was extracted with Trizol reagent and alcohol-precipitated in the presence of glycogen and treated with DNase (#1906, Ambion). Original protocol kindly provided by M.Sc. Jenni Anttila, Dr. Kert Mätlik, and Dr. Mikko Airavaara.

#### 4.2.8 RNA sequencing

Altogether 30 ng of total RNA was first subjected to rRNA and mitochondrial rRNA removal using a RiboGone Mammalian kit (Clontech, Mountain View, CA, USA) and then used as a starting material for RNA-Seq. Indexed cDNA libraries from 200 pg of the ribosomal/mitochondrial RNA-depleted RNA were generated using a SMARTer® Stranded RNA-Seq Kit (Clontech). Strand-specific sequencing data from the synthesized cDNA was obtained from the Illumina next-generation sequencing system. NextSeq High SE 1x75 bp run was utilized.

Initial quality control on sequenced reads was performed with FastQC v0.11.3. Based on the quality reports, Illumina adapters were removed by Trimmomatic v0.33 and the read files were preprocessed as follows: reads with the first 5 bases were trimmed from the start of the read. Base quality scores less than 30 were trimmed from the 5' end. Reads of length less than 20 bases were excluded. When samples had less than 70% of reads surviving trimming, they were discarded from the analysis.

The processed reads were mapped (reference genome Rat Ensembl5) with Tophat v2.1.0. Up to 2 mismatches were allowed (both in the initial read and the segmented read alignment), and enforcing rules for first-strand synthesis library type were used. The mapped reads were then quantified in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) with Cufflinks v2.2.1. Multiple read correction and upper-quartile normalization were used. Differential expression analysis was executed on a gene level using CuffDiff v2.2.1 with multiple read corrections. Original protocols kindly provided by Functional Genomics Units, Biomedicum Helsinki and Dr. Katherine Icaj.

## 4.2.9 Experimental design

The design of the experimental settings of the individual studies is described in brief in Table 3 and in detail in the original publications.

Table 3. Experimental design.

Research question	Experimental setting	Method
<b>Study I</b>		
Acute effect of spironolactone on morphine-induced antinociception and concomitant concentrations of morphine and its metabolites in brain, serum, and liver	Administration of one or two injections of spironolactone (50 mg/kg, i.p.) with morphine (4 mg/kg, s.c.)	Hot plate, Tail-flick and Liquid chromatography–mass spectrometry
Effect of chronic spironolactone on morphine-induced antinociception in morphine-naïve rats and effect of acute spironolactone on morphine-induced antinociception in morphine-tolerant rats. Effects of spironolactone on development of morphine tolerance	Combined treatment of morphine and spironolactone	Hot plate and Tail-flick
Acute effect of spironolactone on the antinociceptive effect of loperamide	Administration of loperamide (10 mg/kg, s.c.) with two injections of spironolactone (50 mg/kg, i.p.)	Hot plate and Tail-flick
<b>Study II</b>		
Acute antinociceptive effect of spironolactone, eplerenone, furosemide, and chlorothiazide individually and with oxycodone and morphine	Administration of spironolactone (100 mg/kg, i.p.), eplerenone (100 mg/kg, i.p.), furosemide (100 mg/kg, i.p.), and chlorothiazide (50 mg/kg, s.c.) with and without oxycodone (0.75 mg/kg, s.c.) or morphine (3 mg/kg, s.c.)	Hot plate, Tail-flick
Concentrations of oxycodone, morphine, and their metabolites when spironolactone is present	Administration of spironolactone (100 mg/kg, i.p.) or eplerenone (100 mg/kg i.p.) one or two days after a rat model of postoperative pain	Hot plate, Tail-flick, Liquid chromatography–mass spectrometry
Antinociceptive effect of spironolactone or eplerenone on postoperative pain	Administration of spironolactone (100 mg/kg, i.p.) or eplerenone (100 mg/kg i.p.) one or two days after a rat model of postoperative pain	Mechanical gauge test
<b>Study III</b>		
Effect of pregabalin on oxycodone- and morphine-induced antinociception and sedation	Administration of pregabalin (50 mg/kg, i.p.) with oxycodone (0.6 mg/kg, s.c.) and morphine (2.5 mg/kg, s.c.)	Hot plate, Tail-flick, and rotatod
	Administration of smaller dose of pregabalin (10 mg/kg, i.p.) before the oxycodone and morphine	Hot plate and Tail-flick
Concentrations of oxycodone, morphine, their metabolites, and pregabalin in brain when oxycodone or morphine is co-administered with pregabalin	Morphine (2.5 mg/kg, s.c.) and oxycodone (0.6 mg/kg, s.c.) co-administered with pregabalin (50 mg/kg, i.p.)	Hot plate, Tail-flick, and Liquid chromatography–mass spectrometry
Effect of pregabalin on the development of morphine tolerance and the acute effect of pregabalin on morphine-tolerant rats	Combined treatment of pregabalin and morphine	Hot plate, Tail-Flick
Evaluation of possible cross-tolerance between morphine and pregabalin		Hot plate
<b>Study IV</b>		
Development of tolerance to the antinociceptive effect of morphine and opioid-induced hyperalgesia	Administration of morphine b.i.d. for 14 days	Hot plate, Cold Plate, Dynamic Plantar Aesthesiometer
Effect of chronic morphine treatment on microglia and astrocyte reactivity in spinal cord and brain		Immunohistochemistry, flow cytometry
Effect of chronic morphine treatment on microglia transcriptome in the spinal cord		Transcriptomics

### 4.3 Statistical analyses

The results of the hot plate test are expressed as percentage of the maximum possible effect (MPE%), calculated as  $MPE\% = [(post\text{-}drug\text{ latency} - baseline\text{ latency}) / (cut\text{-}off\text{ time} - baseline\text{ latency})] \times 100\%$ . The results of the tail-flick test are expressed either in MPE% (Studies I-III) or latency in seconds (Study IV). The results of the cold plate test are expressed in latency in seconds (Study IV). The rotarod test results are expressed as the percentage change from the baseline values (Study II). The results of the DPA (Study IV) and mechanical gauge (Study II) are expressed as threshold in grams. In the text and figures, results are presented as mean of the sample values (+SEM when applicable) with minimum-maximum values (when applicable) or with individual values.

