

Opioid receptor efficacy during normal and pathological conditions

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ABBREVIATIONS

aCSf	artificial cerebrospinal fluid
amg	Amygdala
ANOVA	Analysis Of Variance
BCA	Bicinchoninic acid
Bmax	maximum number of binding sites
Ca ²⁺	Calcium
CaCl ₂	Calcium chloride
cAMP	cyclic Adenosine monophosphate
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin gene-related peptide
Cpu	Caudate putamen
CYT	Cytoplasm
DAMGO	[D-Ala ² , N-Me-Phe ⁴ , Gly-ol]-enkephalin
dg	Dentate gyrus
DRG	Dorsal root ganglion
ECL	Enhanced chemiluminescence
EGTA	Ethylene glycol tetraacetic acid
Emax	maximal response of an agonist
FE	Fentanyl
FITC	Fluorescein isothiocyanate
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GRK	G receptor protein kinase
GTP	Guanosine triphosphate
GTP γ S	Guanosin 5'-O-(3-thiotriphosphat)
G α_i /or G α_o	G protein α_i or α_o subunit
h	hour
HCL	Hydrochloride
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IR	Immunoreactive

i.pl.	intraplantar
i.t.	intrathecal
i.v.	intravenous
ipsi	ipsilateral
IR	Immunoreactive
K ⁺	Potassium
KCl	Potassium chloride
Kd	Dissociation constant
KDa	Kilodaltons
KH ₂ PO ₄	Potassium phosphate
KOR	Kappa opioid receptor
L	Lumbar dorsal root ganglia
Lamp-1	Lysosomal– associated membrane protein-1
LOP	Loperamide
LSM	Laser scanning microscopy
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulfate
min	minute
MO	Morphine
MOR	Mu-opioid receptor
MPE	Maximal possible effect
mRNA	messenger ribonucleic acid
Na ₂ HPO ₄	Sodium phosphate
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NC	Negative control
NGF	Nerve growth factor
NLX	Naloxone
NLXM	Naloxone-methiodide
p75 ^{NTR}	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP9.5	Protein gene product 9.5
PM	Plasma membrane

PPT	Paw pressure threshold
Rab4	Ras-related protein Rab-4
Rab7	Ras-related protein Rab-7
RM-ANOVA	Repeated-Measures ANOVA
RT-PCR	Real Time Quantitative PCR
s	seconds
s.c.	subcutaneous
SD	Standard deviation
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
siRNA	small interfering RNA
STZ	Streptozotocin
thl	Thalamus
Tris	Trishydroxymethylaminomethane
Tris-HCl	Tris hydrochloride
TrkA	Tyrosine Receptor Kinase A
v/v	volume / volume
VDCCs	Voltage-dependent Ca ²⁺ channels
w/v	weight / volume

General summary

Along the pain pathway, i.e. from the peripheral sensory neuron via the spinal cord to the thalamus and sensory cortex, mu-opioid receptors (MOR) are accessible for the modulation of incoming painful stimuli. In this thesis the relative contributions of MOR to the systemic antinociceptive effects of MOR agonists at the level of the peripheral sensory neuron, spinal cord, and brain was examined. Moreover, since previous work has suggested that sensory neuron opioid receptors may vary in their number, G protein coupling and efficacy depending on the presence of a normal or pathological condition (e.g. cancer, inflammation), this thesis examined the underlying mechanisms of a reduced opioid responsiveness and explored possible therapeutic approaches in the animal model of streptozotocin-induced diabetic neuropathic pain.

Chapter I – In rats with unilateral complete Freund’s adjuvant hindpaw inflammation antinociceptive effects of the systemically active MOR agonists fentanyl and morphine versus the peripherally restricted MOR agonist loperamide were compared following their systemic and peripheral administration. In addition, antinociceptive effects of systemically applied MOR agonists were antagonized by site-directed application of naloxone-methiodide at the level of the brain, spinal cord and peripheral sensory neuron. Antinociceptive effects of systemically applied MOR agonists were two- to threefold greater and longer lasting compared to their peripheral application. Antagonism of systemic opioid antinociception by site-directed naloxone-methiodide showed superior involvement of cerebral and spinal compared to peripheral MOR.

Chapter II – In previous studies using the animal model of streptozotocin (STZ)-induced diabetes, antinociceptive effects of opioids were significantly impaired, however, their explanations of the underlying mechanisms focussing on the brain and spinal cord were somewhat inconsistent. Since painful diabetic neuropathy is primarily a disease of the peripheral nervous system, the focus of this work was to examine putative changes of the opioid receptor system in the peripheral sensory neuron. Impaired peripheral antinociceptive effects of opioids are associated with a loss in the number, coupling and efficacy of sensory neuron MOR in rats with STZ-induced diabetic neuropathic pain. This loss is due in part to the enhanced Rab7 dependent lysosomal targeting and degradation of MOR. Importantly, down-regulation of the enhanced Rab7 expression by siRNA-mediated gene silencing or by normalization of the reduced nerve growth factor concentration in diabetic rats prevented

MOR targeting to lysosomes, restored MOR density on the cell membrane of sensory neurons, and rescued opioid responsiveness towards better pain relief.