The behavioral data were tested for significant differences in mean values by two-way analysis of variance followed by a Holm–Sidak correction for multiple comparisons (Studies I, II, IV), One-way analysis of variances followed by a multiple-comparison-adjusted Tukey *post hoc* analysis (Study III) or unpaired T-test with Welch’s correction (Study IV). For the concentration data, one-way analysis for variance followed by the Holm–Sidak correction (Studies I, III) or two-way analysis of variance followed by a Holm–Sidak *post hoc* test with correction for multiple comparisons (Study III) was used. The immunohistochemistry data were tested for significant differences in mean values by two-way analysis of variance followed by a two-tailed Holm–Sidak correction for multiple comparisons or unpaired two-tailed T-test with Welch’s correction (Study IV). The fluorescence-activated cell sorting (FACS) data were tested for significant differences in mean values with unpaired two-tailed T-test with Welch’s correction. The data were analyzed using GraphPad Prism, version 6.0 a-c for Mac OS X (GraphPad Software, La Jolla, CA, USA). In the iPathway analysis (Study IV), the differential expression threshold for the transcript expression fold change was set at 0.5 (log<sub>2</sub>) and

the false discovery rate adjusted  $p$ -value ( $q$ -value) at 0.05. The false discovery rate  $p$ -value correction was applied for GO-enrichment analysis and impacted phenotype analysis. The difference was considered significant at  $p < 0.05$  (or  $q < 0.05$  in Study IV) in all of the tests.

## 5. RESULTS

### Studies I and II

#### 5.1 Individual antinociceptive effect of spironolactone, eplerenone, furosemide, and chlorothiazide

None of the studied diuretics (spironolactone 50 mg/kg or 100 mg/kg i.p., eplerenone 100 mg/kg i.p., furosemide 100 mg/kg i.p., or chlorothiazide 50 mg/kg, i.p.) had antinociceptive effects in either the hot plate or tail-flick tests (Study I: Fig. 1 and Study III: Fig. 1 and Table 1).

#### 5.2 Antinociceptive effect of spironolactone, eplerenone, furosemide, and chlorothiazide in co-administration with oxycodone and morphine

Spironolactone co-administration (50 mg/kg i.p.) enhanced the antinociceptive effect of morphine 2.5 mg/kg and 5 mg/kg s.c. in naïve rats (Study I: Fig. 1) and 4 mg/kg in tolerant rats (Study I: Fig. 2) in the thermal tests. Spironolactone also enhanced the antinociceptive effect of oxycodone 0.75 mg/kg s.c. in naïve rats in the thermal tests, whereas furosemide caused only a negligible effect in the hot plate test when combined with morphine (Study II: Fig. 1 and Table 1). Eplerenone and chlorothiazide did not enhance the antinociceptive effect of either of the opioids in the thermal tests (Study II: Table 1).

### 5.3 Spironolactone increases the morphine brain concentration and inhibits the elimination of morphine

When spironolactone (50 mg/kg i.p.) was administered both 60 min and 0 min prior to morphine 4 mg/kg s.c., the antinociceptive effect of morphine was increased significantly in the hot plate at 30 min and 90 min time points (Fig. 12A), (Study I: Results). Concurrently, in the brain the morphine concentration was greatly elevated (Fig 12B). In spironolactone co-treatment, the morphine serum concentrations were elevated only in the 90 min time point (Fig. 12C), however. In the liver, the morphine concentrations were elevated at both time points (Fig. 12D). The concentrations of the main metabolite of morphine, M3G, in the liver did not decrease at the 30 min time point, but increased at the 90 min time point in the spironolactone co-treatment (Study I: Fig. 3). In addition, spironolactone co-treatment significantly decreased CYP3A produced normorphine concentrations in the liver (Study I: Results).

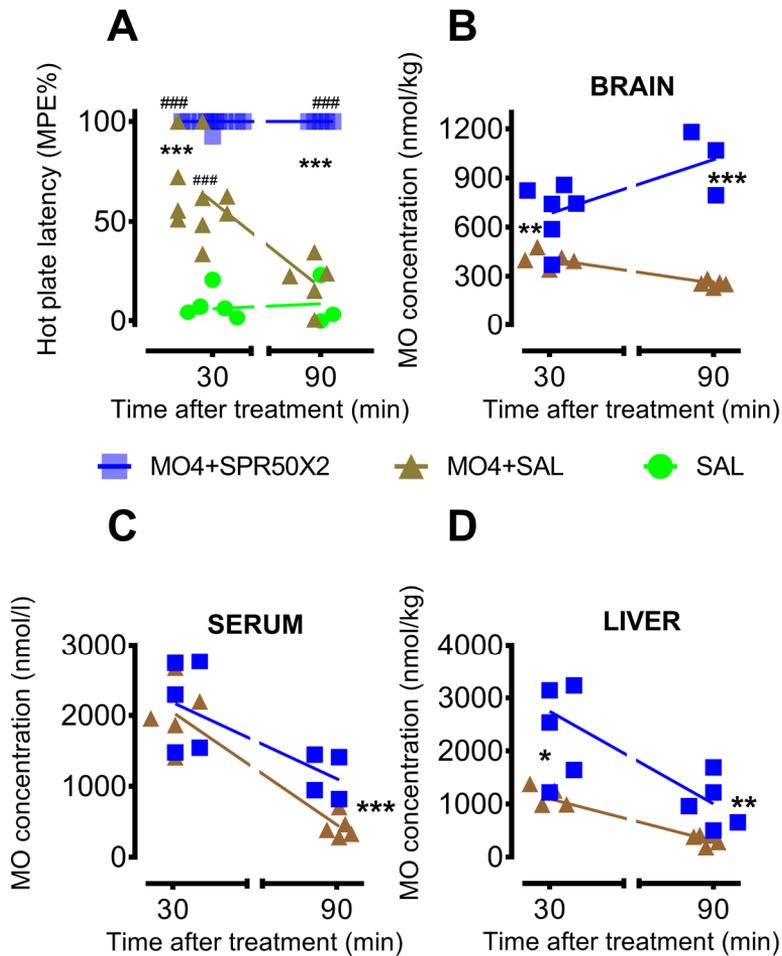


Figure 12. Effect of spironolactone (50 mg/kg i.p.) on morphine (4 mg/kg s.c.) (MO4) antinociceptive effect in the hot plate test when spironolactone was administered 60 min and 0 min before (MO4+SPR50X2) morphine. The results expressed as the mean of the maximum possible effect (MPE%) including the individual values are plotted at 30 and 90 min after morphine administration (A). The corresponding brain (B), serum (C), and liver (D) morphine concentrations are presented as mean in addition to the individual values. \*, \*\*, \*\*\* Statistically significant difference ( $p < 0.05$ , 0.01, 0.001) between the treatment groups. ### Statistically significant difference ( $p < 0.001$ ) when compared against the saline group (SAL). N = 6-10 at 30 min and 5 at 90 min time point.

#### 5.4 Spironolactone increases oxycodone brain concentrations and inhibits elimination of oxycodone

Spironolactone 100 mg/kg i.p. administered 30 min before oxycodone 0.75 mg/kg increased the antinociceptive effect of oxycodone 68% and 50% MPE in the hot plate test at the 30 min and 90 min time points, respectively (Fig. 13A). In the brain, spironolactone increased the concentration of oxycodone 46% and 140%, respectively (Fig. 13B). Also, the concentrations of oxycodone in the liver and serum were increased at both time points (Fig. 13C-D).

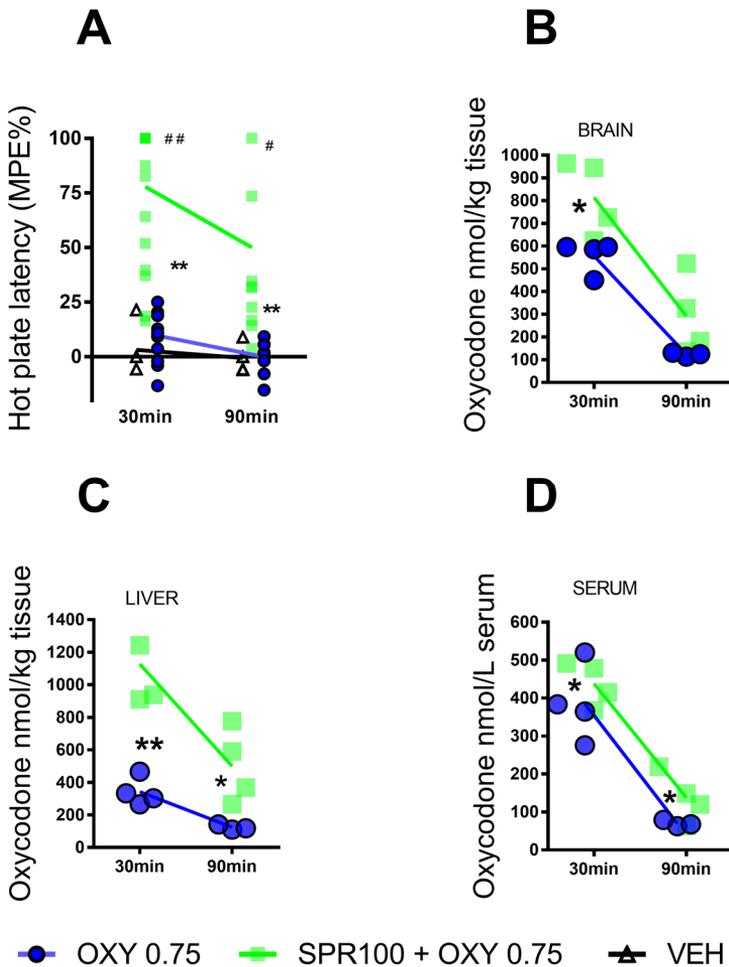


Figure 13. Effects of spironolactone 100 mg/kg i.p., oxycodone 0.75 mg/kg s.c. (OXY 0.75), vehicle (VEH), and the combined effect of spironolactone with oxycodone (SPR100 + OXY 0.75) in the hot plate tests (A). The mean of the maximum possible effect (MPE%) including the individual values in the hot plate test are plotted at the 30 min and 90 min time points. The respective oxycodone concentration in brain (B), liver (C), and serum (D). #, ##, Statistically significant difference ( $p < 0.05$ ,  $0.01$ , respectively) as compared with the vehicle control. \*, \*\*, Statistically significant difference ( $p < 0.05$ ,  $0.01$ , respectively) between treatment groups. N = 5-15 in the behavioral test and 3-6 in the concentration measurements per group.

### 5.5 Loperamide shows antinociceptive effect when co-administered with spironolactone

Loperamide did not show any antinociceptive effect on its own in the thermal tests (Study I: Fig. 4). However, when spironolactone 50 mg/kg i.p. was administered 60 min and 0 min before loperamide 10 mg/kg s.c., loperamide demonstrated antinociceptive effects (Study I: Fig. 4).

### 5.6 Spironolactone does not prevent the development of morphine tolerance

Chronic spironolactone co-treatment (50 mg/kg i.p. b.i.d.) did not prevent the development of tolerance to the antinociceptive effect of morphine, when tested with morphine 4 mg/kg s.c. in the tail-flick and hot plate test. Chronic spironolactone pretreatment (50 mg/kg i.p. b.i.d., four days) did not cause changes in the acute antinociceptive effect of morphine 4 mg/kg in morphine-naïve rats in the thermal tests (Study II: Results).

### 5.7 Spironolactone and eplerenone do not have acute antinociceptive effects in a model of incision pain

After surgery, the threshold for mechanical hyperalgesia decreased approximately 40% on the first and 50% on the second postoperative day. Eplerenone 100 mg/kg i.p. was administered on the day after surgery and spironolactone 100 mg/kg i.p. on the second postoperative day (Study II: Fig. 3). There was no significant difference between the treatment and vehicle groups on either of the days at any time point in the mechanical threshold test (Study II: Fig. 3).

## Study III

### 5.8 Pregabalin differentially potentiates the antinociceptive effect of oxycodone and morphine

Pregabalin (10 mg/kg or 50 mg/kg i.p.) alone did not produce significant antinociception in either the tail-flick or hot plate tests at the 30 min time point (Fig. 14A-H). At the 90 min time point, pregabalin 50 mg/kg caused a significant antinociceptive effect of 43% MPE in the hot plate test (Study III: Results). Pregabalin (50 mg/kg i.p.) increased the acute antinociceptive effect of oxycodone (0.6 mg/kg s.c.) in the hot plate test (Fig. 14A) but not tail-flick (Fig. 14B), and of morphine 2.5 mg/kg (s.c.) in the hot plate test (Fig. 14C) but not tail-flick (Fig. 14D). When pregabalin (10 mg/kg s.c.) was administered 30 min before the opioids, the effect of oxycodone (Fig. 14E, F) but not morphine (Fig. 14G, H) was potentiated in the hot plate and tail-flick test. In the rotarod test, pregabalin (50 mg/kg i.p.) alone did not significantly reduce the time that the rats stayed on the rod, but its combination with both oxycodone (Fig. 14I) and morphine (Fig. 14J) reduced the time significantly.