Chapter III – Interestingly in parallel to the changes at the peripheral nerve terminal, similar changes occurred at the central presynaptic nerve terminal of sensory neurons within the spinal cord of diabetic rats. In STZ treated animals with diabetic neuropathic pain antinociceptive effects of i.t. MOR agonists were significantly impaired. This was due to a loss in the number and G protein coupling of spinal MOR. Pretreatment with NGF reversed these changes and recovered the antinociceptive efficacy of intrathecal opioids.

Zusammenfassung

Entlang der Schmerzbahnen, d.h. vom peripheren sensorischen Neuron via Rückenmark hin zum Thalamus und sensorischen Kortex, sind Mu-Opioidrezeptoren für die Modulation hereinkommender schmerzhafter Reize zugänglich. In dieser Arbeit wurde die relative Beteiligung zerebraler, spinaler und peripherer MOR an den analgetischen Wirkungen systemischer Opioide untersucht. Unter Berücksichtigung früherer Arbeiten, die angeregt hatten, dass Opioidrezeptoren sensorischer Neurone in Abhängigkeit eines vorhandenen normalen oder pathologischen Zustandes (z.B. Tumor, Entzündung) in ihrer Anzahl, G Protein Kopplung und Wirksamkeit variieren, untersucht diese Arbeit die zugrunde liegenden Mechanismen einer reduzierten Ansprechbarkeit auf Opioide und deren mögliche therapeutischen Konsequenzen in einem Tiermodell der Streptozotocin-induzierten schmerzhaften diabetischen Neuropathie.

Kapitel I – In Ratten mit einer unilateralen, durch komplettes Freund's Adjuvans ausgelösten Entzündung einer Hinterpfote wurden die antinocizeptiven Wirkungen der systemisch aktiven MOR Agonisten Fentanyl und Morphin mit dem peripher selektiven MOR Agonisten Loperamid nach ihrer systemischen bzw. peripheren Applikation miteinander verglichen. Zusätzlich wurden die antinocizeptiven Wirkungen systemisch applizierter MOR Agonisten mittels ortsspezifischer Applikation von Naloxone-methiodid im Gehirn, Rückenmark und Peripherie antagonisiert. Die antinocizeptiven Wirkungen der systemisch applizierten MOR Agonisten waren zwei-bis dreifach größer und länger andauernd als die Wirkungen nach ihrer peripheren Applikation. Der lokale Antagonismus der antinocizeptiven Wirkungen systemisch applizierter MOR Agonisten mittels ortsspezifischer Applikation von Naloxone-methiodid im

Gehirn, Rückenmark und Peripherie involvierte in erster Linie zerebrale und spinale gegenüber peripheren MOR.

Kapitel II – In früheren Studien des STZ-induzierten Diabetes waren antinozizeptive Wirkungen von Opioiden signifikant reduziert; jedoch ihre Fokussierung auf das Gehirn und Rückenmark als mögliche Orte der zugrunde liegenden Mechanismen war in ihren Ergebnissen inkonsistent. Unter Berücksichtigung, dass die diabetische Neuropathie primär eine Erkrankung des peripheren Nervensystems ist, lag der Fokus dieser Arbeit hauptsächlich auf den mutmaßlichen Veränderungen des Opioidsystems im peripheren sensorischen Neuron diabetischer Tiere. Verminderte periphere antinozizeptive Wirkungen von Opioiden waren mit einem Verlust der Anzahl, G Protein Kopplung und antinozizeptiven Wirksamkeit von MOR sensorischer Neurone in Ratten mit STZ-induzierter diabetischer Neuropathie assoziiert. Dieser Verlust beruhte zum Teil auf einem vermehrten, Rab7 abhängigen, lysosomalen Transfer und Abbau von MOR. Zudem kann die Herunterregulation der erhöhten Rab7 Expression durch siRNA- vermitteltes „Gene-Silencing“ oder durch die Normalisierung der reduzierten Konzentration an Nervenwachstumsfaktor (NGF) in diabetischen Tieren den lysosomalen Transfer verhindern, die Dichte membranständiger MOR wiederherstellen und die Ansprechbarkeit auf Opioide hin zu einer verbesserten Schmerzlinderung retten.

Kapitel III – Interessanterweise geschehen parallel zu den Veränderungen am peripheren Nervenende ähnliche Veränderungen am zentralen präsynaptischen Nervenende sensorischer Neurone im Rückenmark diabetischer Tiere. In STZ behandelten Tieren mit einer schmerzhaften, diabetischen Neuropathie war die antinozizeptive Wirksamkeit i.t. MOR Agonisten signifikant reduziert. Dies beruhte auf einem Verlust in der Anzahl und funktionellen Kopplung spinaler MOR diabetischer Tiere. Eine Vorbehandlung mit dem Nervenwachstumsfaktor (NGF) hob diese Veränderungen auf und stellte die ursprüngliche antinozizeptive Wirksamkeit intrathekaler Opioide wieder her.

1. General Introduction

Opioids are highly effective in inhibiting pain. However, evidence is accumulating that sensory neuron opioid receptor responsiveness may vary depending on the existing state of pain (Mousa et al., 2007; Yamamoto et al., 2008). For example while opioids appear to have enhanced analgesic efficacy during inflammatory pain, their analgesic effects might be impaired during cancer or neuropathic pain. The principle aim of this thesis was evaluate the relative contributions of central versus peripheral MOR to the antinociceptive effects of opioids and to determine possible alterations in opioid responsiveness during diabetic neuropathic pain. Moreover, this study aimed to elucidate some of the mechanisms that may contribute to these alterations and identify possible strategies that may prevent these alterations.

Opioids and opioid receptors

Opioids are the most effective drugs to alleviate pain, even in patients with severe pain. They were originally derived from the milky juice of the opium poppy that contains several different alkaloids including morphine. Today, numerous naturally occurring, semisynthetic and synthetic opioids are in clinical use to treat patients with acute or chronic pain. While different opioid receptors (μ -, δ -, κ -opioid receptor) have been identified and cloned (Kieffer and Gavériaux-Ruff, 2002), opioids in clinical use are predominantly ligands of the MOR.

MOR belong to the family of the G protein coupled receptors. Upon ligand binding, MOR couple with inhibitory G proteins such as $G\alpha_i$ and/or $G\alpha_o$ and subsequently decrease the intracellular cAMP concentration by inhibiting adenylyl cyclase (Mostany et al., 2008). This leads to diminished release of neurotransmitters and -peptides by inhibition of voltage-gated Ca^{2+} channels (Christie et al., 2000), and/or to reduced excitation of sensory neurons by hyperpolarizing the cell membrane through opened K^+ channels (Christie et al., 2000). All these mechanisms impair the generation and transmission of painful stimuli, leading to a relief of pain perception.

The activation of opioid receptors is limited by their desensitization which occurs upon ligand binding and subsequent G receptor protein kinase (GRK)-mediated phosphorylation (homologous desensitization). Alternatively, it can occur through other intracellular signalling pathways, such as protein kinase C (PKC) or protein kinase A (PKA)-mediated phosphorylation (heterologous desensitization) (Chen and Yu et al., 1994; Rodríguez-Muñoz,

et al., 2007). Once phosphorylated, the opioid receptors become internalized and are thought to have several potential fates. One is the dephosphorylation of the receptor in endosomes followed by recycling back to the plasma membrane in a fully sensitized state (Zhang et al., 2008; Klaasse et al., 2008). Another is that internalized receptors are often sorted through endosomes and delivered to lysosomes, where receptor degradation by cysteine proteases occurs (Tanowitz and Zastrow, 2002). The delivery of membrane proteins from the plasma membrane to lysosomes is a multiple-step process that is mediated by different Rab proteins. Rab proteins are monomeric Ras-related GTPases that are associated with distinct intracellular membranes, where they control vesicle trafficking between intracellular compartments (Hirota et al., 2007). The trafficking of internalized receptors from multivesicular bodies to lysosomes is dependent on the Ras-related Rab7 GTP-binding protein (Dale et al., 2004; Ceresa and Bahr, 2006). Proteolysis of endocytosed receptors in lysosomes contributes to GPCR downregulation in mammalian cells (Marchese et al., 2008; Zhang et al., 2008).

Antinociceptive effects of opioids

Originally opioid receptors were identified in specific pain related brain areas (Simon and Hiller, 1978) and it was believed that opioids exert their analgesic action primarily within the central nervous system which led to their characterization as “centrally acting analgesics”. Indeed, previous studies demonstrated that the intracerebroventricular (i.c.v.) injection of MOR agonists resulted in potent analgesia in animals and humans (Dib and Duclaux, 1982; Lazorthes, 1988). However, in 1976 Yaksh and Rudy showed that intrathecal administration of opioids was also effective in reducing pain (Yaksh and Rudy, 1976). Consistently, MOR were demonstrated in the dorsal horn of the spinal cord (Arvidsson et al., 1995). The activation of these was shown to reduce nociceptive signal transmission by inhibition of presynaptic voltage-gated Ca^{2+} channels (Heinke et al., 2011). This new approach of opioid application was subsequently introduced into daily clinical practice for the treatment of acute (Rathmell et al., 2005) and chronic pain (Kedlaya et al., 2002). More recently, MOR have been identified in the cell body and on the central and peripheral nerve endings of sensory neurons (Mousa et al., 2007; Stein et al., 1990). In line with these findings, peripheral application of low, systemically ineffective doses of a MOR agonist into painful inflamed paw tissue of rats was shown to elicit potent antinociception (Khalefa et al., 2012; Stein et al., 1988). This effect is dose-dependent, stereoselective and antagonized by MOR selective antagonists indicating its receptor specificity (Stein et al., 1988). Moreover, the data from