## Results

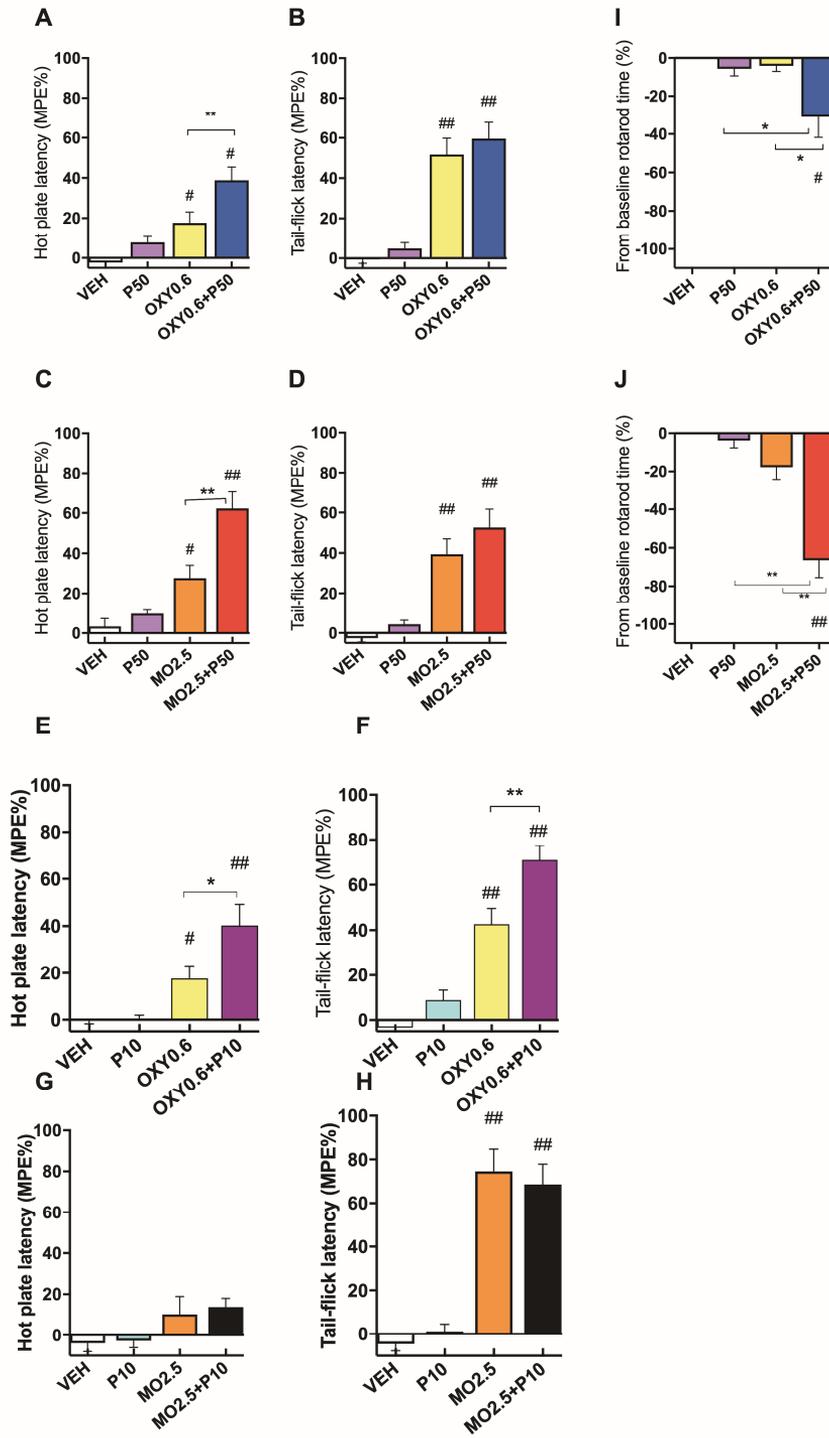


Figure 14. Effects of pregabalin 50 mg/kg i.p. (P50) or 10 mg/kg i.p. (P10), vehicle (VEH), oxycodone 0.6 mg/kg s.c. (OXY0.6), morphine 2.5 mg/kg s.c. (MO2.5), and the combined effect of either pregabalin 50 mg/kg i.p. or 10 mg/kg i.p. with oxycodone (OXY0.6+P50), (OXY0.6+P10) or morphine (MO2.5+P50), (MO2.5+P10), respectively, in the hot plate test (A, C, E, and G), tail-flick (B, D, F, and H), and the rotarod test (I and J), rotarod only P50. P50 was administered with the opioids and P10 was administered 30 min before the opioids. The means of the maximum possible effects (MPE%) or the latency from the baseline rotarod time (%) after 30 min of administration of the opioids are plotted with SEM. #, ##, Statistically significant difference ( $p < 0.05$ ,  $p < 0.01$  respectively) as compared with the vehicle control. \*, \*\*, Statistically significant difference ( $p < 0.05$ ,  $p < 0.01$ ) as compared between the indicated groups. N = 10–16 rats per group.

### 5.9 Pregabalin does not have an effect on brain concentrations of oxycodone or morphine or vice versa

Pregabalin 50 mg/kg i.p. was administered 30 min before oxycodone 0.6 mg/kg s.c. Brain samples were collected immediately after the behavioral measurements at the 30 min time point. Pregabalin did not change the brain concentrations of oxycodone, noroxycodone, or oxymorphone; nor were the brain concentrations of pregabalin affected by co-administration of oxycodone (Study III, Fig. 3).

Pregabalin 50 mg/kg was administered with morphine 2.5 mg/kg s.c., and the thermal antinociceptive tests were performed 90 min later (Study III: Results). The brain samples were collected after the behavioral measurements. The brain concentrations of morphine, M3G, or pregabalin were not affected by co-administration of pregabalin and morphine (Study III, Figure 3).

### 5.10 Pregabalin does not prevent morphine tolerance

Co-administration of pregabalin 50 mg/kg s.c. with morphine during the tolerance treatment did not affect the development of morphine tolerance (Study III, Fig. 4). In morphine-tolerant animals, pregabalin 50 mg/kg s.c. did not potentiate the antinociceptive effect of acute morphine 5 mg/kg s.c., as measured by the hot plate test at the 30 min time point (Study III, Fig. 4). In addition, the effect of acute pregabalin 50 mg/kg was assessed in both morphine-naïve and -tolerant rats to study potential cross-tolerance between morphine and pregabalin. However, prior morphine treatment did not affect the hot plate test latencies induced by acute pregabalin treatment at 120 min.

## Study IV

### 5.11 Chronic morphine treatment induces tolerance and hyperalgesia

Morphine 10 mg/kg s.c. administered b.i.d. induced tolerance to the antinociceptive effect of morphine in 10 days in the tail-flick test (Fig. 15A). Opioid-induced hyperalgesia was detected in both the cold plate and DPA tests during the morphine treatment (Study IV: Fig. 2).

### 5.12 Chronic morphine increases microglial reactivity in the spinal cord but not in the brain

Chronic morphine treatment induced a 32-33% and 22-24% increase in microglial immunoreactivity in the dorsal horn and ventral horn of the spinal cord, respectively (Fig. 15B-C). Also, immunoreactivity of substance P and CGRP in the dorsal horn was

## Results

increased (Study IV: Fig. 5). However, the treatment did not change the immunoreactivity of astrocytes in the dorsal horn (Study IV: Fig. 4). In flow cytometry, the proportion of M2-polarized (anti-inflammatory) microglia was increased (Study IV: Fig. 7). However, in the brain no increase in microglia or astrocyte reactivity after chronic morphine treatment was detected (Fig. 15D-E).