behavioural experiments were further substantiated by electrophysiological experiments which demonstrated a reduction in neuronal activity following local MOR agonist treatment (Moshourab and Stein, 2012; Wenk et al., 2006; Andreev et al., 1994). The evidence of a peripheral application of opioids was soon introduced into clinical practice in the way that patients undergoing painful knee surgery reported about significant pain relief following the intraarticular application of a low dose of morphine (Stein, 1991). The advantage of this approach is that it is devoid of severe central side effects such as respiratory depression, sedation, cognitive dysfunction, dependence and addiction. Thus, pain can be inhibited by MOR agonists not only through their corresponding receptors in certain brain areas, but also through their receptors expressed at different levels of the neuraxis of pain transmission, i.e. the spinal cord and the peripheral sensory neuron. However, the most common way of opioid treatment in patients is by systemic application either by the oral, intravenous or subcutaneous route. To date no studies have been reported that assessed the relative contributions of the supraspinal, spinal and peripheral MOR to the overall antinociceptive effect of systemically given opioids. Therefore the first aim of my work was to investigate this objective in an animal model of inflammatory pain.

Antinociceptive effects of peripherally applied MOR agonists are pronounced under inflammatory pain conditions (Mousa et al., 2007; Hartwig et al., 2003; Schäfer et al., 1994). Consistent with these findings, studies have demonstrated an increased number and coupling of MOR in dorsal root ganglia (Núñez et al., 2007). Furthermore, enhanced peripherally directed axonal transport of MOR in CGRP-immunoreactive nerve fibres and a higher density of MOR-immunoreactive nerve endings arborising into the epidermis have been shown. (Shaqura et al., 2004; Zöllner et al., 2003). Indeed, reducing the number of functional sensory neuron MOR by local application of β -funaltrexamine, which selectively and irreversibly binds to MOR, resulted in diminished antinociceptive effects of peripherally applied MOR agonists (Schäfer et al., 1994). Recent studies have identified nerve growth factor (NGF) as a potential mediator of MOR up-regulation (Mousa et al., 2007), since it has been shown that local concentration of the former is significantly increased within inflamed tissue (Safieh-Garabedian et al., 1995). Neutralization of the locally enhanced NGF concentration resulted in decreased numbers and G protein coupling of sensory neuron MOR (Mousa et al., 2007).

In contrast to inflammatory pain, in the rat model of bone cancer pain - following the implantation of sarcoma cells into the intramedullary space of the femur - the doses of morphine required to block painful behaviour were ten times higher than those required to inhibit inflammatory pain (Luger et al., 2002). In another study, it was shown that this is

related to a significant reduction in the number of MOR mRNA, protein and immunoreactive DRG cells costaining for CGRP which is a marker for peripheral sensory neurons (Yamamoto et al., 2008). Since peripheral application of low doses of morphine and loperamide at the site of tumor infiltration inhibited pain successfully (Menendez et al., 2003; Menedez et al., 2005), the reduction in peripheral sensory neuron MOR will lead to impaired pain inhibition. Because sensory neuron MOR expression and antinociceptive efficacy is enhanced during inflammatory pain and impaired during bone cancer pain, it appears that the peripheral sensory neuron is particularly susceptible to a distinct regulation of MOR, depending on different local pathomechanisms caused by various types of disease.

In an animal model of neuropathic pain following ligation and subsequent transection of the L5 spinal nerve root, MOR mRNA and protein were down-regulated in the injured L5 dorsal root ganglion of the innervated skin and the spinal cord (Lee et al., 2011). This down regulation was not observed in the neighboring uninjured dorsal root ganglia and their nerve fibers (Lee et al., 2011; Kohno et al., 2005). Following a chronic constriction injury of the sciatic nerve, diminished sensory neuron MOR expression resulted in a significant loss of antinociceptive effects (Obara et al., 2009). In another animal model of neuropathic pain induced by partial nerve ligation of the sciatic nerve the significant loss in the number of MOR-immunoreactive dorsal root ganglion neurons correlated with the reduced antinociceptive effects of locally applied MOR agonists (Rashid et al., 2004). Therefore, it seems that similar to bone cancer pain, however in contrast to inflammatory pain, neuropathic pain resulting from nerve injury also leads to a loss of sensory neuron MOR and antinociceptive effects. Nevertheless, local application of MOR agonists directly at the site of nerve injury apparently is able to elicit some degree of pain inhibition (Truong et al., 2003; Labuz et al., 2009).