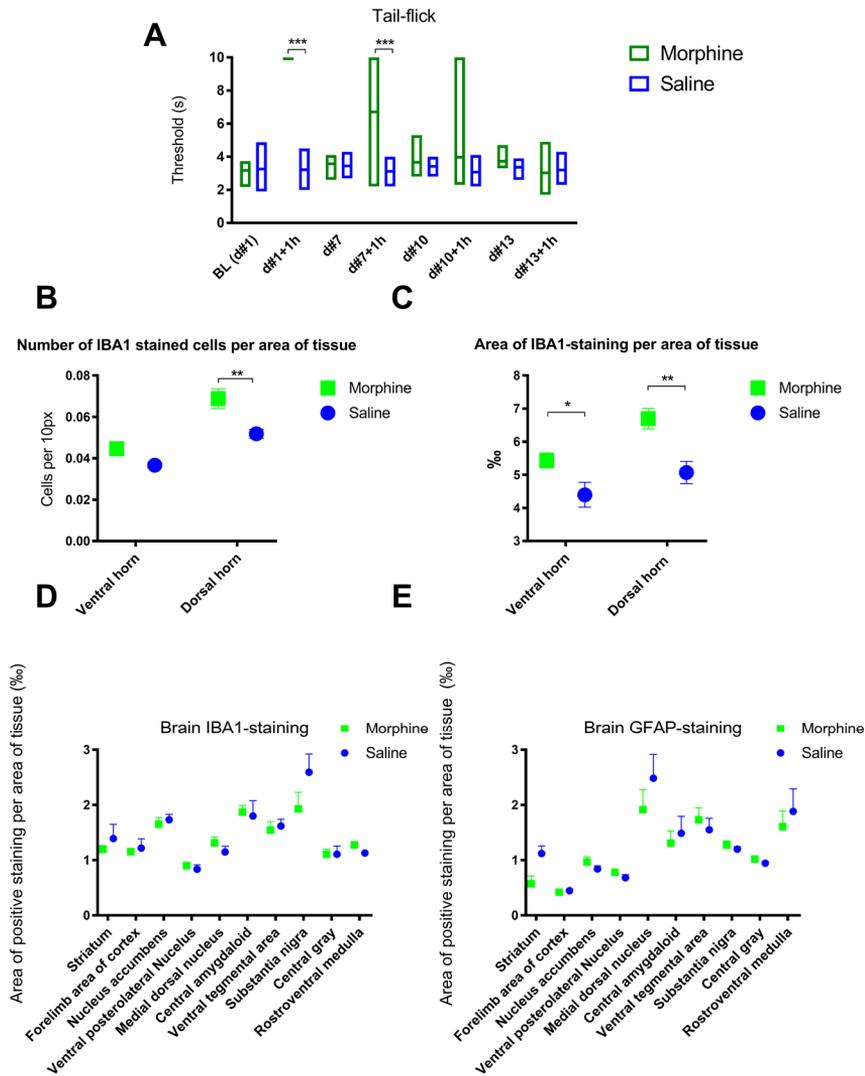


Figure 15. Mean effects of morphine in the tail-flick (A) tests in minimum-maximum-value-box. The rats were administered morphine 10 mg/kg s.c. or saline twice daily for 14 days. The tail-flick test was performed at baseline at day (d#) 1, 12 hours after the latest morphine injections from the previous day at d#7, 10, and 13, and one hour after the morphine injection at d#1, 7, 10, and 13. The samples for immunoreactivity tests were gathered at d#14. The mean+SEM of the IBA1 (B-C) immunoreactivity in the spinal cord and IBA1 (D) and GFAP (E) immunoreactivity in the brain. \*, \*\*, \*\*\* Statistically significant difference ( $p < 0.05$ , 0.01, 0.001 respectively) as compared with indicated groups. N = 14-15 rats per group, except in immunohistochemistry group, where n = 4-5 rats per group.

### 5.13 Chronic morphine treatment induces transcriptional changes in spinal microglia

Altogether 2454 differentially expressed genes were detected after chronic morphine treatment in spinal microglial. Gene ontology enrichment analysis showed that genes in biological processes, including 'immune system' and 'response to stress', were enriched. Also, genes related to chronic pain in previous studies, like in the DAP12 pathway, were upregulated. Other significantly upregulated individual genes included *Kcnn4*, *Fkbp5*, *Fcgr1a*, and *Cd244* (Study IV: Results).

## 6. DISCUSSION

### 6.1 Pharmacokinetic interaction between diuretics and morphine or oxycodone

Studies I and II demonstrated that the diuretics spironolactone, eplerenone, furosemide, and chlorothiazide lacked acute antinociceptive effects of their own in the thermal tests. Spironolactone, but not eplerenone, however, potentiated the antinociceptive effect of oxycodone and morphine, and spironolactone concomitantly increased the brain concentrations of both opioids. The results indicate that the increased antinociception in Studies I and II is due to the evident increase in opioid concentrations in the brain when spironolactone is co-administered.

Both oxycodone and morphine are eliminated mostly in the liver by metabolism. In humans, UGT2B7 (2b1 in rat) metabolizes approximately 50% of the morphine dose to M3G and 10% to M6G (Andersen et al., 2003), while the rat forms no M6G (Kuo et al., 1991). In a parallel route, a minor fraction of morphine is metabolized to normorphine by CYP3A4 (Andersen et al., 2003; Projean et al., 2003). The CYP3A also metabolizes oxycodone to its major metabolite noroxycodone, whereas parallel metabolism to oxymorphone via 2D6 is a less significant route (Lalovic et al., 2006). CYP3A metabolizes oxymorphone and 2D6 noroxycodone to noroxymorphone (Lalovic et al., 2004).

In Study I, spironolactone treatment did not decrease hepatic M3G concentrations, which indicates that spironolactone does not inhibit the major morphine metabolizing enzyme, UGT2b1. It, however, decreased hepatic concentrations of normorphine, suggesting that spironolactone might inhibit CYP3A, a significant metabolizing enzyme

of oxycodone. Interestingly, in Study I, spironolactone indeed decreased the concentration ratio of noroxycodone to oxycodone. Also, the concentrations of noroxymorphone were reduced, which could also be explained by spironolactone-induced inhibition of CYP3A metabolism.

The increased morphine serum concentrations are likely due to decreased excretion, as the metabolism of morphine was shown not to decrease in the experiment (Study I). However, the elevated serum concentrations alone were unlikely to cause the increased morphine brain concentration, as under spironolactone, the brain/serum ratio of morphine was elevated. Both morphine (Letrent et al., 1999; Xie et al., 1999) and spironolactone (T. Nakamura et al., 2001) are substrates for P-gp, a drug efflux transporter expressed in the BBB and peripheral tissues, including the liver and kidney. A plausible hypothesis for the presented results could be that spironolactone inhibits P-gp, which would cause both impaired efflux of morphine at the BBB but also reduction of elimination by decreasing excretion of morphine in bile and urine. Supporting this hypothesis, the results of this thesis demonstrated that the P-gp substrate loperamide exerted an antinociceptive effect only in co-administration with spironolactone. The finding implies that spironolactone increased brain distribution of the otherwise peripherally restricted loperamide, and thus, spironolactone was able to produce effective depression of P-gp function in the present study setting. The approach was, however, indirect and confirmation of the theory would require *in vitro* modeling of morphine transport in P-gp containing cells challenged with spironolactone. Loperamide is metabolized by CYP3A4 and 2C8 (Baker, 2007), and it is possible that spironolactone also increased its concentrations.

Unlike with morphine (Studies I and II), the increased oxycodone (Study II) concentrations in the serum could be explained by a spironolactone-induced decrease in metabolism. However, whether spironolactone-induced inhibition of the P-gp at the

BBB contributes to the increased oxycodone brain concentrations, like it probably did with morphine, is unclear. The brain/serum ratio of oxycodone was increased, but the literature remains controversial regarding whether oxycodone is a substrate of P-glycoprotein, see for; Hassan et al. (2007) and against; Boström et al. (2005). The hypothesis of P-gp inhibition would, however, explain the results of this thesis regarding the increased oxycodone brain disposition.

Both spironolactone and eplerenone are MR antagonists. Activation of MR can promote pro-inflammatory signaling, diminishing the GR-dependent anti-inflammatory activity (Ibrahim et al., 2016). Indeed, eplerenone has been shown to reduce pain-related behavior and excitability of afferent neurons in a back-pain model in the rat (Dong et al., 2012; Ye et al., 2014). In addition MR has been demonstrated to mediate rapid effects of glutamate transmission in hippocampal neurons (Karst et al., 2005). The hippocampus, on the other hand, affects acute pain processing in rats (Ford et al., 2011). It is therefore possible that spironolactone and eplerenone could have exerted an acute pharmacodynamic effect, which was not detected in both the model of postoperative pain (Study II) and acute thermal tests (Studies I and II). In the low back pain studies (Dong et al. 2012; Ye et al. 2014) pain behaviour was provoked by locally inflaming the L5 dorsal root ganglion. In the study by Dong et al., eplerenone decreased excitability of sensory neurons of inflamed but not uninflamed dorsal root ganglion. Therefore, it is possible that the antinociceptive effect of MR blocking can not be generalized to reduce pain with different underlying mechanisms. It must be noted, however, that brain or spinal concentrations of spironolactone were not measured in the acute co-administration experiments with opioids in Studies I and II. Therefore, the possibility of increased CNS concentration of spironolactone contributing to the increased antinociceptive latencies in the behavioral results cannot be fully excluded.

An important conclusion from the results is that an enhanced acute antinociceptive effect of opioids with spironolactone cannot be attributed to increased diuresis, as furosemide, a more potent diuretic than spironolactone, caused only a negligible antinociceptive effect with morphine. Moreover, the spironolactone-induced diuresis was beyond the timeframe of the acute study in this thesis, as the natriuretic effect of spironolactone begins 3-5 days after treatment (Yeung and Wong, 2002).

The clinical relevance of the demonstrated interaction between spironolactone and morphine or oxycodone remains elusive. Spironolactone and opioids could be co-used in conditions such as malignant ascites and cause heightened opioid brain concentrations and predispose to opioid adverse effects. On the other hand, the increased brain disposition of an opioid might also reduce the peripheral adverse effects. In the study of Heiskanen et al. P-gp inhibitor itraconazole failed to increase the pharmacodynamic effect of morphine in humans, although the brain concentrations could not be assessed (Heiskanen et al., 2008). The effect of spironolactone on opioid pharmacokinetics should also be investigated in human volunteers.

## 6.2 Pharmacodynamic interaction between pregabalin and morphine or oxycodone

Study III demonstrated that acute administration of pregabalin potentiated both thermal antinociceptive and sedative effects of oxycodone and morphine. The measured changes in the brain concentrations of oxycodone, morphine, their major metabolites, and pregabalin failed to explain the antinociceptive results. In addition, pregabalin administration did not affect the development of morphine tolerance. The antinociceptive results are in agreement with clinical studies that have shown that co-administration of gabapentinoids has reduced the amount of opioids needed to alleviate

experimental nociceptive pain in healthy volunteers (Eckhardt et al., 2000) and also postoperative pain (Clarke et al., 2009; Fassoulaki et al., 2012; Mahoori et al., 2014; Tiippana et al., 2007; Yücel et al., 2011.). Pregabalin is eliminated renally (Bockbrader et al., 2010a), but an interaction via the poorly understood drug transporters at the BBB level is possible. It seems that pharmacokinetic interactions between oxycodone, morphine, and pregabalin have not been previously studied. The unchanged brain concentrations of the studied drugs and their major metabolites after concomitant administration indicated, however, that the behavioral changes cannot be explained by altered brain disposition of the drugs. Thus, the results imply that the drug interaction between pregabalin and opioids at the CNS level is pharmacodynamic.

Pregabalin is considered to exert its analgesic effect via binding to the  $\alpha 2\text{-}\delta\text{-}1$  subunit of voltage-gated calcium channels (VGCCs) (Field et al., 2006). VGCCs can be subdivided into low voltage-activated Cav 3.1-3.3 (T-) and high voltage-activated Cav 1.1-1.4 (L-), Cav 2.2 (N-), Cav 2.1 (P/Q-), and Cav 2.3 (R-types) (Dolphin, 2016). All of the high-voltage channels have been shown to be expressed in DRGs and the spinal cord (Lee, 2013) and to be associated with the  $\alpha 2\delta$  subunit (Dolphin, 2016).

Opioids exhibit their inhibition of calcium currents via N- (2.2) and P/Q-type (2.1) channels (Bourinet et al., 1996; Seward et al., 1991), but presumably not via L-channels (1.1-1.4) (Rhim and Miller, 1994). Interestingly, in the study of Omote et al. a selective N-type calcium channel blocker  $\omega\text{-CgTx}$  produced a synergistic interaction with morphine in nociceptive tests (Omote et al., 1996). Thus, it could be hypothesized that pregabalin exerts its interaction by inhibiting the same presynaptic calcium channels as opioids (N and/or P/Q), which ultimately leads to a synergistic decrease in the secretion of excitatory neurotransmitters. Surprisingly, also L-type calcium channel inhibitors verapamil, diltiazem, and nifedipine have been shown to have synergistic antinociceptive interaction with morphine (Omote et al., 1993). However, whether

verapamil, diltiazem, and nifedipine actually exert their synergistic effect by selectively blocking the non-opioid-receptor-coupled L-channel could be argued, as verapamil has also been demonstrated to block P- and possibly N- and Q-channels (Dobrev et al., 1999), nifedipine N-type calcium channels (S. N. Li et al., 1999), and diltiazem P-type calcium channels (Dobrev et al., 1999). Diltiazem and verapamil are P-gp substrates and in the study of Shimizu et al potentiated the morphine-induced antinociception while not, however, markedly increasing the morphine brain concentrations (Shimizu et al., 2004). The role of pharmacokinetic interaction between morphine and the discussed calcium channel blockers cannot, however, be excluded from the mechanism behind the observed potentiation of analgesia. To summarize, it seems plausible that the potentiating effect of pregabalin on the opioids is mediated via inhibiting the common (N and/or P/Q) presynaptic calcium channels. However, interaction associated with L-type channel inhibition is also possible.