Diabetic neuropathic pain

Diabetes mellitus is one of the major clinical problems worldwide. It is characterized by chronic hyperglycemia, i.e. abnormally elevated blood glucose levels, due to defects in the secretion and/or action of insulin. The common symptoms of diabetes mellitus are increased thirst, polyuria, polyphagia, blurred vision, and loss of body weight. These symptoms arise as a consequence of abnormalities in carbohydrate, fat, and protein metabolism. Diabetes mellitus can be divided into two main types according to the etiology, Type 1, “Juvenile Diabetes Mellitus” (Insulin Dependent Diabetes Mellitus) and Type 2, “Adult type” (Non-Insulin Dependent Diabetes Mellitus). Type 1 diabetes occurs mainly in childhood due to a

complete insulin deficiency resulting from autoimmune-mediated eradication of pancreatic β -cell islets. In contrast, diabetes mellitus type 2 is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (reviewed by Patel et al., 2012).

Diabetic polyneuropathy is a common long term complication of diabetes mellitus. Every fifth patient with diabetes develops diabetic neuropathy during the course of the disease and half of these patients suffer from different forms of pain (Boulton et al., 2004). Patients with diabetic polyneuropathy often complain of sensory disturbances (paresthesia), hypersensitivity to sensory stimuli (hyperesthesia), a feeling of numbness and unpleasant burning (burning feet), as well as cramping, stabbing, or shooting pain (Gimbel et al., 2003). Primary sensory afferent hypersensitivity in diabetic neuropathy has been attributed to altered expression, membrane insertion, and function of voltage-gated sodium, potassium and calcium channels (Cao et al., 2010). Interestingly, patients suffering from diabetic neuropathic pain respond poorly to opioid analgesics (Dworkin et al., 2010). In effort to elucidate possible mechanisms, different studies have concluded with controversial results (Hall et al., 2001; Shangguan et al., 2003; Chen et al., 2002; 2003).

Experimental models of diabetes

In order to better understand the disease, the pathophysiology, and the complications of diabetes and to test various therapeutic agents for its treatment, appropriate experimental models are needed. Diabetes animal models can be obtained through chemically-induced or surgically-evoked manipulations. Since 1943, alloxan has been used for inducing experimental diabetes by selectively eradicating the pancreatic beta islets leading to insulin deficiency and hyperglycaemia (Srinivasan and Ramarao, 2007). Like alloxan, streptozotocin (STZ), a glucosamine–nitrosourea compound derived from the *Streptomyces achromogenes* causes hyperglycaemia principally by direct cytotoxic action on the pancreatic beta cells (Melanie et al, 2011). This animal model has been shown to reveal the typical signs of type I diabetes (polydipsia, polyuria, weight loss, etc.) within a relatively short time period. In the majority of experimental studies STZ was injected intraperitoneally, however in addition to the diabetes, animals suffered also from accompanying peritonitis. Therefore following the advice of the local animal care committee (LAGeSo), STZ (45mg/kg) was given by i.v. injection which resulted in a similar loss of insulin producing pancreatic beta cells without accompanying peritonitis. As a consequence, animals showed elevated blood glucose concentrations (hyperglycemia), reduced body weight gain, polyuria, polydipsia, increased

food and water intake, and reduced mechanical thresholds (hyperalgesia, allodynia). In recent trends new genetically modified animal models including transgenic, generalized knockout and tissue specific knockout mice have been used as animal models of diabetic disease (Fröde and Medeiros, 2008). However, in this study all analyses were performed using the conventional animal model of STZ-induced diabetes, thereby comparing our data directly with those reported by other investigators.

Antinociceptive effects of opioids in diabetic neuropathic pain

Several experimental studies in diabetic animal models suggested a reduced opioid responsiveness as reflected by the loss in antinociceptive effects following the systemic (Courteix et al. 1998), spinal (Chen and Pan, 2003; Zurek et al., 2001) or supraspinal (Zurek et al., 2001; Ohsawa et al., 1997) application of opioids. Recently, few studies investigated alterations in opioid receptor expression and signaling at the spinal level (Chen et al., 2002; Chen and Pan, 2003), however, the mechanisms by which diabetes impaired opioid antinociception were not entirely clear and the results were conflicting. Previous experiments by Courteix et al. (1998) and Zurek et al., (2001) suggested that the decreased antinociception of systemic morphine at an early phase of diabetes (5 weeks) may be related to alterations in the opioid distribution and clearance of diabetic rats. But, Hall et al. (2001) and Shangguan et al. (2003) at 4 and 8 weeks of i.p. STZ treatment could not detect a change in [³H]Naloxone binding sites within the spinal cord of diabetic rats. However, they identified a diminished inhibitory G protein activity. Also, other investigators observed a reduced spinal MOR G protein coupling without changes in the number of MOR or G protein subunits at early phase (between 3-5 weeks after SZT injection) (Chen et al., 2002; Chen et al., 2003). In addition, a reduction in distinct G protein subunits (Hajjalizadeh et al., 2010) or an increase in the concentrations of the pronociceptive peptides pronociceptin, nociceptin/orphanin FQ and nocistatin (Liu et al., 2012) have been reported. The majority of these investigations were performed after 4 weeks and only few studies after 8 weeks of STZ-induced diabetes. Therefore, this work set out to systematically investigate the number of G protein coupling and antinociceptive efficacy of spinal MOR in rats during a more advanced stage (12 weeks) of streptozotocin-induced diabetes. Because, diabetic neuropathy is primarily a disease of the peripheral sensory neuron, this project aimed at investigating how opioid responsiveness of these peripheral sensory neurons changed with the development of diabetes.

Objectives

The principle aims of the present study were:

1. Investigate the relative contributions of the supraspinal, spinal and peripheral MOR to the overall antinociceptive effects of systemically given opioids. This was addressed using an animal model with persistent inflammatory pain and to compare the effects of peripherally restricted versus systemically active opioids.
2. Examine the putative mechanisms of loss in opioid receptor numbers, coupling and antinociceptive efficacy of opioids in an animal model of streptozotocin-induced diabetic neuropathy. In addition determine the relative contributions of an enhanced local Rab7 and/or a reduced NGF expression to the lysosomal targeting of sensory neuron MOR.
3. Assess whether the loss in sensory neuron MOR also affects the number of central, presynaptic MOR within the dorsal horn of the spinal cord. And also investigate the antinociceptive effects of intrathecally delivered opioids.

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Chapter I of this dissertation is based on the following manuscript:

Khalefa, B.I., Shaqura, M., Al-Khrasani, M., Fürst, S., Mousa, S.A. and Schäfer, M. Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. **Eur J Pain** 2012, **16(5):690-705**
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Chapter III of this dissertation is based on the following manuscript:

Shaqura M, Khalefa B.I., Shakibaei M, Winkler J, Al-Khrasani M, Fürst S, Mousa SA, Schäfer M. Reduced Number, G protein coupling, and antinociceptive efficacy of spinal μ -opioid receptors in diabetic rats are reversed by nerve growth factor. **J Pain** 2013,**14(7)720-30**
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5. General discussion

In the first part of my thesis antinociceptive effects of MOR opioid agonists were studied along the neuraxis of pain transmission, i.e. at the level of the peripheral sensory neuron, the spinal cord and the brain. In a series of immunohistochemical experiments in rats MOR were identified at these three levels. Thereafter, the antinociceptive effects of systemically active versus peripherally restricted MOR agonists were compared in rats with inflammatory pain. Also the relative contributions of MOR at these three levels to the overall antinociceptive effect were determined. Since recent evidence suggests a distinct regulation of MOR expression, coupling and function depending on different pain states, the second part of my thesis investigated whether in contrast to inflammatory pain peripheral antinociceptive effects of the MOR agonist fentanyl were impaired in an animal model of STZ-induced diabetic neuropathy. In a series of immunohistochemical experiments accompanied by Western blot analysis a loss of total and membrane bound MOR in dorsal root ganglia of diabetic versus control rats was examined. Since MOR were removed from the plasma membrane to the perinuclear region and colocalized with multivesicular- and lysosome-like structures the experiments focused on a possible colocalization of MOR with Rab7 and the lysosomal marker LAMP-1. Following i.t. treatment with Rab7 specific siRNA a functional link between Rab7 and lysosomal targeting of sensory neuron MOR was assessed. To rescue sensory neuron MOR from enhanced lysosomal targeting, the reduced NGF concentration of peripheral sensory neurons during diabetic neuropathy was normalized and immunohistochemical, Western blot and behavioral experiments were repeated. The third part of my thesis tested whether the loss in peripheral sensory neuron MOR in rats with diabetic neuropathy also affected the number and functional coupling of presynaptic MOR as well as the spinally mediated antinociception of a MOR agonist.

Main findings

Chapter I – Although it has been demonstrated that site-directed application of mu-opioid agonists at the level of the brain, spinal cord and peripheral subcutaneous tissue activated MOR and elicited potent antinociceptive effects, it is unclear which of the three sites along the pain pathway with their local MOR contribute most to the overall antinociception following systemic opioid administration. Moreover, it is unknown whether there are differences between systemically active and peripherally selective MOR agonists. Therefore, this project examined using an animal model of painful Complete Freund's Adjuvant (CFA)-