Surprisingly, when pregabalin was administered before the opioids at a smaller dose, only the antinociceptive effect of oxycodone, not morphine, was potentiated. Therefore, it was hypothesized that the difference in the results between the opioids could be related to functional selectivity, i.e. different agonists elicit different downstream signaling despite binding to the same  $\mu$ -opioid receptor. Although it has been shown that MOR can demonstrate functional selectivity to morphine and oxycodone (A. Nakamura et al., 2013), the specific intracellular pathway that could explain the discussed result in the present study remains unknown.  $Ca^{2+}$ -dependent endocytosis is highly complex and varies in response to the array of unique stimulation patterns, for review, see Leitz and Kavalali (2016). Therefore, it seems possible that even subtle differences in MOR downstream signaling might explain the differences between opioids regarding the pharmacodynamic interactions with pregabalin.

In the meta-analysis of Tiippana et al. (2007) the figures for number-needed-to-harm (the number of patients needed to be treated to reveal a particular adverse effect) were relatively high, 35 and 12 for perioperative gabapentinoid to cause strong sedation or dizziness, respectively, in humans. Consistent with this, in the meta-analysis of Peng et al. (2007), the severity of dizziness caused by perioperative gabapentin was often classified as mild. By contrast, Jokela et al. (2008) reported an increased incidence of adverse effects, including dizziness, after perioperative pregabalin and oxycodone co-administration. In the present study, the amount of sedation, measured as the time that rats were able to stay on a rotating rod, in concomitant administration with opioid was greatly increased. The results could be interpreted so that, from pharmacological point of view, gabapentinoids and opioids predispose to increased sedation, as shown by this study. Whether such sedation is experienced postoperatively might be dependent on the factors related to a specific clinical setting, such as type of surgery or timing of the drug administration. On the other hand, discrepancy between the experienced sedation in the discussed studies might also be explained by differences in the sensitivity of the methods of measuring sedation. Besides the analgesic effect, it should be noted that the supraspinally mediated hot plate test used in the present study is also affected by sedation and the associated motor impairment. Therefore, the observed potentiation of the opioids in the hot plate test was probably not solely analgesic in nature.

In the postmortem toxicology study conducted in Finland in 2010-2011, 91% of pregabalin abusers also showed concomitant opioid use (Häkkinen, 2014). Hence, it was interesting also to determine whether the deaths could be attributed to the pharmacological interaction between pregabalin and opioids. Pregabalin did not, however, prevent the development or reverse morphine tolerance. Moreover, a synergistic effect on antinociception and sedation in morphine-tolerant animals was not detected. In contrast to the hypothesis, the results indicate that the combined use of pregabalin and an opioid does not predispose opioid-tolerant individuals to increased

effects of opioids. However, a limitation of the study was that respiratory depression measurements in chronic morphine administration was not performed. Moreover, the studies were performed with only one dose. It should also be noted that the mechanism of death is not necessarily related to respiratory depression, as both opioid receptors (Sobanski et al., 2014) and L-type calcium channels (Zhao et al., 2017) are known to regulate, for example, also myocardial function.

### 6.3 Chronic morphine treatment increases microglial reactivity

Study IV shows that chronic morphine treatment induced tolerance and hyperalgesia. Microglia immunoreactivity increased concomitantly at the spinal but not the supraspinal level. In flow cytometry, the proportion of anti-inflammatory spinal microglia was increased. Upregulation of many pain-related genes in the transcriptome of the spinal microglia after chronic morphine treatment were also detected.

The mechanism of the opioid-induced activation of microglia remains to be elucidated. Hutchinson et al first suggested that glial activation would be mediated via TLR4 signaling (Hutchinson et al., 2010) but the role of TLR4 signaling in opioid tolerance and OIH has also been challenged (Mattioli et al., 2014). Spinal microglia and astrocytes have been shown not to express MORs (Corder et al., 2017.; Kao et al., 2012). In the present study, chronic morphine treatment increased the immunoreactivity of substance P and CGRP in the dorsal horn, both of which have been reported to activate microglia (Reddington et al., 1995; Zhu et al., 2014). Therefore, the activation of spinal microglia in chronic morphine treatment might be related to an increase in neuron-derived peptides.

Chronic morphine treatment did not have an effect on spinal M1-polarized (pro-inflammatory) microglia, but it increased the proportion of M2-polarized (anti-

inflammatory) microglia of the total microglia. The result could be interpreted as transition to the resolution phase of the neuroinflammatory process or a sign of a process opposing the onset of inflammation. A reduction of microglia in the medulla and substantia nigra was also detected, but it is difficult to speculate about implications.

In the present study, immunoreactivity of microglia or astrocytes was not significantly changed after chronic morphine treatment in such brain regions as the nucleus accumbens, ventral posterolateral nucleus, central amygdala, ventral tegmental area, central gray, or rostroventral medulla. Other studies have demonstrated both increases and decreases in immunoreactivity of the glial cells in individual brain regions after chronic morphine treatment (Hutchinson et al., 2009). For example, Hutchinson et al. reported that the count of microglial cells was decreased in the nucleus accumbens, while (Hutchinson et al., 2009) it was increased in the work of Zhang et al (X.-Q. Zhang et al., 2012). The finding in this study implies that the pathophysiology of opioid tolerance or opioid-induced hyperalgesia is not dependent on microglia or astrocyte activation in the studied supraspinal structures.

The transcriptome of the spinal microglia revealed enrichment of genes in the sets related to the 'immune system process', 'response to stress catabolic process', and 'defense response'. In addition, individual genes including *Kcnn4*, *Fkbp5*, and members of the TREM2/DAP12-signaling pathway were upregulated. The calcium-activated potassium channel *KCa3.1* (*Kcnn4*) is known to contribute to microglia activation via functional linkage to the intracellular p38 MAPK pathway, and many investigations have asserted that its blockade on glia attenuates neuroinflammation (Bouhy et al., 2011; Y.-J. Chen et al., 2016; Kaushal et al., 2007; Reich et al., 2005). Interestingly, several *KCa3.1* blockers are also in clinical use, making *KCa3.1* a feasible target for pharmacological intervention, for review, see Wulff and Castle (2010).

Fkbp5, a component of a steroid receptor complex, has been shown to have a significant role in pain regulation by modulating glucocorticoid signaling (Maiaru et al., 2016). Study of Maiaru et al. indicated that Fkbp5 was expressed exclusively in neurons of the superficial dorsal horn of the spinal cord, and thus, the role of Fkbp5 in microglia in morphine tolerance remains unknown. Interestingly, glucocorticoid signaling in microglia is suggested to govern the inflammatory state in inflamed CNS (Carrillo-de Sauvage et al., 2013). The TREM2/DAP12 signaling pathway is involved in cell activation and phagocytic activity, for review, see Xing et al. (2015). Kobayashi et al. have demonstrated that TREM2/DAP12 signaling in microglia promotes proinflammatory phenotype and aggravates neuropathic pain. (Kobayashi et al., 2016). According to the present results, it possibly also facilitates nociception in opioid treatment.