induced hindpaw inflammation the relative contributions of these MOR to the antinociception of i.v. versus i.pl. fentanyl, morphine and loperamide, representing a highly lipophilic full, a hydrophilic partial and a peripherally restricted opioid agonist, respectively. The results showed that antinociceptive effects of i.v. fentanyl and morphine were two- to three-fold higher and longer lasting than the antinociceptive effects of their i.pl. injection. In contrast, the antinociceptive effects of i.v. and i.pl. loperamide were not significantly different. Site-directed application of the opioid antagonist NLXM, which commonly does not penetrate the blood-brain-barrier, revealed that the contributions of supraspinal and spinal MOR to the antinociceptive effects of i.v. fentanyl were approximately 80% and 20%, respectively, and to those of i.v. morphine 70% and 30%, respectively. Apparently, at these high doses of systemic fentanyl and morphine peripheral MOR in inflamed subcutaneous tissue did not seem to play a major role. This might not be the case with lower, but still systemically effective doses of MOR agonists which may allow more easily the occurrence of peripheral antinociceptive effects. In contrast, site-directed application of the opioid antagonist NLXM revealed that supraspinal and spinal MOR did not have an impact on the antinociception of i.v. loperamide, whereas peripheral MOR fully contributed to it. These findings imply that a peripherally restricted opioid agonist is inferior compared to systemically delivered opioids. However, they suggest that any development of novel peripherally restricted opioid compounds must consider among other factors the lipophilicity, the P-glycoprotein substrate specificity and the intrinsic efficacy of new drug.

Chapter II – In painful diabetic neuropathy experimental and clinical studies have shown a loss in opioid responsiveness and thus, antinociceptive efficacy. The putative mechanisms that were investigated in these studies were inconsistent, partly because they focused mainly on the brain or the spinal cord. Since diabetic neuropathy is a disease of the peripheral nervous system, this project focused on possible changes in sensory neuron MOR expression, membrane targeting and functional coupling in animals with painful diabetic neuropathy. In rats with STZ-induced diabetes local injection of the mu-opioid agonist fentanyl at doses, which are systemically ineffective, showed a significant loss in antinociception. In parallel, the number and functional coupling of MOR in peripheral sensory neurons were significantly reduced. Subcellular fractionation and subsequent Western blot analyses revealed that this was due to a shift of MOR from the cell membrane to the cytosol of DRG neurons in diabetic rats. Consistently, MOR specific immunoreactivity at the cell membrane disappeared and accumulated mostly in the perinuclear region. Here they increasingly colocalized with the

lysosomal markers LAMP-1 and Rab7, which led to the approach of the i.t. injection of Rab7 small interference RNA in diabetic rats. Rab7 knockdown by i.t. siRNA prevented the lysosomal targeting of sensory neuron MOR and supported their redistribution from the perinuclear region to the cell membrane. Consistent with previous studies of others which have demonstrated an inverse relationship between NGF and Rab7, in this animal model of STZ-induced diabetes NGF concentrations of DRGs were low, whereas Rab7 expression was high. Following i.t. substitution of NGF enhanced Rab7 expression, increased MOR targeting to lysosomes, and diminished fentanyl-induced antinociception were prevented. The findings from this project present for the first time a systematic examination of possible mechanisms in peripheral sensory neurons for a loss in opioid antinociception. Moreover in addition to *in vitro* studies, they show for the first time *in vivo* that MOR can be subjected to lysosomal targeting and degradation under pathological conditions. But also they give a therapeutic approach on how to prevent these mechanisms. Thus in contrast to enhanced peripheral opioid analgesia in inflammatory pain, in the animal model of diabetic neuropathic pain antinociceptive effects of opioids were impaired suggesting a distinct regulation of the number, functional coupling and efficacy of sensory neuron MOR in different pathological conditions.

Chapter III – It is widely accepted that the spinal cord is an important site for pain modulation and that MOR are crucial presynaptic modulators of dorsal horn pain transmission. Since the number of sensory neuron MOR is reduced during STZ-induced diabetic neuropathy, I examined whether this affects the number and functional coupling of presynaptic MOR as well as the spinally mediated antinociception of a MOR agonist and if this could be prevented by intrathecal pretreatment with NGF. Therefore in the animal model of STZ-induced diabetic neuropathy, i.t. fentanyl-induced antinociception was significantly reduced in diabetic rats. In parallel, it was shown that a significant reduction in MOR localized on sensory neuronal marker CGRP-immunoreactive nerve terminals within the dorsal horn of the spinal cord is concomitant with the loss in the number of membrane spanning MOR binding sites as well as with the functional MOR G protein coupling. These changes in the spinal cord were accompanied by a MOR down-regulation in the dorsal root ganglia of diabetic animals. Since diabetes affects the peripheral sensory neurons in depriving them of NGF, i.t. NGF treatment of diabetic rats restored MOR density in both DRG and the superficial dorsal horn of the spinal cord, improved functional MOR coupling and rescued opioid antinociceptive efficacy.

Summary of findings

This study provides first authoritative data showing that opioid efficacy and its receptor regulation in peripheral sensory neurons varies as a function of pain condition. In chronic pain states such as inflammatory pain, there is an increase in the number and coupling of sensory neuron MOR which consequently leads to enhanced opioid antinociceptive effects. This is in contrast to other pathological pain states such as STZ-induced diabetic neuropathy in which an enhanced Rab7 expression leads to increased lysosomal targeting and degradation of MOR which impaires opioid antinociceptive effects. The blockade of the enhanced Rab7 expression in diabetes, e.g. by Rab7 gene silencing or by normalization of the NGF deprivation in diabetes, prevented MOR targeting to lysosomes, restored MOR density in peripheral sensory neurons, and rescued opioid responsiveness towards better pain relief.

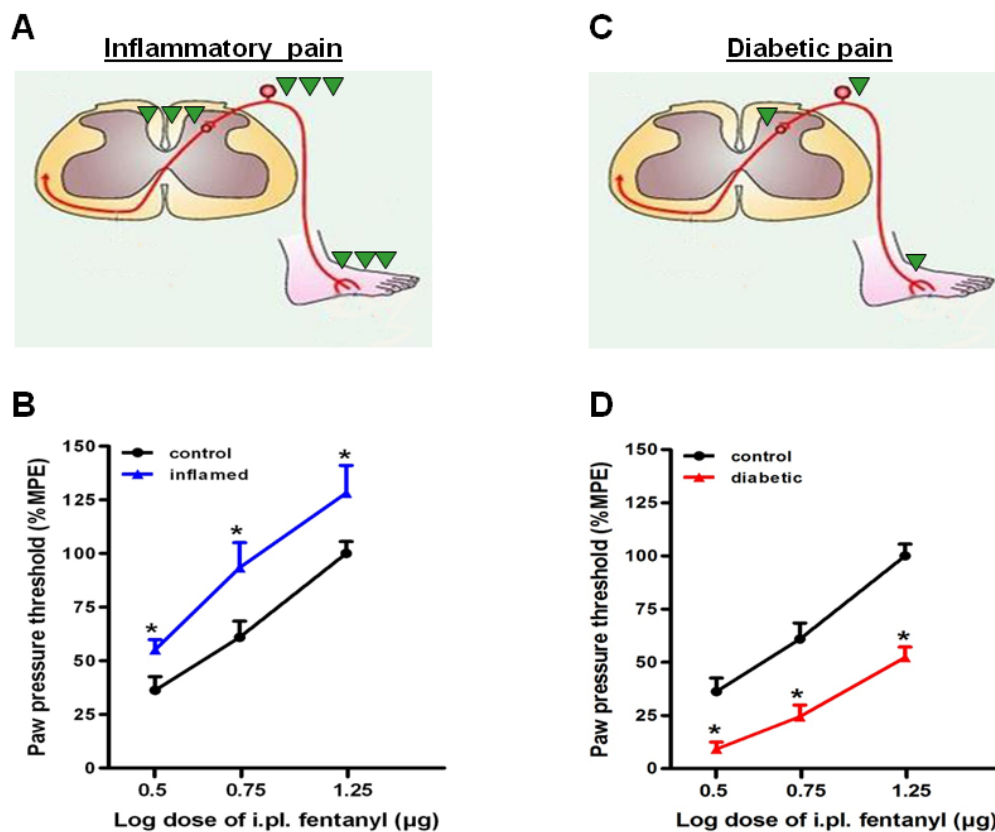


Figure 1. Sensory neuron Mu-opioid receptor (MOR) number and antinociceptive efficacy during inflammatory and diabetic neuropathic pain. (A): Increases in MOR number (green triangles) during inflammatory pain described at the spinal cord, DRG and peripheral sensory neuron. (B): Dose-response curves of increased paw pressure thresholds (antinociceptive effects) following i.pl. fentanyl were significantly shifted towards improved antinociceptive potency and efficacy in rats with an inflamed hindpaw compared to control animals. (C): Decreases in MOR number (green triangles) during diabetic painful conditions at the spinal cord, DRG and peripheral sensory neuron. (D): Dose-response curves of increased paw pressure thresholds (antinociceptive effects) following intraplantar fentanyl were significantly shifted towards impaired antinociceptive potency and efficacy in rats with diabetic neuropathic pain compared to control animals.

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Publications

Papers

- **Khalefa, B.I.**, Shaqura, M., Al-Khrasani, M., Fürst, S., Mousa, S.A. and Schäfer, M. Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. *European Journal of Pain* 2012; 16(5): 690–705; Impact factor: 3.94.
- Mousa SA, Shaqura M, **Khalefa B.I**, Zöllner C, Schaad L, Schneider J, Shippenberg TS, Richter JF, Hellweg R, Shakibaei M, Schäfer M. Rab7 silencing prevents μ -opioid receptor lysosomal targeting and rescues opioid responsiveness to strengthen diabetic neuropathic pain therapy. *Diabetes* 2013; 62(4): 1308-19; Impact factor: 8.30.
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Abstracts

- **Khalefa, B.I.**, Shaqura, M., Al-Khrasani, M., Fürst, S., Mousa, S.A. and Schäfer, M. Superior antinociception of centrally penetrating opioids fentanyl and morphine over the peripherally restricted opioid loperamide in pain modulation. Abstracts of Pain in Europe VII 7th Congress of the European Federation of Pain® Chapers (EFIC®), 2011; 5(1): 90.
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- In the printed version, the articles are included as pages 1-78.