## 6.4 Methodological and ethical considerations

Pain is a product of a very complicated biological system. Due to the inherent complexity of the phenomenon, no *in vitro* or *in silico* methods exist today to model the pain in humans. Behavioral animal models are therefore still necessary despite their shortcomings. Indeed, the murine behavioral models are known to be sensitive to laboratory factors, including the individual conducting the experiments, air humidity, diet, and environmental stress factors (Chesler et al., 2002; Vissers et al., 2003). For instance, the olfactory stimuli associated with exposure to male but not female experimenters has recently been shown to cause stress and antinociception in rodents (Sorge et al., 2014.). Internal factors of the animals, such as genotype, age and gender, can also affect the results (Vissers et al., 2003). Controlling all possibly relevant factors can be difficult, which explains some of the discrepancy in the preclinical results in the literature.

This thesis was limited by its utilization of male rats only. The gender of the animals used in preclinical research has received special attention. The literature regarding humans suggests, although the issue is complex, that women are more sensitive to pain than men (Mogil, 2012) and might also show significant qualitative differences to men in their pain physiology, such as in some aspects of the immune system related to pain modulation (Sorge et al., 2015). The majority of preclinical studies of pain have, however, been conducted in male rats (Mogil, 2012), which can cause bias and reduce the applicability of the preclinical results to women.

Ethical aspects are of particular importance when nociception is elicited in conscious animals. The Finnish act for the use of animals for scientific purposes is based on the implementation of the EU directive 2010/63/EU. Besides legislation, also the scientific community develops contemporary guidelines for ethical issues and to promote welfare of animals (Knopp et al., 2015). Interestingly, a preclinical trial register to tackle the significant problem of negative publication bias, which might be a leading cause of unnecessary use of animals, has also been recently discussed (Kontinen, 2015). This research was conducted in accordance with the guidelines of the local authorities and the International Association for the Study of Pain. The Three Rs principle of animal testing (replacement, reduction, and refinement) was considered in the design of the study settings. In the nociceptive behavioral tests, only the threshold of withdrawal was measured and the animals were allowed to discontinue the administration of the gradually increasing stimulus. The National Animal Experiment Board of Finland approved the study protocol.

An ideal behavioral animal model for nociception has been suggested to possess the following qualities: input specificity, validity, and sensitivity (Le Bars et al., 2001). The administered stimulus should be nociceptive (input specificity) and the measured behavioral sign should be a selective response to nociception (validity) (Le Bars et al.,

2001). The model should also be able to quantify the response and to discern the effect of possible pharmacological interventions (sensitivity) (Le Bars et al., 2001). In this thesis, frequently used behavioral tests were utilized, of which the heat tests (tail-flick and hot plate test) are validated tools in the study of opioid pharmacology, for review, see Le Bars et al. (2001). In the study of hyperalgesia, the cold plate in freely moving animals is reviewed by Allchorne et al. (2005) and the evaluation of mechanical hyperalgesia using methods similar to the mechanical gauge and dynamic plantar aesthesiometer is reviewed by Le Bars et al. (2001). All of the tests used are inherently prone to error due to potential non-specific responses, which could be related to, for example, learning in repeated measurements or drug-induced confounding effects. Interestingly, marked advances in the neuroimaging of pain (Morton et al., 2016) foster the hope that the problem of validity could be bypassed with functional imaging measuring the activation of pain-related brain regions directly. In this thesis, immunohistochemistry to measure glial reactivity, which necessitates the sacrificing of the animal, was employed. Non-invasive positron emission tomography imaging can, however, quantitatively measure glial activation (Imamoto et al., 2013) and may in the future replace less versatile immunohistochemical methods.

## 6.5 Future perspectives

The results of this thesis using the rat as a model organism, suggest that drug interactions can alter opioid brain concentrations and the possibility of increased opioid brain disposition should be acknowledged in clinical pharmacology. Although animal models are routinely used to predict pharmacokinetic behaviour of drugs in the human, interspecies differences of CYP-mediated metabolism and limited understanding of interspecies differences in drug transporters prevent direct extrapolation of the results (Chu et al., 2013; Martignoni et al., 2006). Therefore, further human studies are

warranted. In clinical trials, the drug brain concentrations cannot be, however, measured directly, but surrogate methods like analysis of the cerebrospinal fluid might be used (Nagaya et al., 2014). Non-invasive pharmacokinetic measurement methods, including positron emission tomography (Gupta et al., 2002), should, however, be further developed. Interestingly, magnetic resonance imaging could be used in the future to detect the distribution of magnetically labeled drugs (S. K. Li et al., 2008). Future pharmacogenetic studies could reveal relevant interindividual differences in the drug transporter molecule profiles, which could predispose to the effects of such interactions. The mechanism of antinociceptive potentiation between pregabalin and opioids seems to be mediated via voltage-gated calcium channels. The role of VGCCs has gained attention in pain signaling studies (Gribkoff, 2006), and VGCCs have been a target for the development of pharmacological inhibitors (S. Lee, 2013). As the understanding of the function of the subunit  $\alpha 2\delta 1$ , the binding site of pregabalin, remains incomplete, further basic research characterizing its function might provide a basis for future drug development. In addition, the role of functional selectivity of  $\mu$ -opioid receptor in pharmacodynamic drug interactions between opioids and other drugs should be studied more extensively as it might prove to have a clinical impact. The detailed view of the distinct glial cell populations involved in opioid-induced neuroinflammation and the time scale of its progression remain to be elucidated. The endeavour to develop effective treatments for opioid tolerance and opioid-induced hyperalgesia would benefit from focused studies on the molecular mechanisms and identification of new druggable targets.

## Conclusions

Conclusions addressing the aims of this thesis using the rat as a model organism:

- I. Spironolactone did not display antinociceptive effects of its own, but acutely enhanced the morphine antinociception, most likely by inhibiting P-gp and increasing morphine brain concentrations. Spironolactone did not prevent the development of morphine tolerance. Spironolactone increased the antinociceptive effects and brain concentrations of oxycodone. Unlike with morphine, the mechanism of the potentiation is probably through inhibition of metabolism. The increased brain disposition might be associated with P-gp inhibition.
- II. Pregabalin enhances the antinociceptive and sedative effects of both oxycodone and morphine. The interaction, however, differs between the opioids as it depends on dose or/and temporal scheme of the drug administration. The behavioral results could not be explained by pharmacokinetic interaction at the CNS level. Pregabalin did not prevent or reverse morphine tolerance.
- III. Chronic morphine treatment induced tolerance and hyperalgesia. The associated increase in microglia activation took place at the spinal but not the supraspinal level, including the relevant brain regions related to pain processing. The transcriptome of the spinal microglia cells indicated upregulation of pro-inflammatory processes and genes associated with pathological pain.

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